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Ruzanna HAYRAPETYAN

REVÊTEMENTS EN POLYESTER DE BOITES METALLIQUES EN CONTACT AVEC LES DENRÉES ALIMENTAIRES : IDENTIFICATION ET ÉVALUATION DES DANGERS/RISQUES DES SUBSTANCES NON INTENTIONNELLEMENT AJOUTÉES

POLYESTER COATINGS USED WITH METALLIC CANS IN CONTACT WITH FOODSTUFFS: IDENTIFICATION AND HAZARD/RISK ASSESSMENT OF NON-INTENTIONALLY ADDED SUBSTANCES

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Composition du Jury :

Mme	FESSARD Valérie	Docteur, ANSES	Présidente
M.	KASS Georges	Professeur, EFSA	Rapporteur
M.	PARINET Julien	Docteur, ANSES	Rapporteur
Mme	OMER Elsa	Docteur, Nestlé	Examinatrice
Mme	CHAGNON Marie-Christine	Professeur, L'Institut Agro Dijon	Directrice de thèse
Mme	SEVERIN Isabelle	Professeur, L'Institut Agro Dijon	Codirectrice de thèse
M.	CARIOU Ronan	Docteur, LABERCA, Oniris	Invité
Mme	MONNERAYE Véronique	Responsable R&D Matières premières, MASSILLY HOLDING	Invité

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Article 1

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Other articles

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ÉCOLE DOCTORALE
Environnements - Santé

Titre : REVÊTEMENTS EN POLYESTER DE BOITES METALLIQUES EN CONTACT AVEC LES DENRÉES ALIMENTAIRES : IDENTIFICATION ET ÉVALUATION DES DANGERS/RISQUES DES SUBSTANCES NON INTENTIONNELLEMENT AJOUTÉES.....

Mots clés : Matériaux au contact des denrées alimentaires, vernis en polyester, extraction, tests de migration, substances ajoutées non intentionnellement, évaluation des dangers et des risques

Résumé : Cette thèse traite des aspects clés de l'identification et de l'évaluation de la toxicité des substances non intentionnellement ajoutées (NIAS en anglais) provenant des revêtements en polyester utilisés dans les matériaux au contact des aliments. Une stratégie a été mise en œuvre pour identifier les NIAS volatiles, semi-volatiles et non volatiles avec des études d'extraction et de migration en utilisant un simulant alimentaire (éthanol à 95 %) et un solvant d'extraction (acétonitrile, ACN). Les substances identifiées ont été analysées à l'aide de techniques analytiques avancées, puis soumises à une évaluation des dangers *via* des modèles prédictifs *in silico*. Pour les composés disposant de standards commerciaux, des biotests pertinents *in vitro* ont été réalisés afin de vérifier les alertes prédictives identifiées *in silico.*

La thèse porte également sur le devenir gastro-intestinal des NIAS non volatiles grâce à une nouvelle méthode d'extraction à l'ACN appliquée à des plaques d'acier revêtues de polyester. Ces extraits ont été soumis à une digestion intestinale simulée afin de controler les substances initiales et leurs éventuels produits de transformation dans des conditions physiologiques.

Enfin, une première étude de pré-validation d'une nanométhode du test d'Ames (NanoAmes — une version miniaturisée du test d'Ames réglementaire) a été réalisée. Cette méthode a été développée pour détecter des substances génotoxiques à de très faibles concentrations, cas des NIAS. Des substances génotoxiques et non génotoxiques de référence ont été testées, avec et sans activation métabolique (mélange S9 mix), en utilisant les souches

Au-delà de l'identification et de la caractérisation chimique des NIAS, la génotoxicité et le potentiel cancérogène des extraits et migrats des revêtements ont été évalués à l'aide de biotest *in vitro* pour vérifier les effets mélanges.

Salmonella typhimurium TA98 et TA100 dans deux laboratoires (Packtox et GenEvolution). L'analyse comparative des résultats a permis d'évaluer la fiabilité, la reproductibilité et potentiel de ce biotest.

Title: POLYESTER COATINGS USED WITH METALLIC CANS IN CONTACT WITH FOODSTUFFS: IDENTIFICATION AND HAZARD/RISK ASSESSMENT OF NON-INTENTIONALLY ADDED SUBSTANCES..... .....

Keywords: Food contact materials, polyester coatings, extraction, migration testing, non-intentionally added substances, hazard and risk assessment

Abstract: This thesis addresses key aspects of the identification and toxicity assessment of non-intentionally added substances (NIAS) originating from polyester coatings used in food contact materials. A strategy was implemented to identify volatile, semi-volatile, and non-volatile NIAS through extraction and migration studies using a food simulant (ethanol 95%) and an extraction solvent (acetonitrile, ACN). The identified substances were analyzed using advanced analytical techniques and subsequently subjected to hazard evaluation via *in silico* predictive models. For compounds with available commercial standards, relevant *in vitro* bioassays were conducted to verify predictive alerts identified *in silico*.

Beyond the identification and chemical characterization of NIAS, the genotoxicity and carcinogenic potential of the extracts and migrates from the coatings were assessed using *in vitro* biotests to verify mixture effect.

The thesis also focuses on the gastrointestinal fate of non-volatile NIAS through a new ACN-based extraction method applied to polyester-coated steel plates. These extracts underwent simulated intestinal digestion to monitor the initial substances and their possible transformation products under physiological conditions.

Finally, a preliminary pre-validation study of a nanomethod version of the Ames test (NanoAmes—a miniaturized version of the standard regulatory Ames test) was conducted. This method was developed to detect genotoxic substances at very low concentrations, as is the case for NIAS. Reference genotoxic and non-genotoxic substances were tested, with and without metabolic activation (S9 mix), using the *Salmonella typhimurium* strains TA98 and TA100 in two laboratories (Packtox and GenEvolution). A comparative analysis of the results was carried out to evaluate the reliability, reproducibility, and potential of this biotest.

ABBREVIATION LIST

AA: Adipic acid

2AA: 2-Aminoanthracene

ACN: Acetonitrile

ADMET: Absorption, distribution, metabolism, excretion, and toxicity

AFB1: Aflatoxin B1

AhR: Aryl hydrocarbon receptor

APCI: Atmospheric pressure chemical ionization

4AP: 4-Aminophenol

AR: Androgen receptor

AU: Arbitrary units

BADGE: 2,2-Bis(4-hydroxyphenyl)propane bis(2,3-epoxypropyl) ether

BaP: Benzo-α-pyrene

BD: 1,4-Butanediol

BFDGE: Bis(hydroxyphenyl)methane bis(2,3-epoxypropyl) ethers

BHT: 2,6-Di-tert-butyl-4-methylphenol

BPA: Bisphenol A

BrdU: 5-Bromo-2'-deoxyuridine

Bw: Body weight

CAD: Charge aerosol detector

cAMP: Cyclic adenosine monophosphate

CAR-3: Constitutive androstane receptor-3

CHDM: 1,4-Bis(hydroxy-methyl) cyclohexane

CoMSAS: Complex mixture safety assessment strategy

CPA: Cyclophosphamide monohydrate

CTA: Cell transformation assay

CV: Cristal violet

cytoB: Cytochalasin B

Da: Dalton

DEG: Diethylene glycol

DDI: DNA damage–inducing

DDR: DNA damage response

DI: Drawn and ironed

DMBA: 7,12-Dimethylbenzanthracene

DMSO: Dimethyl sulfoxide

DPBS: Dulbecco's phosphate buffered saline

DRD: Drawn and redrawn

DSB: Double-strand break

DWI: Drawn and wall-ironed

E2: 17β-Estradiol

ECCS: Electrolytic chromium/chromium oxide coated steel

ECHA: European Chemicals Agency

ED: Endocrine disruptor

EFSA: European food safety authority

EG: Ethylene glycol

EI: Electron ionization

ENU: N-Nitroso-N-Nethylurea

ER: Estrogen receptor

ESI: Electrospray ionization

EtOH: Ethanol

ETP: Electrolytic tinplate

EU: European Union

EURL ECVAM: EU Reference Laboratory for Alternatives to Animal Testing

FBS: Bovine serum

FCA: Food contact article

FCM: Food contact material

GADD45a: Growth arrest and DNA damage-inducible alpha

GC: Gas chromatography

GC-HRMS: Gas chromatography coupled to high-resolution mass spectrometry

GLP: Good Laboratory Practice

HESI: Health and Environmental Sciences Institute

HD: 1,6-Hexanediol

HHA: Hexahydrophtalic acid

HPLC-DAD: High-performance liquid chromatography-diode array detector

HPTLC: High-performance thin-layer chromatography

HRMS: High-resolution mass spectrometry

hrTPO: Human recombinant TPO

IAS: Intentionally added substances

ICR: ICR 191 acridine mutagen hydrochloride

IPDI: Isophorone diisocyanate isocyanurate

iPA: Isophthalic acid

IS: Internal standard

LAGDA: Larval Amphibian Growth and Development Assay

LC: Liquid chromatography

LC-HRMS: Liquid chromatography–high-resolution mass spectrometry

LDH: Lactate dehydrogenase

LEC: Lowest effective concentration

LOD: Limit of detection

MA: Maleic acid

MCA: 3-Methylcholanthrene

mESCs: mouse embryonic stem cells

MMS: Methyl methane sulfonate

MMT: Mini Mutagenicity Test

MN: Micronuclei

MPD: 2-Methyl-1,3-propanediol

MS: Mass spectrometry

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

m/z: Mass to charge ratio

NA: Nadic acid

NADPH: Nicotinamide adenine dinucleotide phosphate

2NF: 2-Nitrofluorene

NIAS: Non-intentionally added substances

NMDR: Non-monotonic dose-response

NOGE: Novolac glycidyl ethers

NPG: Neopentyl glycol

4NQO: 4-Nitroquinoline 1-oxide

NRU: Neutral red uptake

OECD: Organization for economic co-operation and development

PA: Terephthalic acid or Isophthalic acid

Pa: Polyacids

PET: Polyethylene terephthalate

PG: Propanediol, 1,2- and 1,3-isomers

p-NPB: 4-Nitrophenyl butyrate

p-NP: p-Nitrophenol

PO: Polyols

(Q)SAR: (Quantitative) structure-activity relationships

REACH: Registration, Evaluation, Authorisation, and Restriction of Chemicals

SA: Sodium azide

SAR: Structure-activity relationships

SeA: Sebacic acid

SD: Standard deviation

SHE: Syrian hamster embryo

SHE CTA: Syrian hamster embryo cell transformation assay

SML: Specific migration limit

STTA: Stably transfected transactivation

SVHC: Substances of very high concern

T: Testosterone

T4: Thyroxine

T3: Triiodothyronine

TA: Transactivation assays

tBuPh: 4-Tert-butylphenol

TCDDM: Tricyclodecanedimethanol

TDI: Tolerable daily intake

TEG: Triethylene gycol

TG: Test guideline

TOF: Time-of-flight

tPA: Terephthalic acid

TPA: 12-O-Tetradecanoylphorbol 13-acetate

TPO: Thyroid Peroxidase

tR: Retention time

TTC: Threshold of toxicological concern

U: Units

WST-1: Water-soluble tetrazolium salt-1

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GENERAL INTRODUCTION

Imagining a modern world without food packaging is challenging, given its indispensable global significance. It plays a crucial role in providing physical and barrier protection of food, preventing it from chemical and microbial contamination, facilitating transport, averting quality decline, and enabling effective communication with consumers. Beyond these roles, packaging also contributes to marketing, security, convenience, and portion control. Moreover, it acts as a tool to extend shelf life and reduce food waste, further enhancing its utility.

Food contact materials (FCMs) are all articles and materials that are intended to come into contact with food. Food contact articles (FCAs) refer to items such as equipment, containers, packaging, and various utensils that are specifically designed or presented for use in the production, preparation, storage, transportation, or handling of food (ILSI, 2015). FCMs are not inert, for example interactions between food and FCMs can lead to migration (transfer) of chemicals from packaging into food. The migration of these chemicals may change the quality of food and/or cause human exposure to undesirable chemicals. The migration of chemicals from packaging is monitored by producers of packaging. In addition, manufacturers of food should control the absence of hazard in the food to provide the safety of consumers.

Within the European Union (EU), there are two primary regulations concerning FCMs: the framework Regulation No 1935/2004 and Commission Regulation (EC) No 2023/2006 (EU, 2023; EC, 1935). Regulation No 1935/2004 concerns 17 groups of materials and states that the manufacturing of materials and articles, including active and intelligent materials and articles intended to come into contact with food should be in compliance with good manufacturing practice (EC, 1935). It ensures that these materials must be sufficiently inert in order do not release their components into food at hazardous levels for human health nor adversely modify food composition, taste, or odor beyond acceptable limits. Commission regulation (EC) No 2023/2006 provides detailed rules on good manufacturing practice applicable across all stages of FCMs manufacturing chain (EU, 2023).

Chemicals able to migrate from packaging to food can be classified into two categories: intentionally added substances (IAS) and non-intentionally added substances (NIAS). While IAS are all chemicals included in the formulation of packaging (e.g., monomers or additives), mostly well -regulated for the plastic materials, and some of them are authorized with restrictions (e. g.,

with a defined specific migration limit (SML)). NIAS in contrast represent more often unpredictable chemicals not added intentionally in the packaging formulation but present in the final product. High proportion of chemicals such as NIAS can migrate from FCAs (Grob *et al*., 2010). Regulation EU 10/2011 (Article 3) on plastic packaging, defines NIAS as impurity in the substances used or a reaction intermediate formed during the production process or a decomposition or reaction product or contaminants (mostly due to recycled FCMs, e. g., paper-board) (EU, 2011).

NIAS are present often in trace quantities with a large scale of physico-chemical properties, a low molecular weight and can be undesirable for the consumer. Regarding plastics, Regulation EU 10/2011 which is one of the most harmonized regulation states (Article 19) that substances not listed in Annex I must undergo a risk assessment in accordance with Article 3 of Regulation (EC) No. 1935/2004 and in accordance with internationally recognized scientific principles (EU, 2011). Risk assessment of NIAS remains challenging as their origin and formation is often unknown, identification and quantification remain difficult due to the lack of analytical standards, and very often with no data regarding their toxicities (Kato and Conte-Junior, 2021).

NIAS can migrate from various types of materials used in food packaging, including coatings. Coatings are applied in the internal part of cans to prevent the interaction between metal and food which can lead to metal plates corrosion and migration of metal ions into the food. This dual function ensures the preservation of both structural integrity of packaging and food safety. Coatings are produced from varnishes, which are complex materials and vary from one manufacturer to another. It is worth mentioning that to date, there is no harmonized regulations for coatings intended to come into contact with foodstuffs. The Council of Europe has established a Resolution ResAP 1 on this topic (ResAP(2004) 1, 2009). Manufacturers are recommended to use Resolution ResAP 1 as a guideline, however, there is no obligation (ResAP(2004) 1, 2009). In addition, some countries designed their own national regulation applied to coatings.

Epoxy coatings were largely and traditionally used worldwide thanks to their technical performance and low-cost production before increased concern of scientific society about the adverse health effects of bisphenol A (BPA). At the EU level, a restriction was made in 2011 on

use of BPA in plastic infant bottles (Commission Directive, 2011). France was even stricter forbidding completely the use of BPA in all kinds of FCMs (LOI n° 2012-1442).

Regulation EU 2024/3190 extends the ban, within the EU, to BPA, its salts, other hazardous bisphenols and hazardous bisphenol derivatives in the manufacture of all FCMs and FCAs: plastics, inks, rubbers, adhesives, ion-exchange resins, silicones, varnishes and coatings. Not only packaging but also articles/professional equipment are in the scope of the ban (EU, 2024).

Furthermore, Commission Regulation (EC) No 1895/2005 states that the sum of the migrations of 2,2-bis(4-hydroxyphenyl)propane bis(2,3-epoxypropyl) ether (BADGE), BADGE.H2O, BADGE.2H2O shall not exceed 9 mg/kg (9 mg/6 dm^2) in food or food simulants and the sum of the migrations of BADGE.HCl, BADGE.2HCl, BADGE.H2O.HCl shall not exceed 1 mg/kg (1 mg/6 dm^2) in food or in food simulants. In addition, the use and/or presence of novolac glycidyl ethers (NOGE) and bis(hydroxyphenyl)methane bis(2,3-epoxypropyl)ethers (BFDGE) in the manufacture of materials and articles have been prohibited (EC, 2005).

To date there is a need to develop alternative coatings to replace epoxy-phenolic ones. Recently polyester coatings gained widespread use all over the world, however, there are few studies conducted on identified NIAS able to migrate from these types of coatings, to measure the level of exposure and to assess their risk.

Several studies showed the migration of different NIAS from polyester coatings intended to come into contact with food. For example, numerous oligoesters have been showed to migrate from polyesters coatings (Hayrapetyan *et al*., 2024; Cariou *et al*., 2022; Lestido-Cardama *et al*., 2022a; Omer *et al*., 2019). However, there is a need to perform additional studies to fill this gap of knowledge and to conclude on their safety.

This thesis work is a result of a collaboration between academia and manufacturer of food metallic packaging to provide the safety of polyester coatings. Massilly Holding, which is one of the leaders in metal packaging production, provided samples. Extractions and migration tests of metal plates and further chemical analysis were conducted in the laboratory of Food Residue and Contaminant (LABERCA, Oniris, Nantes) specialized in research activities in the field of chemical safety of food. It is a technical platform dedicated to sample preparation and analytical chemistry analysis (good laboratory practice (GLP) accreditation). The hazard evaluation of identified NIAS

from coating's extracts and migrates was conducted in the 'Center for Translational and Molecular Medicine' (CTM), INSERM-UMR1231, platform "Packtox" of the NUTox team and in the Laboratory of Genetic Toxicology of Institut Pasteur de Lille. NUTox is specialized in the FCMs safety, particularly in hazard identification/characterization of NIAS present in FCMs/FCAs extract and migrates (representing a complex mixture) using *in vitro* bioassays as a whole approach (www//packtox.fr). The Laboratory of Genetic Toxicology of Institut Pasteur de Lille is specialized to assess the genotoxic, mutagenic, and hormonal activities of many categories of substances.

Objectives

The objectives of the thesis were:

- to identify and characterize NIAS originating from polyester food-contact coating extracts and migrates;
- to assess the hazard and the risk posed by NIAS capable of migrating into food;
- to investigate their fate in the gastrointestinal tract,

in order to ensure the safety of consumers exposed *via* canned food.

The thesis is composed of several parts each dedicated to cover a specific aspect of this issue.

Part I is **the bibliographic report** (state-of-the-art) and is focused on several aspects related to FCMs. It provides a comprehensive information about the production of cans and coatings used to cover internal surface of can, migration phenomenon from food packaging, defined migration test conditions for FCMs, food packaging legislations, identification and quantification methods of NIAS, challenges associated with risk assessment of NIAS, description of NIAS from FCMs, especially from polyester coatings, etc. In addition, relevant *in silico* and *in vitro* hazard identification and characterization approaches and risk assessment methods reliable for NIAS are presented.

Part II focuses on **the research strategy** employed in this thesis, detailing the approaches and methodologies applied for this research.

Part III is dedicated to **the results** obtained in this thesis. It is composed of 6 chapters.

Chapter 1 covers *in silico* hazard assessment of NIAS (fenozan and four cyclic oligoesters) identified in polyester coatings acetonitrile (ACN) extracts and ethanol (EtOH) 95% migrates (refer to Chapter 2 and 3) highlighting the need to perform *in vitro* bioassays.

Chapter 2 is a published article based on identification of hazard linked to the migration of non-volatile NIAS from a single polyester-coated tin plate (5 batches) to canned food. In the frame of this chapter, extractions and migration tests were performed using ACN and EtOH 95% and samples were analysed by non-targeted approach using liquid chromatography–high-resolution mass spectrometry (LC-HRMS). Hazard assessment was performed for identifed NIAS (four cyclic oligoesters) able to migrate from polyester coatings. In addition to chemical analysis FCMs extracts and migrates were tested on *in vitro* Mini Mutagenicity Test (MMT) and DNA Damage Response (DDR) tests to check the absence of genotoxic effect.

Chapter 3 is another published article dedicated to the identification of volatile and semi-volatile NIAS able to migrate from polyesters coating. ACN extracts and EtOH 95% migrates were analysed using non-targeted approach on gas chromatography coupled to high-resolution mass spectrometry (GC-HRMS). Relevant *in vitro* bioassays, such as the Ames test (to test point mutation), the micronucleus assay (to detect clastogenic and aneugenic genotoxicity), and the iodide uptake assay (to check *in silico* alerts for sodium/iodide symporter (NIS1) activity) were conducted on an identified NIAS (namely fenozan).

Chapter 4 addresses potential carcinogenicity of polyester coatings ACN extracts and EtOH 95% migrates. In this chapter, samples were tested in the Cell Transformation Assay (CTA) to check their potential tumor initiating and tumor promoting activities as alerts were observed *in silico* (from Chapter 1) for some identified non-volatile NIAS (oligoesters) present in these mixtures.

Chapter 5 is focused on identifying non-volatile NIAS arising from polyester coatings and investigating their fate in the gastrointestinal tract. To study gastrointestinal fate of previously identified oligoesters (see Chapter 2) and to identify additional NIAS not detected earlier, another ACN extraction was performed on polyester coatings in order to get more concentrated extract. The ACN extracts were then subjected to simulated intestinal digestion (EFSA, 2008), and both the parent compounds and anticipated transformation products were monitored using LC-HRMS.

Chapter 6 is dedicated to a pre-validation of the NanoAmes bioassay for the detection of genotoxic chemicals present at very low levels. Bioassays were conducted by two laboratories: Packtox and GenEvolution. The initial validation process involved testing several genotoxic and non-genotoxic reference chemicals, along with their negative solvent controls, both with and without a metabolic activation system (S9 mix) using *S. typhimurium* strain TA98 and TA100. The objective was to evaluate the repeatability and reproducibility of the bioassay. The results from the two laboratories were compared to assess the relevance of the NanoAmes bioassay.

Part IV is a **discussion of the results.**

Part V is a **conclusion** about the safety of polyester coatings based on the results of this research.

Part VI presents the **perspectives** for future research.

I. BIBLIOGRAPHIC REPORT

1. The role of food packaging

The main function of food packaging is to protect the contents from physical impacts, temperature fluctuations, and other environmental factors, while also serving as a barrier against oxygen, moisture, and more. This protection helps prevent chemical and microbiological contamination, eases transportation, preserves aroma and quality, aids in containment or grouping, communicates information to consumers, and supports marketing and security (by minimizing the risk of package pilferage). It also enhances convenience and portion control (Marsh and Bugusu, 2007; Bix *et al*., 2004). Furthermore, packaging plays a key role in prolonging shelf life and reducing food waste (Licciardello, 2017).

2. Migration of chemicals from food contact materials

Manufacturers use various chemicals in the production of FCMs to achieve specific mechanical or physicochemical properties and to ensure stability. These IAS are an integral part of FCMs formulations and include components like monomers, plasticizers, and antioxidants.

In addition to IAS, FCMs may also contain NIAS, which are present in the final product but were not deliberately introduced. During the production process, pre-treatment or post-treatment of packaging, various neoformed substances with sometimes unknown origins may develop. For example, neoformed substances may form when warming up the food in the microwave. Moreover, the raw materials used in FCMs production might contain trace amounts of impurities or contaminants. Both IAS and NIAS may migrate from packaging into food or beverage resulting consumers exposure to chemicals (Figure 1-1).

By considering the chemical composition of the packaging, its processing and usage conditions, along with available literature and previous experiences, it is sometimes possible to foresee the formation of certain NIAS (ILSI, 2015). For instance, in coatings intended for food contact, formation of cyclic and linear oligoesters can be predicted (ILSI, 2015).

There are also unpredictable NIAS, whose occurrence could not have been anticipated. These NIAS are mainly linked to FCMs with highly complex compositions and contaminants that can emerge during various stages of the FCM's production and supply chain (ILSI, 2015).

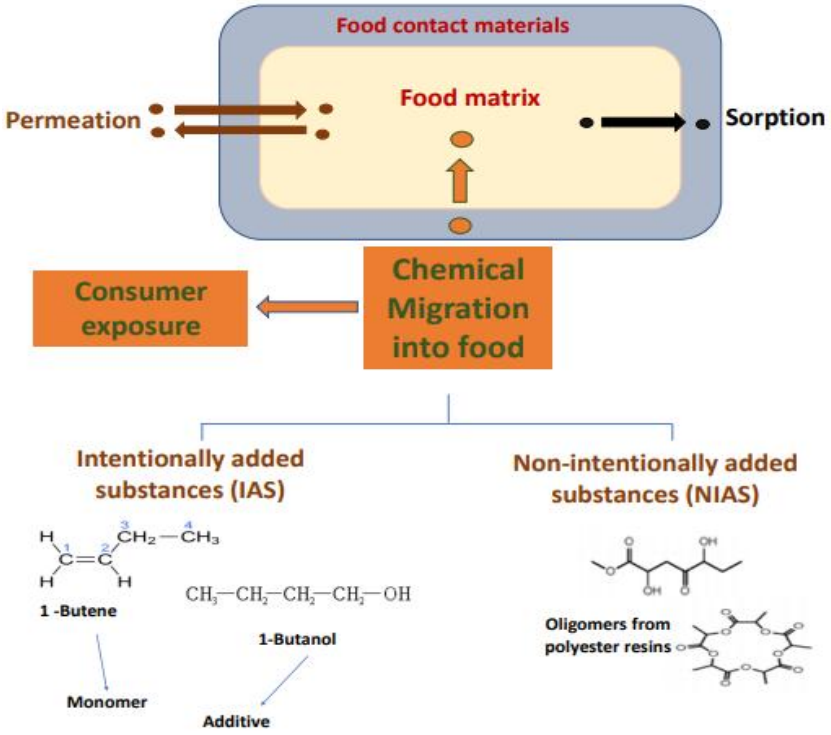


Figure 1-1. Illustration of the migration phenomenon from food contact materials (personal interpretation)

3. Legislation of food contact materials

3.1. Overview of the regulation

There are two main legislations in the EU that pertain to FCMs: Framework Regulation No 1935/2004 and Commission Regulation (EC) No 2023/2006 (EU, 2023; EC, 1935).

Framework Regulation No 1935/2004, which focuses on materials and articles intended to come into contact with food, stipulates that the manufacturing of these materials and articles, including active and intelligent materials and articles, must adhere to good manufacturing practices. The goal is to ensure that these materials and articles do not release their constituents into food at levels harmful to human health or alter food composition, taste, or odor in an unacceptable manner. The regulation encompasses 17 groups of materials that may be subject to specific measures as outlined in the framework regulation (EC, 1935).

On the other hand, Commission Regulation (EC) No 2023/2006 on good manufacturing practices provides comprehensive and detailed rules for implementing good manufacturing practices throughout all stages of the manufacturing chain for FCMs. Business operators must establish both a quality assurance system and a quality control system, in addition to maintaining proper documentation to ensure compliance with this regulation (EU, 2023). These regulations collectively aim to ensure the safety and integrity of materials and articles that come into contact with food within the EU. Certain FCMs are covered by specific legislation, for example plastic materials (Commission Regulation (EU) 10/2011), active and intelligent materials (Commission Regulation (EC) No 450/2009), recycled plastic materials (Commission Regulation (EU) 2022/1616), ceramics (Council Directive 84/500/EFC), and regenerated cellulose film (Commission Directive 2007/42/EC) (EU, 2022, 2011, 2009, 2007; Council Directive, 1984).

Apart from the aforementioned regulations, there are additional substance-specific regulations at the EU level (Figure 1-2).

For the other food packaging for which harmonized regulation does not exist the majority of EU Members States have set their own national regulations.

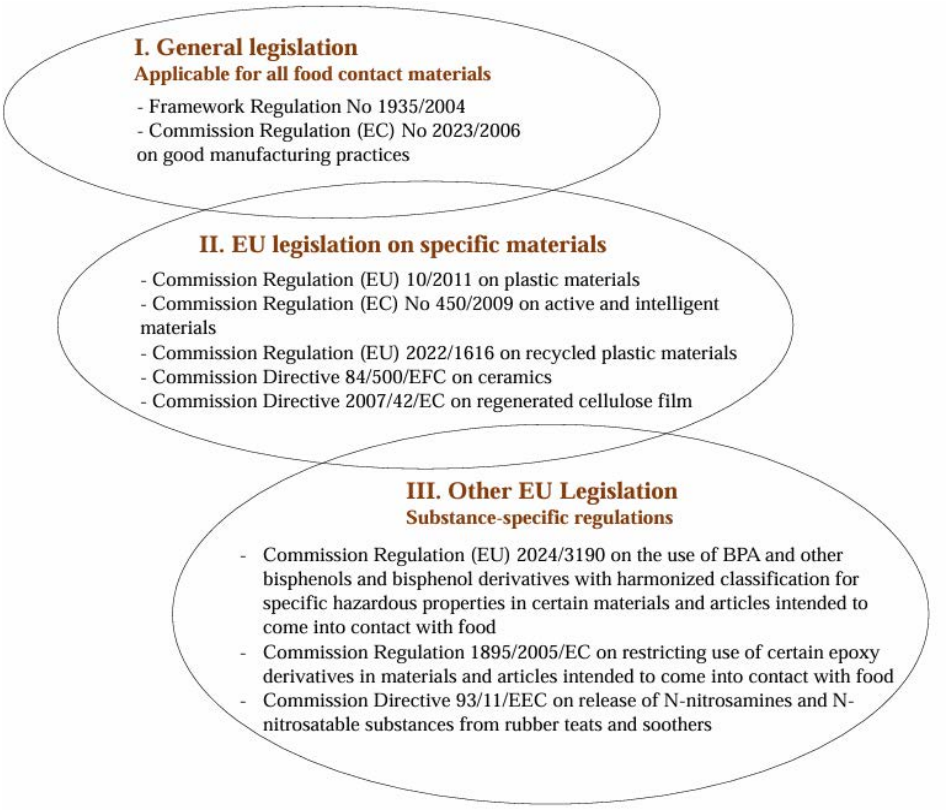


Figure 1-2. The overview of regulations (https://food.ec.europa.eu/food-safety/chemical-safety/food-contact-materials/legislation_en#II)

3.2. Regulation (EU) No 10/2011

3.2.1. Principles

Commission Regulation (EU) No 10/2011 on plastic materials and articles is one of the most important regulations for safety assessment of FCMs. It gives assignment of food simulants to foods for migration testing of materials and articles and sets outs detailed migration testing rules, such as contact time, temperature (EU, 2011). It establishes the overall migration limit of all substances migrating into food which should not exceeds 60 mg/kg food, or 10mg/dm^2 of the contact materials. This regulation also defines (Article 3)

- the 'overall migration limit' as a maximum permitted amount of non-volatile substances released from a material or article into food simulants,
- the 'SML' as the maximum permitted amount of a given substance released from a material or article into food or food simulants (EU, 2011).

This regulation provides a clear definition of NIAS (Article 3), categorizing them as impurities within the substances used, reaction intermediates formed during the production process, or products arising from decomposition or reaction. Furthermore, the risk assessment should be conducted not only on the substance itself but also on NIAS (Article 19). This classification is crucial in ensuring the safety and compliance of FCMs with established EU standards (EU, 2011). In the context of multi-layer FCMs, it is acceptable to use non-authorized substances that are non-carcinogenic, non-mutagenic, or non-reprotoxic (CMR) or in nanoform behind a functional barrier. The migration of these substances should not surpass 0.01 mg/kg (EU, 2011). This 0.01 mg/kg migration level, or a level of interest, can also be adopted for NIAS as a practical guideline for materials lacking a functional barrier.

3.2.2. Migration testing

Chemical migration from packaging to food may represents heath concern for consumers.

Several factors can greatly impact the extent and rate of migration. The most important of these include the nature of the food, the type and duration of contact, the temperature during contact, the characteristics of the packaging material, the properties of the migrating substance, and the amount of the migrates present in the packaging material (Arvanitoyannis and Kotsanopoulos, 2014). Migration can be evaluated through experimental methods or predicted using various mathematical models (refer to the Exposure Assessment of NIAS section). These models can help streamline the process, saving time and resources that might otherwise be required for extensive experimental testing (ILSI, 2023).

Commission Regulation (EU) No 10/2011 stands out as one of the most comprehensive regulations concerning plastic materials and articles intended for food contact. It specifies the conditions for migration testing and provides appropriate food simulants, contact times, and temperatures for quantifying the chemicals that migrate from plastic materials (EU, 2011).

Six different food simulants were selected based on their physico-chemical properties to imitate various types of foods and their behaviour, simulating migration from FCMs. Table 1-1 illustrates the types of food and the corresponding food simulants used in this context.

Table 1-1. Type of food simulants outlined in Regulation EU 10/2011 for different food matrixes (EU, 2011)

Food type	Food simulant	Abbreviation
Aqueous food	EtOH 10 % (v/v)	Food simulant A
Aqueous food (pH below 4.5)	Acetic acid 3 % (w/v)	Food simulant B
Low lipophilicity, alcohol content of up to 20 %	EtOH 20 % (v/v)	Food simulant C
Medium lipophilicity, oil in water emulsions, alcohol content of above 20 %	EtOH 50 % (v/v)	Food simulant D1
High lipophilicity (free fats at the surface)	Vegetable oil	Food simulant D2
Dry	poly(2,6-diphenyl-p-phenylene oxide), particle size 60-80 mesh, pore size 200 nm	Food simulant E

Regulation EU 10/2011 also precises that sample shall be placed in contact with the food simulant under the worst foreseeable conditions (time/temperature) of use. For example, if worst foreseeable condition of use is at 20 °C < T ≤ 40 °C during 5 min < t ≤ 0.5 hours. Then, migration test should be conducted at 40 °C for 0.5h (EU, 2011).

This regulation also states that to assess specific migration and overall migration, food simulants may be substituted with alternative simulants if scientific evidence demonstrates that the substitute simulants provide an overestimation of migration compared to the regulated simulants. For example, EtOH 95% can be used in some case as a substitute for the vegetable oil (D2) food simulant.

Migration testing conditions outlined in Regulation EU 10/2011 can be applied when assessing the migration from coatings intended for food contact, considering that there is no harmonized regulation specifically tailored to such materials.

4. Food metallic packaging

Metals have a wide range of applications in the food industry. They are used in the production of beer, soft drinks, food cans, drums, pails, aerosol containers, tubes, open trays, caps, closures, and lids (see Figure 1-3) (Oldring and Nehring, 2007).



Figure 1-3. Applications of metals in food industry

Metals bring several benefits to food packaging. They provide excellent barriers against light, gas, and moisture, and their heat resistance makes them ideal for withstanding the high temperatures required for sterilizing food and beverages. Additionally, the durability of metals ensures safer and more efficient transportation over long distances. Metals are also recyclable and can be easily shaped into various forms, offering diverse design and decoration options.

However, there are some disadvantages to metal packaging. Its production contributes to carbon dioxide emissions, which exacerbates global warming and depletes natural resources. Moreover, from a safety standpoint, metal packaging may pose risks by potentially releasing chemicals that could endanger consumer health (Deshwal and Panjagari, 2020).

4.1. Cans

Cans are metal containers, typically in cylindrical shapes, although they can also be manufactured in other forms, such as rectangular cans. They are utilized to preserve a wide range of products,

including beverages, food items, aerosol products, paints, medicines, and more (U.S. Environmental Protection Agency, 1998).

4.2. History of canning

Canning is a method of food preservation that involves sealing food in hermetically sealed containers and using heat treatment to eliminate microorganisms. This technique is especially important for people in areas where refrigeration is unavailable or limited. Metal cans are highly durable and protect the contents, and the practice of canning gained popularity in the 18th century when Nicholas Appert devised food preservation methods for Napoleon Bonaparte's army, winning a prize of 12,000 francs. Appert's process involved placing vegetables in hermetically sealed, thick, wide-necked bottles, then immersing them in boiling water at 100°C, which allowed the food to be preserved for several years.

It was not until 1865, about 70 years after Appert's discovery, that Louis Pasteur demonstrated the effectiveness of high temperatures in destroying microorganisms in food. Following this, Philippe de Girard and Peter Durand developed techniques for sealing food in tin cans, which were later refined by Bryan Donkin and John Hall (Vergara-Balderas, 2016).

It has been shown that in 2017, 99.7% of the French population bought at least one canned food item. Approximately 50 kg of canned food is consumed annually per household in France. Of the consumed canned food, 65% consists of vegetables, followed by fish, prepared dishes, fruits, desserts, and meat (La conserve/UPPIA, 2017).

4.3. Metals and alloys used for the production of cans

Cans are manufactured mostly from aluminum and steel, however, in some cases to give resistance against corrosion thin tin layer is applied on the surface of steel plates.

Steel

Steel is an alloy of iron containing a small percentage of carbon, typically ranging from 0.002% to 2.14% by weight. This carbon content imparts strength and fracture resistance to the iron. However, for applications in food packaging, the carbon content in steel should not exceed 1%.

In some cases, steel plates are coated on one or both sides with a thin layer of tin using a process known as electroplating, referred to as electrolytic tinplate (ETP). This tin coating provides

excellent corrosion resistance, as tin acts as an effective oxygen scavenger (Deshwal and Panjagari, 2020; Oldring and Nehring, 2007).

Tin-free steel

Tin –free steel also known as electrolytic chromium/chromium oxide coated steel (ECCS), serves as an alternative to ETP. It effectively prevents sulfur staining of steel when it comes into contact with food products and guards against corrosion. ECCS involves the addition of an extra organic coating and is typically employed for manufacturing drawn food cans, can ends, crown caps, and similar items.

Stainless steel is also utilized in food packaging applications, albeit its usage is somewhat limited due to its higher cost. It is predominantly employed for reusable containers. Stainless steel boasts exceptional corrosion resistance and chemical inertness, primarily attributable to its substantial chromium content, which is approximately 11%. When exposed to atmospheric oxygen, chromium on the steel's surface forms a protective layer of chromium oxide (Cr_2O_3), rendering it inert and shielding it from corrosion (Deshwal and Panjagari, 2020).

Aluminum

Aluminum and its highly corrosion-resistant alloys find extensive applications in the production processes of food and beverage cans. Aluminum, the most abundant element in the Earth's crust, develops a thin, colorless, durable, and non-flaking film of aluminum oxide (Al_2O_3) when exposed to air. Studies have shown that salty, acidic, or neutral aqueous food can cause deterioration of aluminum surface and thereby cause migration of aluminum into food which can have a correlation with Alzheimer's disease (Vela *et al*., 1998; Gramiccioni *et al*., 1996). For packaging purposes, aluminum is typically coated, as its reactivity can vary depending on the pH of the environment (Deshwal and Panjagari, 2020; Oldring and Nehring, 2007).

4.4. Manufacturing of cans

The manufacturing process of cans is briefly illustrated in Figure 1-4.

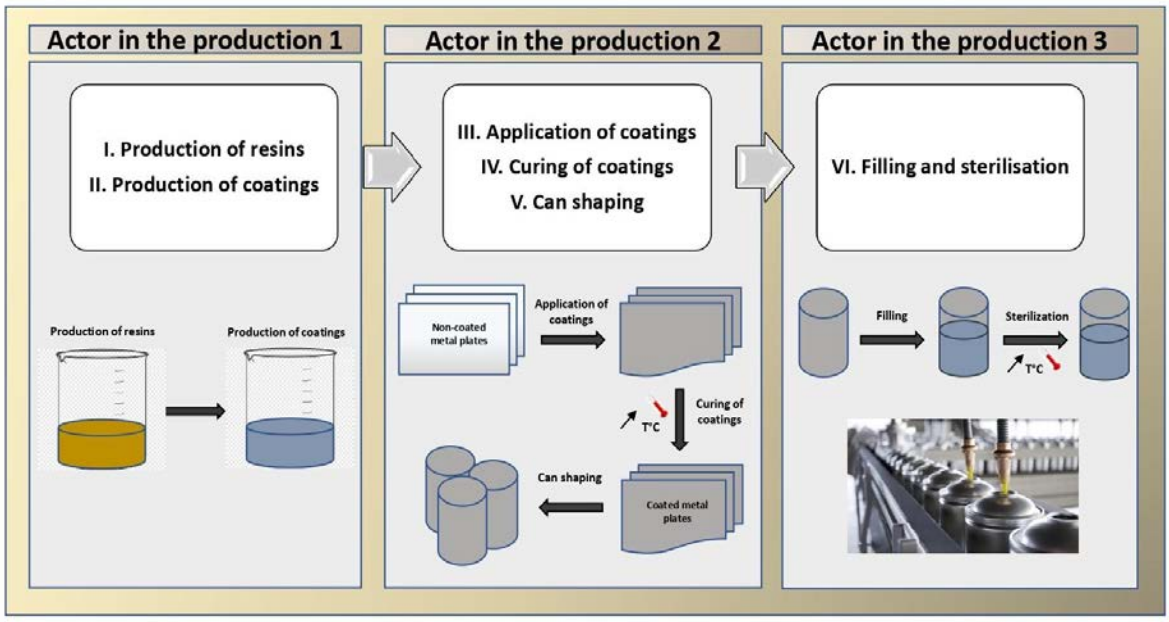


Figure 1-4. Illustration of manufacturing process of cans (personal interpretation)

4.4.1. Production of resins and coatings

The initial stage involves the production of resins, which are then transformed into coatings. Resins can exist in either solid or viscous liquid states. These complex organic compounds predominantly consist of long polymer chains formed through polymerization reactions involving monomers. These reactions occur in the presence of solvents, catalysts, and continuous agitation over a duration of 4 to 15 hours, all conducted at high temperatures (Oldring, 2001). Various polymerization techniques yield diverse resin varieties, each with its unique characteristics. These resins are subsequently employed in the formulation of coatings.

The following components predominantly make up coatings (Oldring and Nehring, 2007):

• **Resin(s):** Resin(s) serve as the primary building blocks for coatings. Depending on the specific application, various types of resins are utilized in the creation of coatings.

• **Cross-linking Agents:** These are chemical compounds that initiate reactions leading to cross-linking among polymer molecules, generating a three-dimensional network. This network contributes to the coating's strength, chemical resistance, and overall performance. Cross-linking agents are almost always included due to their pivotal role in shaping coating characteristics.

• **Additives:** Additives are incorporated into coating formulations and play a crucial role in adjusting coating properties. For instance, antioxidants might be introduced to delay oxidative processes, extending the coating's durability and shelf life. Pigments, such as titanium dioxide, can also be integrated to modify the visual attributes of coatings.

• **Solvents:** When used, solvents are responsible for dissolving resins and other constituents. This dissolution reduces the viscosity of the coating, ensuring an even application over metal surfaces. While aiding in application, solvents evaporate during the curing stage, ultimately leaving behind a solid coating film, hence they are not always present in the final coating.

4.4.2. Application of coatings and curing

Coatings are applied prior to the formation of the can bodies. Typically, coatings are administered as a wet film to both the interior and/or exterior surfaces of flat sheets using a roller or a spray. Applying interior coatings serves to protect the can from corrosion due to its contents and/or prevent the contents from potential contamination caused by dissolved metals from the can itself. Exterior coatings are employed for decorative purposes, corrosion prevention, preserving printed designs from marring or abrasions, and even reducing friction at the base of the can to facilitate easier handling (U.S. Environmental Protection Agency, 1998).

After the coating application, metal plates undergo thermal treatment called curing, which forms a dry (final) film on the metal due to the formation of a three-dimensional cross-linked network and the evaporation of solvents. Multiple internal coatings or multiple layers of the same coating may be used; however, it is essential to ensure proper curing of the previous layer before proceeding with the application of the subsequent layer.

Usually, coatings form a very thin film, typically ranging from 1 to 10 µm in thickness. The industry-defined cure schedule varies according to the specific application but consistently aims to attain the peak metal temperature which represents the critical lower limit of the metal temperature required to maintain the coating at a specified time. It is often expressed as a curing range, for example, 195–205°C for 10–12 minutes for a sheet-fed product, to ensure optimal curing. After the curing process, the coatings are cooled, and the sheets are subsequently transformed into cans through shaping processes (Oldring and Nehring, 2007).

4.4.3. Shaping of cans

Cans come in two primary configurations: two-piece cans and three-piece cans. Two-piece cans consist of a single piece that forms both the walls and the bottom, while the lid is a separate component. On the other hand, three-piece cans are constructed with three separate components: a cylindrical body, a distinct top, and a separate bottom.

4.4.3.1. Two-piece cans

Two-piece cans are commonly used to pack both food and beverages. In the production of two-piece cans, aluminum and steel are the materials of choice. The process begins with the metal blank, which is created either by unwinding a metal coil or using metal panels, and then forming a disk through stamping. Subsequently, these disks are molded into cup-shaped (‘cupped’) containers using a high-pressure press. Finally, the lid is affixed to the body.

Different manufacturing methods are employed to produce two-piece cans, depending on their intended use. Two-piece cans are categorized into four groups based on their formation methods: single drawn, drawn and redrawn (DRD), drawn and wall-ironed (DWI), and drawn and ironed (DI) (Oldring and Nehring, 2007; U.S. Environmental Protection Agency, 1998). For instance, shallow cans, such as those used for sardines and tuna, are produced through a single drawing operation, when taller cans require two or more DRD operations. These cans are referred to as DRD cans. DWI cans, primarily used for beer and beverages, undergo additional deformation of their side walls during the stretching process through dyes with decreasing diameters. This process, which reduces the can's wall thickness and elongates its walls, is also referred to as "wall ironing." Finally, for food applications, DI cans are utilized, representing another variation of DWI cans (Oldring and Nehring, 2007). Figure 1-5 shows the examples of some two-piece cans.



Figure 1-5. Examples of two-piece cans

4.4.3.2. Three-piece cans

Three-piece cans are commonly employed to pack food, juices, spices, and other products. These cans are composed of three distinct components: the bottom, body, and lid. To create the body of the can, metal sheets are cut to the appropriate size after a coating has been applied, and the sheets are rolled to form a cylindrical shape. The seam is welded, and a protective lacquer is applied to it. In certain applications where sterilization is not required, the side seam is crimped. Subsequently, the lid and bottom, equipped with a gasket to ensure a hermetic seal, are affixed to the body (Oldring and Nehring, 2007).

4.4.4. Filling and sterilization

Once the cans have been shaped according to their desired form, they are transported from supplier to food industry which fills them with food and then securely seals. The subsequent crucial step in this process is sterilization, conducted to effectively eliminate microorganisms (Oldring and Nehring, 2007). The specific parameters for sterilization can vary based on several factors, including the type of coatings applied to the cans and the particular characteristics of the packed food. This adaptable approach ensures that the sterilization process is optimally tailored to both the can coatings and the nature of the packaged food product.

The sterilization step holds significant importance in the formation of NIAS. This is because the temperature during sterilization rises above 100°C, which can lead to the degradation of chemicals present in the coating formulation.

5. Coatings

5.1. Different families of resins used to coat metallic packaging

Internal (food contact) coating provides protection of the contents from the metal and protection of the metal can from the contents of the can. In the process of identifying NIAS, gaining a thorough understanding of coating composition is a pivotal step. This comprehension enables the compilation of a list of potential NIAS that could potentially emerge from the coating materials.

Various types of resins, including epoxy-phenolic, organosol, epoxy-anhydride, polyester, oleoresinous, amino resins, and phenolic resins, are employed in food contact applications. The choice among these resins is influenced by the specific requirements of each application scenario as a single coating cannot be compatible with all types of food (Geueke, 2016; Oldring and Nehring, 2007; Simal-Gándara, 1999). The main type of resins used in food contact applications are shown in Table 1-2.

Table 1-2. Types and properties of resins used in internal can coatings (Oldring and Nehring, 2007)

	Nature	Main end-uses
Epoxy-phenolic	High molecular weight epoxy resins cross-linked with phenolic resole resins	Universal gold lacquer for three-piece cans
		Shallow drawn cans
Organosol	PVC dispersed in an appropriate varnish and conventionally stabilised with a low molecular weight epoxy resin or novolac epoxy resin.	Drawn cans
		Easy-open ends
	Epoxidised oils can also be used	Often used over epoxy phenolic basecoat
Epoxy-anhydride	High molecular weight epoxy resins cross-linked with anhydride hardeners	Internal white for three-piece cans

Epoxy-amino	High molecular weight epoxy resins cross-linked with amino resins.	Universal lacquer for beer and beverage cans (water reducible)
	Also epoxy acrylic water- based spray internals for B&B DWI	Side seam stripes Some food systems
Polyester	Polyester resins cross-linked with amino or phenolic resins. May contain lower molecular weight epoxy resin	May not be suitable for very acidic and aggressive foods
Phenolic	Phenolic resin(s), which self-crosslink (cure)	Drums and pails where flexibility is not a critical factor
Oleoresinous	Naturally occurring oils with synthetic modification	Very limited uses

Epoxy coatings have historically been utilized across the globe due to their favorable technical performance and cost-effective production. However, concerns raised by the scientific community regarding the potential adverse health effects of BPA. To date there is a need for the development of alternative coatings which can replace epoxy coatings.

5.2. Production of polyester coatings

Polyester coatings represent a more recent alternative to epoxy-phenolic coatings. They are produced through the esterification reaction between monomeric polyacids (Pa) and polyols (PO) (Oldring, 2001). An example of this esterification reaction, involving 1,4-butanediol (BD) and terephthalic acid (tPA) is illustrated in Figure 1-6.

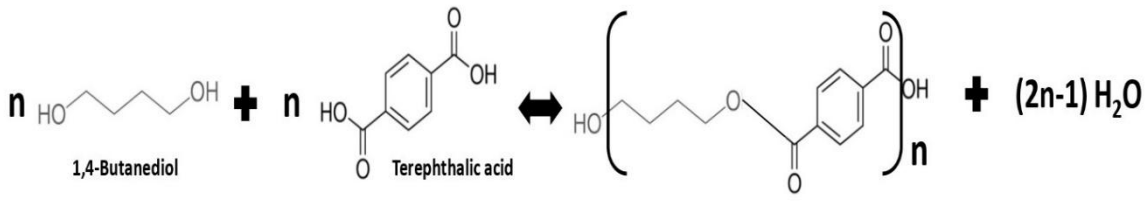


Figure 1-6. Illustration of esterification reaction between 1,4-butanediol and terephthalic acid (personal interpretation)

Numerous studies conducted on polyester coatings showed the possibilities to use several PO and Pa (Cariou *et al*., 2022; Lestido-Cardama *et al*., 2022a; Omer *et al*., 2018, 2019; Bradley *et al*., 2009; Schaefer *et al*., 2004):

- PO: ethylene glycol (EG), propanediol, 1,2- and 1,3-isomers (PG), 2-methyl-1,3-propanediol (MPD), neopentyl glycol (NPG), diethylene glycol (DEG), 1,6-hexanediol (HD), triethylene gycol (TEG), 1,4-bis(hydroxy-methyl)cyclohexane (CHDM), tricyclodecanedimethanol (TCDDM).
- Pa: sebacic acid (SeA), adipic acid (AA), orthophthalic acid, tPA, isophthalic acid (iPA), nadic acid (NA), hexahydrophtalic acid (HHA).

The objective of a polycondensation reaction is to generate a long-chain polymer that subsequently undergoes cross-linking reactions during the curing stage. These reactions are typically driven by the hydroxyl functionality of the polymer. They occur in the presence of poly-isocyanates, which serve as cross-linking agents, connecting polyester chains while forming urethane networks. Examples of cross-linking agents used in the production of polyester coatings include isophorone diisocyanate or isophorone diisocyanate trimer. To prevent premature cross-linking reactions, before applying the coatings to the metal surface and to enable the formation of a three-dimensional network only during the curing stage, several blocking agents, such as ε-caprolactam and methyl ethyl ketone oxime, are added.

These blocking agents form links with the cross-linking agents, effectively blocking their reactivity towards hydrogen. As the temperature increases during the curing stage, it triggers the evaporation of the blocking agents, liberating the cross-linking agents. These freed cross-linking agents can then interact with the hydroxylic functional groups (-OH) of the polymer chains through their isocyanate function (N=C=O) (Wicks and Wicks, 2001).

Figure 1-7 provides illustrations of PO, Pa, cross-linking agents, and blocking agents used during the production of polyester coatings.

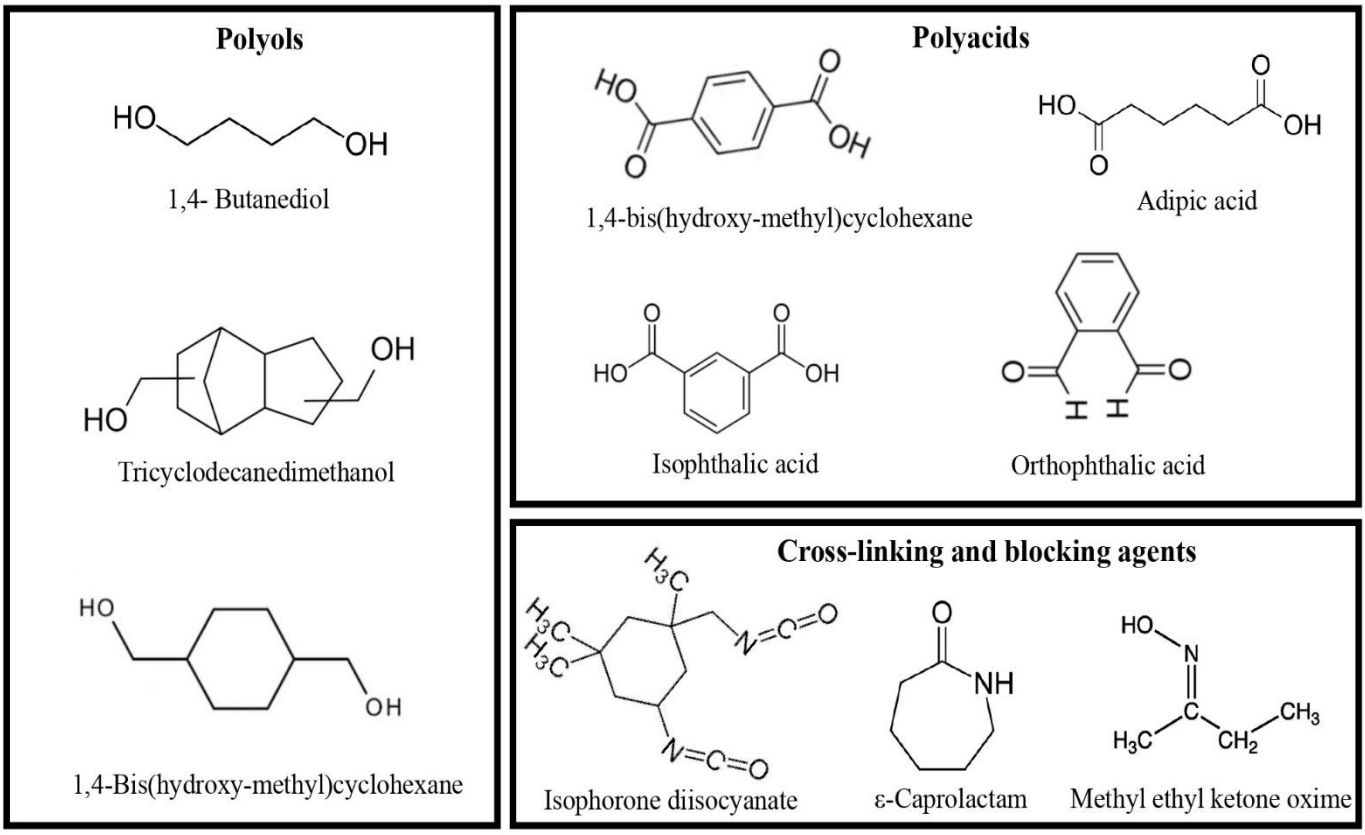


Figure 1-7. Examples of polyols, polyacids, cross-linking and blocking agents used in the polyester coating production (personal interpretation)

It is worth emphasizing that polyester coatings can also exhibit thermoplastic qualities and find application inside seam stripes (Geueke, 2016; Oldring and Nehring, 2007).

5.3. Legislation of coatings

To date, there is no harmonized regulations for coatings intended to come into contact with food. However, the Council of Europe has established a Resolution ResAP 1 on coatings intended to come into contact with foodstuffs (ResAP(2004) 1, 2009). It gives the list of authorized substances (monomers/additives) to be used in the manufacture of coatings intended to come into contact with foodstuffs. Except from these, coatings are covered under national regulations in some EU member countries. For example, in The Netherlands, Dutch Commodities Act Regulation on Packaging and Consumer Articles Coming into Contact with Foodstuffs gives the list of authorized

substances, restriction limits and other requirements for 12 types of FCMs and articles including coatings. In Spain, national regulation Real Decreto 847/2011 covers rubber, adhesives, and coatings. In Belgium, 2016 Royal Decree for Varnishes and Coatings provides definition of varnishes and coatings, as well as gives recommendation and safety rules for the production of coatings (Royal Decree for Varnishes and Coatings, 2016; Commodities Act, 2014; Real Decreto 847/2011).

5.4. NIAS from food contact coatings

5.4.1. General

Growing concerns about adverse health effects of BPA have led to increased security of BPA based epoxy coatings. In 2011, the EU restricted the use of BPA in plastic infant bottles (Commission Directive, 2011), while France implemented even stricter measure by banning BPA in all types of FCMs (LOI n° 2012-1442).

Regulation 2024/3190 bans the use of BPA, its salts, other hazardous bisphenols, and hazardous bisphenol derivatives in the production of all FCMs and FCAs in EU following a re-evaluation of BPA toxicity in 2023 by European Food Safety Authority (EFSA) which derived tolerable daily intake (TDI) of 0.2 ng/kg body weight (bw) per day for BPA, focusing on its effects on the immune system (EFSA, 2023b). This applies to plastics, inks, rubbers, adhesives, ion-exchange resins, silicones, varnishes, and coatings. The restriction covers not only packaging but also articles and professional equipment (EU, 2024).

Epoxy coatings are also associated with the migration of BADGE and its derivatives, which question the safety of these kind of coatings. Commission Regulation 1895/2005/EC on restricting use of certain epoxy derivatives in materials and articles intended to come into contact with food defines SML for BADGE and certain of its derivatives (EC, 2005). According to this regulation the sum of the migrations of the BADGE, BADGE·H2O, and BADGE·2H2O shall not exceed 9 mg/kg in food or food simulants (9 mg/6 dm^2). Also, the sum of the migrations of BADGE·HCl, BADGE·2HCl, and BADGE·H2O·HCl shall not exceed 1 mg/kg in food or in food simulants (1 mg/6 dm^2). In addition, the use and/or presence of BFDGE and NOGE in the manufacture of materials and articles are prohibited.

Some examples of NIAS migrating from different kind of coatings are shown in Table 1-3 (Lestido-Cardama *et al*., 2022b).

Table 1-3. Examples of identified NIAS from different kind of coatings (Lestido-Cardama *et al*., 2022b)

Coating type	Identified NIAS
Epoxy and epoxy-phenolic	BADGE·H2O; BADGE·2H2O; BADGE·HCl; BADGE·2HCl; BADGE·H2OHCl; BFDGE·2HCl, BFDGE·2H2O; BADGE·BuOH; BADGE·PrOH; BADGE·tBuPhe; DiBADGE; BADGE·BuEtOH/tBuPhe; BADGE·2tBuPhe; Cyclo-Di-BADGE; Tri-BADGE; BADGE·H2O·EtOEtOH; BADGE·H2O·BuOEtOH; BADGE·H2O·BuOH; BADGE·H2O·PrOH; BADGE·H2O·BPA; BADGE·2EtOEtOH; BADGE·BuOEtOH; linear or cyclic BADGE·BPA; BADGE·EtOEtOH·BuOH; BADGE·MeOEtOH·BuOH; BADGE·MeOMeEtOPrOH; BADGE·EtOEtOH·BuOEtOH; BADGE·BuOEtOH·HCl; BADGE·EtOEtOH; BADGE (n = 2)·H2O·BuOEtOH; BADGE·2BuOEtOH; BADGE (n = 2)·MeOEtOH; BADGE·BuOH·BuOEtOH; BADGE·BPA·BuOEtOH; BADGE·HexOH; BADGE (n = 2) ·2BuOEtOH; BADGE· H2O· BuEtOH; BADGE·H2O·Ph; BADGE· H2O· BPA; BADGE· Ph; BADGE· 2BPA; BADGE·BPA·BuEtOH; BADGE·BuEtOH; BADGE·2BuEtOH; BADGE·PrOH; BADGE (n = 2)·H2O; BADGE (n = 2) ·PrOH; BADGE· BuEtOH·BuOH,
Vinylic organosol	iBFDGE·MeOH; iBFDGE·HCl; BADGE· MeOH; BADGE·HCl,
Acrylic-phenolic	BADGE·2H2O

5.4.2. Specific from polyester coatings

Several linear and cyclic oligoesters have been identified from different polyester coatings. Table 1-4 summarizes NIAS identified from different polyester coatings detailing extraction or migration test conditions and analytical methods used to identify these substances.

Previous studies reported the presence of several cyclic and linear oligoesters able to migrate from food polyester can coatings. Quantification of oligoesters migrating from packaging to the food is an important step in the risk assessment of FCMs as it will allow estimating consumers' exposure level to these chemicals. The amount of migrated oligoesters in polyester-coated enamel's EtOH migrates was assessed by different groups. It has been shown that the amount of

identified oligoesters varies largely depending on the extraction solvents, food simulants used, extraction or migration test conditions, coating's formulation, as well as the type of oligoesters.

There is no toxicological data available in the literature for the majority of oligomers identified in different studies. However, the toxicity of monomer involved in the coating formulation has been evaluated. To date, no migration limits have been established in the legislation for these molecules and special attention should be paid to assess the safety of these NIAS. It is important to mention that NIAS with a molecular weight below 1000 Dalton (Da) are considered toxicologically relevant, as these molecules may be absorbed by the human body (EFSA, 2008). Additionally, EFSA states that a log $K_{O/w}$ value below 3 provides sufficient evidence of a lack of accumulation potential in the mammalian body. However, a log K_O/w value of 3 or higher does not confirm that a substance will accumulate in the body, and the absence of accumulation potential must be further evaluated (EFSA, 2008).

In the study conducted by Ubeda *et al*. (2020), the endocrine activity of two cyclic oligoesters composed by AA, DEG, and iPA (AA-DEG and AA-DEG-iPA-DEG) migrating from multilayer packaging was assessed through *in vitro* assays. The findings revealed a mild androgen receptor (AR) antagonism activity, with no observed aryl hydrocarbon receptor (AhR) activity or binding to the thyroid hormone transport protein.

Moreover, several studies have shown that cyclic oligoesters may undergo hydrolysis in the gastrointestinal tract resulting in their linear forms with probably different molecular weights, accumulation potency and toxicity (Ubeda *et al*., 2020; Eckardt *et al*., 2019).

The threshold of toxicological concern (TTC) concept can be used for food safety assessment when toxicological data are missing and if the NIAS are fully characterized in terms of their chemical structure (refer to the paragraph Threshold of Toxicological Concern approach). The TTC approach classifies substances in three Cramer classes based on their toxicity potency, however, potentially DNA-reactive mutagen and carcinogen substances, organophosphates or carbamates have specific TTC values. Furthermore, the TTC approach is not applicable to substances that fall under the exclusion categories (EFSA, 2019b; Cramer *et al*., 1976).

In most studies identified cyclic oligoesters were classified as Cramer class III (potential significant toxicity) substances and linear one as Cramer class I (low order of oral toxicity) substances (Eckardt *et al*., 2020, 2019)

Table 1-4. Examples of NIAS identified in different polyester coatings

Samples	Extraction or migration	Analytical method	Identified NIAS	Reference
Polyester coatings	ACN extraction at 40 °C for 24 h	LC/ high-resolution mass spectrometry (HRMS)	Oligoesters with cyclic (n = 65), linear (n = 18) or undetermined (n = 1) structures composed of 8 diols (EG, DEG, PD, BD, NPG, HD, CHDM and TCDDM) and 4 diacids (AA, terephthalic acid or isophthalic acid (PA), HHA and SeA)	Cariou *et al*., 2022
Polyester-polyurethane coatings	ACN extraction at 40 °C for 24 h	LC-HRMS and GC- mass spectrometry (MS)	Several cyclic and linear oligoesters based on PA, NPG, HD, DEG, EG, SeA, TEG, and caprolactam	Omer *et al*., 2019, 2018
Polyester-polyurethane coatings	ACN extraction at room temperature for 18 h	LC–time-of-flight (TOF)-MS, GC–MS	12 cyclic and 1 linear (tetramers and hexamers) oligoesters composed of PA, EG, NPG, DEG, CHDM, and PG	Bradley *et al*., 2009
Polyester coatings	ACN extraction at 40 °C for 24 h	GC-MS, high-performance liquid chromatography-diode array detector (HPLC-DAD) and MS, HPLC-DAD/charge aerosol detector (CAD), and ultra-high performance LC-tandem HRMS	22 linear and 7 cyclic oligoesters composed of EG, PA, NA, 4-tert-butylphenol (tBuPh), MPD, CHDM.	Paseiro-Cerrato *et al*., 2016a
Polyester–phenolic coatings	EtOH and ACN extraction at 60°C for 24 h, migration with water, EtOH 20% and 50% at 121°C for 1 h	RP-HPLC– DAD/MS	Several cyclic oligoesters composed of different combinations of iPA, tPA, BD, TCDDM, EG, MPD, DEG, NPG	Eckardt *et al*., 2020
Polyester-urethane and polyester-phenolic coatings	Migration test with EtOH 95% at 60°C for 4 h	HPLC-ESI-MSD/UVD and HPLC-ELSD/ UVD	cyclic oligoesters composed of dicarboxylic acid, PO, PO-1, PO-2, PO-3, PO-4, PO-5, PO-6	Schaefer *et al*., 2004
Polyester coatings	ACN extraction at room temperature for 24 h. Migration test with water, 10% aqueous EtOH (v/v) and 50% aqueous EtOH (v/v)) processed at 121°C for 1h and then stored at 40°C for 10 days.	LC-TOF-MS	Several oligomers	Driffield *et al*., 2018

Polyester coatings	Migration test with EtOH 50% at 60 °C for different times from 4 h to 30 days	UHPLC-Q-Orbitrap-MS, UFLC-MS/MS, HPLC-DAD	Two trimers, one tetramer based on iPA, MPD, and CHDM were identified	Paseiro-Cerrato *et al*., 2019a
Polyester coatings	Migration test with EtOH 50% for 10 days and EtOH 95% for 4 h both at 60 °C	HPLC-ESI MS	Linear and cyclic oligoesters were identified in two polyester-coated enamels extracts made up of iPA, HD, NPG, MPD, and tPA	Pietropaolo *et al*., 2018
Polyester coatings	Long-term (from 1 day to 515 days) migration test at 40 °C with several food simulants (water, 3% acetic acid, 10% EtOH, 50% EtOH, and isooctane) were performed	HPLC-DAD/CAD and HPLC-MS	Linear and cyclic oligoesters composed of iPA, NA, MPD, tBuPh, and COH	Paseiro-Cerrato *et al.,* 2016b

Schaefer *et al*. (2004) showed that the amount of oligoesters in EtOH 95% migrates varies from 100 to 600 µg/dm^2 (600–3600 µg/kg food) when the migration tests were carried out at 60°C for 4h. Paseiro-Cerrato *et al*. (2019a) followed the migration of three oligomers from polyester cans into EtOH 50% throughout 30 days of migration test performed at 60 °C. The migration of oligomers increased during 30 days of incubation reaching 121-271 µg/dm^2 (726-1626 µg/kg food) in the migration solvent.

Migration of polyester oligoesters into different foodstuffs was also investigated by different authors. The concentration of identified individual cyclic oligoesters in polyester–phenolic coated plate's extracts was in a range of 0.6-32.9 µg/dm^2 (3.6-197.4 µg/kg food) in infant food (Eckardt *et al*., 2020). Paseiro-Cerrato *et al*. (2019b) revealed the presence of two identified trimers with a concentration ranging from non-detect to 99.2 µg/dm^2 (595 µg/kg) in food samples (chicken noodle soup, cream of chicken, sauerkraut, mushrooms, green beans, tomatoes, pumpkin, coconut milk, chicken rice no salt) packed in polyester-coated cans. In another study, the presence of oligoesters was confirmed in 19 out of 24 foodstuffs tested. Around 1-35 oligomers were detected in different foodstuffs with an estimated concentration ranging from 3.3-157.2 µg/dm^2 (20 to 943 µg/kg food) (Driffield *et al*., 2018). Oligoesters with an average concentration of 330 µg/kg food were detected also in the drained vegetables (43–1600 µg/kg) through microwave-assisted extraction using a mixture of acetone/dichloromethane 3:7 (*v/v*, 10 mL) at 80 °C for 10 min (Cariou *et al*., 2022).

6. Chemical analysis of NIAS

6.1. Identification of non-intentionally added substances

There are two primary analytical approaches for analyzing both predicted and unpredicted NIAS.

6.1.1. Targeted Approach

The targeted approach is mainly concerned with analyzing known substances of interest. In our case, this approach is used to examine predicted NIAS. However, depending on the methodology applied, it may also detect unpredictable NIAS. This method involves using internal standards (IS) that have either identical or closely related chemical structures, often utilizing isotope-labeled compounds. The main goal is to achieve a consistent and comparable detector response between the IS and the NIAS being analyzed.

6.1.2. Non-Targeted Approach

The non-targeted approach functions as a screening method mainly aimed at detecting unpredicted NIAS. However, it can also identify predictable NIAS and IAS. This approach offers a comprehensive overview of the sample, often referred to as a "fingerprint." To ensure the detection of all potential substances—on of various analytical techniques is typically employed during the implementation of this method (ILSI, 2015).

To detect volatile compounds, the primary analytical method used is gas chromatography (GC) combined with MS. This technique identifies compounds by comparing their mass spectra with commercially available libraries. When such libraries are unavailable, HRMS can be employed to obtain accurate mass measurements and detailed full-scan spectra, aiding in the targeted identification of compounds. Additional confirmation can be achieved by closely matching retention time (tR) or Kovats index values with data from published authentic standards analyzed under similar conditions.

For the analysis of non-volatile NIAS, liquid chromatography (LC) coupled with MS is a highly effective method. However, identifying these chemicals becomes more challenging due to the limited availability of mass spectral databases. Despite this, the fragmentation patterns of the analyzed compounds play a crucial role in deducing their structural characteristics (Nerin *et al*., 2013).

Identification can be performed using library spectra or databases that contain precise masses of known FCMs substances, as well as databases listing potential NIAS originating from FCMs. When comprehensive databases are unavailable, identification can rely on analyzing fragmentation patterns and calculating molecular formulas.

To confirm the identity of the identified NIAS, analytical standards—either purchased or synthesized—can be used. However, the identification process can be complicated by factors such as the lack of appropriate databases or the unavailability of suitable analytical standards (ILSI, 2015).

6.2. Analytical prerequisites

Analyzing NIAS requires the use of techniques that are both precise and selective. Various analytical methods can be employed to detect and measure NIAS, but the predominant approach for assessing trace compounds involves the integration of chromatography with MS

detection, resulting in a two (or three)-dimensional methodology. Figure 1-8 demonstrates the underlying concept of this approach.

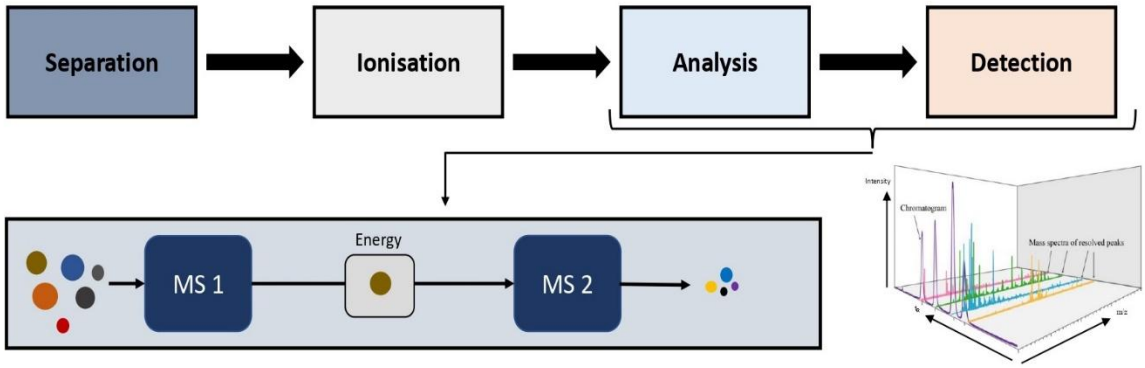


Figure 1-8. Illustration of principle of chromatography coupled with mass spectrophotometry and the principle of fragmentation technique when using two consecutive mass analyzers (MS1 and MS2) (personal interpretation)

First of all, substances are separated in the analytical column depending on their affinity for the stationary phase. The mobile phase, responsible for transporting substances through the column, can be either gaseous in the context of GC-MS or liquid for LC-MS (Guillarme and Veuthey, 2017; Sparkman *et al*., 2011). After separation, substances are ionized within the ion source. Multiple ionization modes are available, some of them are described below.

- Electrospray ionization (ESI) which mostly used when LC-MS is employed. During ESI a liquid sample is subjected to a high voltage when it emerges from a small needle resulting a fine spray of charged droplets which generates ions.
- Atmospheric pressure chemical ionization (APCI) is used in both GC-MS and LC-MS. During APCI sample is heated to form a vapor, and then it interacts with ions produced by a corona discharge to form gas-phase ions.
- Electron ionization (EI) commonly used in GC-MS where gas-phase sample molecules are bombarded with high-energy electrons, leading to the removal of electrons from the sample molecules and the consequent creation of positively charged ions.

While ESI and APCI are considered as ‘soft’ ionization techniques leading to formation of adduct, EI is ‘strong’ ionization technique leading to formation of mostly fragments. Following the ionization stage, mass analyser separates ions according to their mass to charge ratio (m/z). Various types of mass analyzers are applicable in GC-MS or LC-MS, including

Quadrupole, TOF, and Orbitrap. A majority of TOF and Orbitrap devices are categorized as HRMS with a resolution of ≥ 20,000 and mass precision of ± m/z 0.001, capable of distinguishing ions with extremely similar m/z values. The final phase involves converting the ionic signal into a three-dimensional electrical signal comprising three parameters: tR, m/z, and intensity (as illustrated in Figure 1-8).

The utilization of two consecutive mass analyzers can provide valuable insights into a chemical's structure. In such cases, the chemical is initially ionized within the source and subsequently selected based on its m/z ratio by the first analyzer. This selection is followed by fragmentation within a collision cell, where energy is supplied. The resulting fragments are then analyzed by the second mass analyzer (as depicted in Figure 1-8). This process of fragmenting molecules enables the proposal of a potential structure for the targeted molecule, a critical step in investigating unforeseen NIAS.

6.3. Sample preparation for chemical analysis regarding coatings

Sample preparation is a crucial step for the identification of NIAS, and it should be as non-selective as possible.

When investigating migration phenomena from coatings, samples are either in the form of coated metal plates (Bradley *et al*., 2009, 2008) or fully finished empty cans (Paseiro-Cerrato *et al*., 2016a; Vaclavikova *et al*., 2016). Migration tests can be carried out utilizing food simulants and adhering to the migration test conditions outlined in Regulation 11/2011 (as detailed in previous paragraph) (EU, 2011). Apart from migration tests, the preparation of samples for FCMs might involve complete material dissolution (Bignardi *et al*., 2014), as well as extraction of samples using different organic solvents, such as ACN (Paseiro-Cerrato *et al*., 2016a; Bradley *et al*., 2009).

The utilization of ACN is prevalent due to its intermediate polarity properties, enabling the extraction of a broad spectrum of chemicals on a considerable scale (Cariou *et al*., 2022; Omer *et al*., 2019, 2018; Driffield *et al*., 2018; Bradley *et al*., 2009). Nonetheless, employing this solvent tends to overestimate migration in comparison to suitable food simulants, and it does not accurately replicate realistic usage conditions. It is important to highlight that a concentration step is indispensable during sample preparation, given that NIAS are commonly present in trace quantities, necessitating concentration before analysis.

6.4. Quantification of oligomers

Quantifying NIAS poses challenges due to the lack of available analytical standards. Furthermore, the substantial quantity of NIAS typically present in FCMs makes individual quantification impractical. To tackle this issue, a semi-quantitative approach can be utilized, allowing the evaluation of numerous chemicals.

This methodology has been detailed in the literature as effective for estimating oligoesters, which are predictable NIAS arising from different coating types. In this framework, three main methods are employed to quantify oligoesters in FCMs.

The first approach entails the complete hydrolysis of the extract, which results in the breakdown of oligoesters into their individual monomers (Brenz *et al*., 2016). Following this, the monomers are measured, and by assuming equal concentrations of Pa and PO, it becomes possible to assess the amount of oligoesters present. Nonetheless, a limitation of this method is that it does not account for differences in linear and cyclic oligoesters, variations in the size of the cyclic structures, or the presence of various isomers within the oligoester composition.

An alternative method entails the use of a chemically similar compound to the target oligoester as an IS. For instance, Zhang *et al*. (2018) utilized Anthracene-d10 as an IS to achieve semi-quantification of oligoesters derived from food packaging lamination adhesives.

The final approach involves utilizing one or more specific oligoesters to quantify the target oligoesters and achieve semi-quantification of others. This can be accomplished using commercially available standards (Abe *et al*., 2016) or oligoesters obtained through organic synthesis (Ubeda *et al*., 2018; Úbeda *et al*., 2017), or alternatively, oligoesters extracted and purified from FCMs (Abe *et al.*, 2016; Kim and Lee, 2012). Evidently, this strategy stands out as the most suitable for oligoester quantification, offering the highest level of precision.

7. Risk assessment

The risk assessment process starts with problem formulation and consists of four subsequent steps: hazard identification, hazard characterization, exposure assessment, and risk characterization (WHO, 2010). Description of each step is shown in the Table 1-5.

Table 1-5. Paradigm for risk assessment, including problem formulation (WHO, 2010)

Step	*Description*	*Content*
Problem formulation	Establishes the scope and objective of the assessment	Defining the question Prior knowledge Desired outcomes
Hazard identification	Identifies the type and nature of adverse health effects	Human studies Animal-based toxicology studies In vitro toxicology studies Structure–activity studies
Hazard characterization	Qualitative or quantitative description of inherent properties of an agent having the potential to cause adverse health effects	Selection of critical data set Modes/mechanisms of action Kinetic variability Dynamic variability Dose–response for critical effect
Exposure assessment	Evaluation of concentration or amount of a particular agent that reaches a target population	Magnitude Frequency Duration Route Extent
Risk characterization	Advice for decision-making	Probability of occurrence Severity Given population Attendant uncertainties

Source: Adapted from IPCS (2009).

7.1. Hazard identification/ characterization

In *vitro* methods

Characterizing hazards using *in vivo* experiments for all NIAS is impractical and unethical. Animal models have historically been used to assess the toxicity of chemicals. However, there is a growing effort among researchers to minimize the use of animals due to ethical concerns and adherence to the 3R principles (Replacement, Reduction, and Refinement) (Directive 2010/63/EU). As an alternative to traditional animal testing, various *in vitro* bioassays have been developed to detect different toxicological endpoints, enabling the understanding of different modes of action of chemicals.

The EFSA reported that for each FCMs starting materials whatever the level of migration genotoxicity should be assessed using at least two genotoxicity tests—namely, the bacterial reverse mutation test and the *in vitro* mammalian cell micronucleus test (EFSA, 2008). Although there is no specific guidance for assessing the safety of NIAS, the approach described by EFSA for IAS can be applied also for NIAS. It is important to mention that the approach described by EFSA does not consider endocrine disrupting activity of starting materials,

however, it is important to consider this endpoint when characterizing NIAS as endocrine disruptors (ED) can act even at very low concentrations (Groh and Muncke, 2017).

In silico methods

Despite the potential of *in vitro* methods for facilitating advanced high-throughput screening of chemicals, these methods can become time-consuming and labor-intensive, particularly when dealing with a large number of substances, as is the case with NIAS from FCMs. To address these challenges, *in silico* toxicology, or computational toxicology, has emerged as a solution to prioritize substances of greatest concern. Substances showing positive alert in *in silico* tools can be tested in different *in vitro* bioassays and later *in vivo* (if needed) experiments. The following paragraphs will describe different *in silico* and *in vitro* methods used for hazard assessment of NIAS individually and FCMs mixtures as a whole approach.

7.1.1. Computational or *in silico* toxicology

In silico models, which are computer-based and grounded in diverse scientific knowledge, can only be utilized for chemicals with fully characterized structures. These tools operate on the premise that a chemical's intrinsic nature allows for the prediction of its toxicity based on its molecular structure. This approach represents a significant advancement in addressing the limitations of traditional testing methods.

These models can function as initial screening tools, helping to prioritize substances based on safety concerns and reducing the need for initial *in vitro* or *in vivo* experiments. Their purpose is to complement traditional testing methods by leveraging various computational resources. Examples include toxicity databases that store data on the physicochemical and toxicological properties of molecules, software that generates molecular descriptors, statistical modeling packages, expert systems, toxicity prediction modeling methods, and simulation and visualization tools (Raies and Bajic, 2016).

However, it is important to note that while these models provide useful predictions about a molecule's functionality, they do not offer definitive conclusions regarding its toxicological properties. Confirmation of these properties still necessitates *in vitro* and *in vivo* bioassays. *In silico* tools are not infallible and sometimes yield poor predictions for substance toxicity (Verma *et al*., 2010). The predictive performance of some (quantitative) structure-activity relationships ((Q)SAR) models was assessed for both *in vitro* and *in vivo* tests. While all models

showed statistically significant and reliable results for the Ames test—comparable to experimental variability—their accuracy for other assays or endpoints was notably lower. In addition, two Read Across methods were tested for predicting Ames mutagenicity and *in vitro* Chromosomal Aberrations. Both methods performed well for the Ames test but were notably less effective for Chromosomal Aberrations (Benigni *et al*., 2019).

Nevertheless, using *in silico* tools presents significant benefits by avoiding the need for expensive and time-consuming traditional chemical syntheses or biological assays, some of which require animal testing. In contrast, *in silico* methods allow for the rapid screening of many chemicals in a relatively short time (Verma *et al*., 2010).

Several *in silico* tools were developed, such as OECD QSAR Toolbox, Derek Nexus, Sarah Nexus VEGAHUB, ADMETlab 3.0, Danish (Q)SAR Database, Toxtree, Leadscope Model Applier etc. To give better assessment of toxicity of chemicals several *in silico* tools should be used together.

7.1.1.1. Structure–activity relationships

Structure-activity relationships (SARs) involve a qualitative correlation between a molecular (sub)structure—wherein "substructure" denotes an atom or a group of adjacently connected atoms within a molecule—and the presence or absence of a specific biological activity. This correlation results in a binary response (yes/no), indicating the confirmation or denial of projected toxicity endpoints. SARs typically rely on "expert knowledge," signifying that a particular functional group or substructure within a molecule triggers a distinct toxicological outcome.

In the prediction of toxicity using SARs, it is crucial to identify the fragments within a molecule linked to the toxic effect. Various *in silico* tools have been developed based on SARs, including those addressing mutagenicity and carcinogenicity endpoints in Derek Nexus software, DNA binding endpoints in the OECD (Q)SAR Toolbox, and mutagenicity/carcinogenicity endpoints in the CAESAR model (Schilter *et al*., 2014; Wilson, 2011).

7.1.1.2. Quantitative structure-activity relationship

QSAR are tools that establish a quantitative relationship between the chemical structures of compounds and specific properties, encompassing physicochemical, biological, and fate properties (Schilter *et al*., 2014; Wilson, 2011; ECHA, 2008). These tools are based on expert knowledge or statistical correlations to establish connections between specific toxicological effects and fragments or sub-structures present within a molecule. For instance, QSAR tools

can employ various algorithms, including linear and multiple linear regression, partial least squares for continuous endpoints, among others (Kwon *et al*., 2019). Numerous QSAR-based models have been developed to predict diverse toxicological endpoints, such as the TOPKAT (TOxicity Prediction by Komputer Assisted Technology) model, MulCASE, VISDOM, and VEGAHUB software, among others (Schilter *et al*., 2014).

OECD has established principles for the assessment of (Q)SAR predictions and results based on multiple predictions and provided checklists to perform for the assessment of models, predictions, and results from multiple predictions in practice (OECD, 2023a). Combining multiple (Q)SAR tools has been shown to enhance predictive performance (Benigni *et al*., 2019).

7.1.1.3. Grouping and read across

Grouping/read-across, when combined with (Q)SAR methodologies, proves to be a valuable approach for bridging data gaps in toxicological information. The read-across principle involves extrapolating toxicological properties or endpoints of a chemical with a well-defined structure by utilizing the well-characterized toxicological information of analogues. The similarity may be based on a common functional group (e.g., aldehyde, epoxide, ester, metal ion) or a common precursor and/or breakdown product, that results via physical or biological processes (metabolic pathway similarity). These analogues serve as references whose toxicity is thoroughly understood (Schilter *et al*., 2014; Wu *et al*., 2010; ECHA, 2008).

This method relies heavily on substantial expert knowledge and judgment. By leveraging the existing knowledge about a designated "source chemical" it becomes possible to project specific properties or toxicity onto another identified "target chemical." This practice is commonly referred to as "read across". Read-across can be qualitative or quantitative. Qualitative read-across determines if a property or activity is present or absent in the target chemical based on source chemicals, providing a simple "yes" or "no" answer. Quantitative read-across uses known property values from source chemicals to estimate unknown values for the target, often to derive endpoints like dose-response relationships (OECD, 2023a; Benigni *et al*., 2019; Schilter *et al*., 2014; ECHA, 2008).

Analog chemicals share similarities with the target chemical, forming the foundation for decision-making. However, this similarity is relative, meaning that two molecules cannot be absolutely identical but only in relation to specific quantifiable key features. The relative

nature of this approach introduces challenges in its application. In certain cases, even minor alterations in the chemical structure can result in significant changes in biological activity, a phenomenon known as the "similarity paradox" (OECD, 2023a; Benigni *et al*., 2019; Schilter *et al*., 2014; ECHA, 2008).

It is crucial to emphasize that the effectiveness of the read-across technique relies on the meticulous selection of appropriate analogs possessing reliable experimental data. However, constructing a chemical category, a larger and more robust chemical group, is not always feasible. There are instances where only a limited number of analogs can be identified, thereby reducing the method's robustness.

When conducting read-across, key factors to consider include:

- Data relevance: ensure source data is reliable and meets current standards.
- Chemical functionality: assess impacts of multi-functionality.
- Impurities: examine impurity profiles for toxicity influence.
- Property similarity: compare physico-chemical properties like solubility and molecular weight.
- Toxicokinetics: Account for potential differences in metabolism.

In contrast to SARs and QSARs, this approach is more time-consuming and demands extensive chemical and toxicological expertise (expert judgment). Nevertheless, it becomes a valuable option when scientifically valid and applicable QSARs are unavailable. It can also be used to complement the results of QSAR analysis as part of a weight-of-evidence approach (Schilter *et al*., 2014; ECHA, 2008).

7.1.2. Use of *in vitro* bioassays for hazard identification of non-intentionally added substances

In vitro bioassays play a crucial role in evaluating chemical hazards, providing significant advantages. They are fast, more cost-effective, and address ethical concerns adhering to the 3R principles. In addition, some *in vitro* bioassays allow high throughput screening (DIRECTIVE 2010/63/EU). The European chemical regulation (REACH) encourages the prioritization of *in silico* and *in vitro* approaches for chemical risk assessment, emphasizing their preference over *in vivo* methods.

In order to comprehensively evaluate various toxicological endpoints, it is recommended to use a battery of *in vitro* bioassays. Each of these assays can address different mechanisms of action of toxic substances. While these bioassays were originally designed for testing individual chemicals, they have also served as alternative methods for assessing the hazards posed by complex mixtures of environmental pollutants at low concentrations (Calow, 1998; Walker *et al*., 1996; Depledge and Fossi, 1994).

Nowadays, *in vitro* bioassays are also being employed to assess the toxicity of extracts or migrates from FCMs (Feng *et al*., 2023; Rainer *et al*., 2019; Groh and Muncke, 2017; Séverin *et al*., 2016; Wagner and Oehlmann, 2011).

Conducting *in vitro* bioassays solely on identified NIAS can offer a comprehensive understanding of a chemical's toxicity. However, obtaining pure chemicals is often not feasible due to the absence of commercial standards and the high cost of chemical synthesis. FCMs extracts and migrates are complex mixtures, typically lacking complete chemical characterization. Moreover, extensive bioassay testing for each identified NIAS can become impractical, especially when dealing with a large number of NIAS. For certain NIAS, comprehensive bioassay testing may not be necessary or desirable, particularly when they are present at very low exposure levels and are unlikely to cause adverse health effects.

Thus, assessing complex mixtures as a whole, using various *in vitro* bioassays is a valuable screening method that can provide insights into overall hazard. This approach is advantageous over analytical techniques, which may not detect all components in the mixture, and (Q)SAR methodologies, which rely on known chemical structures and cannot be applied to chemical mixtures with unknown substances. Conducting diverse *in vitro* assays on FCMs extracts and migrates can help identify potential hazards from chemicals that might not have been detected, identified, or quantified (Severin *et al*., 2017).

An integrated approach combining different analytical chemistry methods, *in silico* tools, and a battery of bioassays is advisable for a comprehensive assessment (ILSI, 2015).

If *in vitro* bioassays yield a positive response, fractionating the extracts or migrates of FCMs becomes essential. This process aims to isolate and identify the specific substances responsible for the positive outcome. Identifying these substances is crucial as it necessitates further risk assessment (ILSI, 2015).

Furthermore, subjecting complex mixtures to bioassays allows for the detection of the 'matrix' or 'cocktail' effect. Interactions among the various chemicals within the mixture can influence its overall toxicity, and this phenomenon has been well-documented previously (Muncke *et al*., 2020; Groh and Muncke, 2017; Severin *et al*., 2017). The 'matrix' effect may hide positive effects, making it challenging to assess the risks associated with these mixtures (for more details refer to next paragraph).

While *in vitro* bioassays are valuable tools for detecting hazards associated with NIAS, they have certain limitations. One significant drawback is their limited sensitivity and specificity, which can result in false positives or false negatives. Additionally, the biological sensitivity of these methods may not be sufficient to detect substances at low concentrations, such as parts per billion. This limitation is particularly relevant when identifying genotoxic chemicals, which can be harmful even at trace levels (Pinter *et al*., 2020; Schilter *et al*., 2019; ILSI, 2015). Additionally, selecting a biocompatible solvent respecting their maximum tolerable amount is crucial for ensuring the reliability of the results when using bioassays (Kourkopoulos *et al.,* 2025).

The careful selection of an appropriate biological model plays a pivotal role in ensuring the quality and reliability of the results. Using relevant *in vitro* models, such as mammalian cells, rodents, human cell lines, and bacterial/yeast cultures, is imperative. Human cell lines are preferred for chemical toxicity testing because of the differences in metabolism between human cells and those of other organisms. These models must accurately detect the biological effects of concern. The choice of a suitable model may depend on various factors, including the specific targeted endpoint, the model's origin, its metabolic capabilities, the presence of p53, and its DNA repair proficiency for genotoxicity (Kirkland *et al*., 2007).

The summary of the limitations of *in vitro* bioassays is shown in Table 1-6.

Table 1-6. Limitations of *in vitro* bioassays

Drawback	Description
Limited sensitivity and specificity	May lead to false positives or negatives.
Limited biological sensitivity	Often unable to detect substances at very low concentrations.
Solvent selection challenges	Requires biocompatible solvents within tolerable limits to avoid affecting results.
Biological model selection	Choosing an inappropriate model can compromise the quality and reliability of results.

7.1.2.1. Challenges related to testing of FCMs extracts/ migrates in *in vitro* bioassays

FCMs extracts/migrates can be considered as complex mixtures as they may contain substances with known or unknown identity/origin. Sometimes their composition is not fully defined as to date it is almost impossible to identify all compounds able to migrate from packaging to food. In general, 'forest of picks' is obtained after chemical analysis of FCMs extracts/migrates and the absence of well-established analytical standards coupled with the limited sensitivity of current analytical chemistry techniques hinder the complete identification of all substances capable of migrating from food packaging (Grob *et al*., 2010).

To ensure accurate hazard assessment of FCMs extracts and migrates using bioassays, special attention must be given to the **sample preparation** process (Groh and Muncke, 2017; Severin *et al*., 2017). Sample preparation can significantly impact bioassay outcomes. It is crucial to verify that the extraction or migration method is reliable for FCMs. Harmonizing extraction and migration methods is essential to minimize variability in results, particularly when accounting for different categories of mixtures. This is especially important given the heterogeneous nature of many extracts or migrates, such as those derived from different types of FCMs. It is crucial to **sufficiently pre-concentrate extracts or migrates** prior testing them in different bioassays as dilutions occur during testing. Furthermore, chemical loss takes place during pre-concentration step which is needed to concentrate NIAS present in FCMs in low amounts. Last but not least, selecting **biocompatible solvent or vehicle** for dissolving substances after migration or extraction is important to prevent false positive or negative results (for more details, refer to the section on "Sample Preparation for Bioassays"). To avoid the generation of 'false positive' responses (the substance is incorrectly identified as toxic when it is not), it is crucial to use appropriate concentrations in *in vitro* experiments and avoiding cytotoxic levels.

As mentioned above identifying all chemicals in FCMs extracts and migrates is a highly challenging task. Since not all NIAS can be identified, it is relevant to test the overall toxicity of FCMs extracts and migrates. If positive results are observed, the extracts and migrates can be fractionated to identify the chemicals responsible for the positive response (ILSI, 2015).

Another important factor to consider when dealing with FCMs extracts and migrates is the potential **'cocktail effect'.** This effect occurs due to interactions between substances in the mixture. Different kinds of interactions (synergism, potentiation, or antagonism) may occur

between substances present in the complex mixtures (Martin *et al*., 2021). To address data limitations, the European Chemicals Strategy for Sustainability proposed using a Mixture Assessment Factor for assessing single substances under REACH. This approach assumes that mixtures typically behave additively, with significant synergy being rare. Based on eight reviews over 30 years, synergistic effects exceeding two-fold were observed in about 5% of studied mixtures. Hence, assuming that synergistic effects in real-world mixtures are rare may be premature (Martin, 2023).

Furthermore, when testing mixture in various bioassays, quenching of positive effects of references chemicals may occur due to cumulative/matrix effect (Severin *et al*., 2017). Spiking experiments with a known positive control can be conducted to check matrix effects of mixtures. The reduction in the positive response of spiked samples of FCM extracts compared to the response of the positive control in the respective solvent is an indicator of a potential matrix effect.

Furthermore, it is essential to consider **the sensitivity and specificity of *in vitro* bioassays** to ensure their ability to detect biological effects of FCMs extracts and migrates. Determining **threshold of biological sensitivity** of bioassays is an issue that should be taken into account when dealing with FCMs extracts or migrates, as they contain NIAS at low concentration. When testing FCMs mixtures in different bioassays the question arises whether the bioassay is suitable for FCMs. For example, Rainer *et al*. (2018) showed that the Ames test can detect only a small proportion of genotoxic substances (only potent genotoxins) at concentrations of 0.01 mg/kg (10ppb) and only a small percentage (10%) of genotoxic chemicals linked to FCMs.

In addition, Pinter *et al*. (2020) studied mammalian *in vitro* genotoxicity assays for detecting DNA-damaging substances at biological detection limits aligned with safety thresholds. The results indicated significant variation in detection capabilities across substance classes. The assays were particularly ineffective at identifying most DNA-reactive, direct-acting mutagens below 10 ppb. However, their ability to detect chromosomal damage and assess other mechanisms makes them a valuable complement in a standard test battery for ensuring safety. The study concluded that improvements are needed for these assays to meet regulatory thresholds for FCMs safety evaluation.

Some research projects, such as MigraTox and Packsafe, were aimed at addressing the use of bioassays in the safety assessment of FCMs migrates and extracts.

The project's goal is to enhance the use of *in vitro* bioassays to support the safety assessment of NIAS in FCMs. **MigraTox** focuses on genotoxicity assays, addressing sample preparation, assay sensitivity, validation, and standardization. The aim was to create a workflow where these bioassays can identify and exclude substances with high toxicological potential in FCMs migrates, thereby simplifying the risk assessment of unknown NIAS. By referring to the TTC, a substance can be considered safe without identification and toxicological testing, up to a daily intake of 90 µg/person (Food Packaging Forum, 2017).

Packsafe project aims to develop a strategy for ensuring the chemical safety of FCAs. It combines analytical tools and *in vitro* studies to identify hazards within complex mixtures. The project involves extracting and chemically analyzing various FCAs to establish unique chemical signatures. In addition, these extracts will undergo *in vitro* biotests to assess genotoxicity (using Ames test, micronucleus assay, and DDR test) and also *in vitro* assays to identify endocrine disruption mode of action (using Estrogen/Androgen/Thyroid/Steroidogenesis approaches) (Chagnon *et al*., 2024).

7.1.2.2. Sample preparation for bioassays

Sample preparation for bioassays plays a crucial role in toxicity testing of FCMs. Selecting an appropriate solvent or vehicle for dissolving the substances after migration/extraction is of paramount importance, and it should be compatible with the chosen biological model.

Examples of solvent compatibility with different biological models are shown in Table 1-7.

Table 1-7. Examples of solvent compatibility with some biological models

Biological model (cell line or bacteria)	Compatible solvents	Reference
Salmonella typhimurium strain TA97a and TA98 (tested in Ames bacterial reverse mutation assay)	Dimethyl sulfoxide (DMSO), absolute EtOH, acetone and ACN	Bhadravathi Vedmaurthy *et al*. (2012)
MCF-7 human breast cancer cell line, RAW264.7 mouse macrophage cell line and human umbilical vein endothelial cells (HUVEC) (tested on multi-table tournament (MTT) colorimetric assay)	Acetone, EtOH, DMSO (at concentrations < 0.5% (v/v))	Jamalzadeh *et al*. (2016)

The use of cytotoxic solvents should be avoided, as they have the potential to produce misleading positive or negative results. It is worth noting that the concentration of the solvent in the culture medium should not exceed 1%, which is generally considered as the highest non-cytotoxic concentration for most cell lines (Kourkopoulos *et al.*, 2025; Severin *et al.*, 2017).

Therefore, **pre-concentrating the extracts or migrates** before conducting the tests is essential.

It is important to exercise caution and adhere to certain guidelines:

- Preventing the dilution of chemical concentrations before testing is essential to maintain samples that accurately represent the realistic migration of FCMs components into food and, consequently, human exposure.
- Minimizing chemical loss during the extraction process is vital.
- Checking the solution before testing
- Vigilance is necessary to avoid sample contamination stemming from laboratory equipment (Severin *et al*., 2017).

7.1.2.3. Toxicities of food contact coatings

FCMs extracts and migrates are complex mixtures containing a large number of chemicals able to migrate (Geueke, 2016). Identifying each substance and assessing its toxicity is challenging because the structure of the NIAS must be known, and a pure chemical is required to evaluate toxicity in various bioassays.

Additionally, European regulation requires the assessment of all substances migrating from packaging, including NIAS (EU, 2011). Since it is impractical to assess all NIAS migrating from FCMs, testing the migrate or extract of FCMs is an alternative approach. Extraction of FCM is regarded as a worst-case scenario compared to migration tests, as it tends to overestimate the migration of chemicals from FCMs. Toxicity assessment can be conducted using *in vitro* bioassays, as they are both rapid and cost-effective.

The genotoxicity of polyester and other type of coatings has been examined by different authors using multiple *in vitro* bioassays (Ames II-Test, Ames MPF, reversed phases-high-performance thin-layer chromatography (HPTLC)-UV/Vis/FLD-SOS-Umu-C, Ames test (OECD 471), *in vitro* micronucleus assay (OECD 487)). However, the results consistently showed no

genotoxic effects under conditions tested (refer to 'Discussion' part of Chapter 2 for detailed description of each study) (Meyer, 2020; Rainer *et al.*, 2019; Séverin *et al*., 2016; Mittag *et al*., 2006).

Similarly, negative results were obtained when testing coatings migrates in different endocrine disrupting activity biotests. For example, Séverin *et al.* (2016) conducted migration tests using food simulants on two sol-gel coatings based on organoalkoxysiloxane developed on polycarbonate. Migrates were tested on a transcriptional activation assay using transient transfection human HepG2 cells to study estrogenic activity. None of the samples were estrogenic under tested conditions (Séverin *et al*., 2016).

In another study, migrates of polymeric coatings based on tetramethyl bisphenol F was tested using E-screen assay to detect estrogen activity of migrates. Negative results were obtained when testing migrates in this bioassay (Soto *et al*., 2017).

These results suggest that, under the tested conditions, the extracts and migrates from coatings exhibited no mutagenic or endocrine-disrupting activity.

However, sample preparation is critical, and care should be taken to concentrate samples as much as possible, as dilution during bioassays is unavoidable. Additionally, the matrix effect should be checked by spiking with a known positive control, as it can potentially mask positive effects.

Although polyester coatings were found to be negative for mutagenicity and endocrine-disrupting activity, Yang *et al*. (2011) showed that several commercially used plastic materials may leach chemicals having estrogen activity. Ubeda *et al*. (2020) found that two cyclic oligoesters migrating from multilayer packaging exhibited mild AR antagonism but showed no activity on the AhR or thyroid hormone transport protein.

To date, certain chemicals used in coating production are included in the list of substances of very high concern (SVHC) which are defined under the EU REACH regulation (Registration, Evaluation, Authorisation, and Restriction of Chemicals (Regulation (EC) No 1907/2006)) as chemicals that pose significant hazards, such as carcinogenicity, mutagenicity, reproductive toxicity, persistence, bioaccumulation, or endocrine-disrupting effects. The authorisation process seeks to ensure that SVHCs are gradually substituted with safer substances or technologies whenever technically and economically feasible alternatives exist.

7.1.2.3.1. Cytotoxicity

Cytotoxicity testing is a prerequisite step to establish suitable concentration ranges and confirm that any observed effects are not simply a result of overall cell toxicity.

Before exploring various toxicological aspects like genotoxicity and endocrine-disrupting activity, it is essential to perform cytotoxicity testing, especially when inhibitory effects or antagonism are observed. This step helps to determine a concentration range where cells remain viable, preventing misinterpretation of results. High levels of cytotoxicity can lead to false positives or negatives (OECD, 2018a).

Cytotoxicity refers to the inherent toxic effects of a chemical on cells. When cells exposed to cytotoxic substances, their internal balance can be disrupted, potentially resulting in cell death through necrosis or apoptosis. Necrosis is an uncontrolled and often traumatic form of cell death marked by rapid cell swelling, loss of cell membrane integrity, halted metabolism, and the release of cellular contents into the surrounding environment. Conversely, apoptosis is a programmed form of cell death characterized by specific cellular and molecular changes, including alterations in the refractive index, cytoplasmic shrinkage, nuclear condensation, and the segmentation of DNA into uniformly sized pieces (Figure 1-9) (Prasad *et al*., 2020; Çelik, 2018).

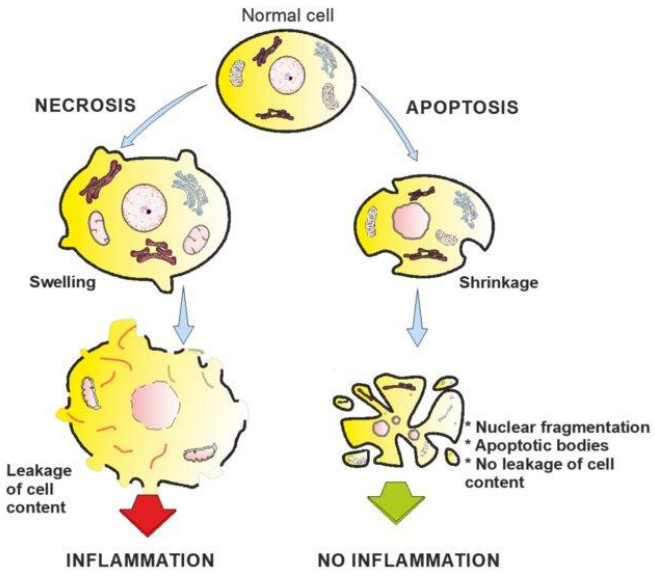


Figure 1-9. Illustration of processes occurring during necrosis and apoptosis (Prasad *et al*., 2020)

Numerous cytotoxicity assessment methods have been developed over time, each focusing on distinct indicators of cytotoxic effects, such as cell membrane permeability, dye absorption, metabolic functions, enzyme release, cell adherence, ATP generation, co-enzyme production, DNA synthesis, and nucleotide uptake (Adan *et al*., 2016). Among the commonly employed cytotoxicity tests, assays based on the breakdown of the cellular permeability are prevalent. Some of these assays involve staining cells that have been exposed to mixtures of FCMs with various dyes, such as trypan blue, neutral red, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), water soluble tetrazolium salt 1, and resazurin. These dyes have the capability to incorporate into either living or dead cells or specific organelles depending on the particular dye used, facilitating the easy detection of cell viability (Groh and Muncke, 2017; Severin *et al*., 2017).

Additionally, there are other bioassays that specifically target the disruption of cell permeability. These assays aim to quantify the release of intracellular "marker" components into the extracellular environment. For instance, enzyme release assays, such as lactate dehydrogenase (LDH). In normal circumstances, LDH enzyme is typically confined within the cell's cytosol. However, when exposed to cytotoxic substances, the integrity of the cellular plasma membrane may be compromised, leading to the release of LDH into the cell culture medium (Adan *et al*., 2016).

Another frequently used bioassay that has been used for FCMs relies on the kinetics of RNA synthesis, known as the "RNA synthesis inhibition test". This technique measures the kinetic of RNA synthesis through the integration of tritiated uridine into cellular RNA after cell treatment (Groh and Muncke, 2017; Valentin-Severin *et al.,* 2002; Valentin *et al.,* 2001; Fauris *et al*., 1985).

Furthermore, techniques based on DNA synthesis or cell proliferation markers have also gained substantial utilization nowadays (Adan *et al.*, 2016).

This European Standard EN 15845:2010 outlines a lab method for evaluating the potential cytotoxicity of paper and board materials using HeLa S3 human cells, it assesses wet contact with food simulant (CEN EN 15845:2010). Standard EN 16418:2014 assesses potential cytotoxicity of paper and board materials using HepG2 cells, which better mimic human oral exposure to xenobiotics due to their metabolic enzyme activity (phases I, II, and III) (CEN EN 16418:2014).

Cytotoxicity of the overall extracts or migrates of FCMs/FCAs was assessed with several *in vitro* bioassays, however, RNA synthesis inhibition assay, MTT assay, neutral red uptake (NRU) assay, *Vibrio fischeri* bioluminescence inhibition or Microtox®, total protein content assay were the most used bioassays for FCMs. Cytotoxicity was detected in various extracts from different FCMs, including paper/paperboard, plastics, and coatings (Groh and Muncke, 2017). Regarding coatings, cytotoxicity was also reported in the literature, for example Mittag *et al*. (2006) evaluated cytotoxicity of EtOH extracts of epoxy and polyester coatings using NRU assay, water-soluble tetrazolium salt-1 (WST-1) assay, 5-bromo-2'-deoxyuridine (BrdU) DNA incorporation assays, and RNA synthesis inhibition assay.

Results from the BrdU assay conducted on HeLa-S3 cells indicated no significant impact of extracts on cell proliferation. The NRU assay performed on HepG2 cells and two colorectal adenocarcinoma cell lines (Caco-2 and HT-29), revealed that both coating extracts were cytotoxic in Caco-2 cells, but only the epoxy extract exhibited significant cytotoxicity in HepG2 and HT-29 cells. Variations in cytotoxicity may arise from the cell lines used and/or due to the intrinsic metabolic activities. In the WST-1 assay conducted on HeLa-S3 cells, both extracts were cytotoxic, with the epoxy coating showing a stronger effect than polyester. However, in RNA synthesis inhibition assay conducted on HeLa-S3 cells, the polyester coating demonstrated greater cytoxicity compared to the epoxy coating (Mittag *et al*., 2006).

7.1.2.3.2. Carcinogenesis

7.1.2.3.2.1. Overview

Genetic changes can interfere with cellular functions, potentially resulting in cancer. Genotoxic substances are considered potential carcinogens due to their ability to initiate cancer. Carcinogenicity refers to a substance's capacity to cause cancer, with some carcinogens causing genetic damage that leads to mutations and uncontrolled cell growth. However, not all genotoxic agents necessarily cause cancer, and some carcinogens can promote cancer through non-genetic damage mechanisms (Koya and Ibrahim, 2025; Tarin, 2023; Baba and Câtoi, 2007).

Key characteristics of carcinogens are shown in the Table 1-8.

Table 1-8. Key characteristics of carcinogens (Smith *et al.*, 2016)

Characteristic	Examples of relevant evidence
1. Is electrophilic or can be metabolically activated	Parent compound or metabolite with an electrophilic structure (e.g., epoxide, quinone), formation of DNA and protein adducts
2. Is genotoxic	DNA damage (DNA strand breaks, DNA–protein cross-links, unscheduled DNA synthesis), intercalation, gene mutations, cytogenetic changes (e.g., chromosome aberrations, micronuclei)
3. Alters DNA repair or causes genomic instability	Alterations of DNA replication or repair (e.g., topoisomerase II, base-excision or double-strand break repair)
4. Induces epigenetic alterations	DNA methylation, histone modification, microRNA expression
5. Induces oxidative stress	Oxygen radicals, oxidative stress, oxidative damage to macromolecules (e.g., DNA, lipids)
6. Induces chronic inflammation	Elevated white blood cells, myeloperoxidase activity, altered cytokine and/or chemokine production
7. Is immunosuppressive	Decreased immunosurveillance, immune system dysfunction
8. Modulates receptor-mediated effects	Receptor in/activation (e.g., ER, PPAR, AhR) or modulation of endogenous ligands (including hormones)
9. Causes immortalization	Inhibition of senescence, cell transformation
10. Alters cell proliferation, cell death or nutrient supply	Increased proliferation, decreased apoptosis, changes in growth factors, energetics and signaling pathways related to cellular replication or cell cycle control, angiogenesis

Abbreviations: AhR, aryl hydrocarbon receptor; ER, estrogen receptor; PPAR, peroxisome proliferator–activated receptor. Any of the 10 characteristics in this table could interact with any other (e.g., oxidative stress, DNA damage, and chronic inflammation), which when combined provides stronger evidence for a cancer mechanism than would oxidative stress alone.

Carcinogenesis is a complex process involving genetic and epigenetic changes, often following the multistage theory (Figure 1-10) (Koya and Ibrahim, 2025; Tarin, 2023; Baba and Câtoi, 2007).

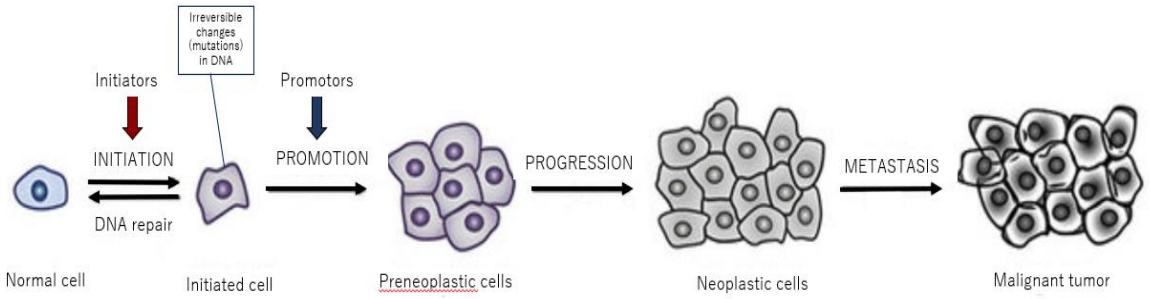


Figure 1-10. Illustration of different stages of carcinogenesis (modified picture from https://graciekruwrusso.blogspot.com/2022/08/carcinogenesis-initiation-promotion.html)

The mechanisms of carcinogenesis can be broadly categorized into the following key steps:

I-Initiation: This step is a key step where exposure to carcinogens causes mutations that disrupt cell cycle regulation and DNA repair. This process results in uncontrolled cell growth, evasion of apoptosis, and the formation of cancerous cell clones with a selective advantage (Koya and Ibrahim, 2025; Saria, 2016; Baba and Câtoi, 2007). Most carcinogens are genotoxic chemicals able to cause direct mutations, however, not all carcinogens are genotoxic chemicals. It is estimated that 10–20% of known human carcinogens classified as Class I by the IARC act through non-genotoxic mechanisms (Hernández *et al*., 2009).

Non-genotoxic carcinogens indirectly cause mutations without directly harming DNA. They operate through various pathways, such as inducing oxidative stress, altering epigenetic regulation, inhibiting DNA repair, and affecting enzyme activity. Additional mechanisms include modulating hormonal signaling through receptors or other methods, promoting tumor growth, causing tissue-specific toxicity, triggering inflammatory responses, suppressing the immune system, and disrupting intercellular communication *via* gap junctions. Understanding these mechanisms is crucial for accurately assessing the risks of exposure to non-genotoxic mutagens and implementing strategies to minimize cancer risk (Stewart, 2019; Hernández *et al*., 2009; Kirsch-Volders *et al*., 2003).

II-Promotion: After initiation, the promotion stage involves the uncontrolled growth and survival of initiated cells, leading to the formation of pre-neoplastic lesions (Koya and Ibrahim, 2025; Saria, 2016; Baba and Câtoi, 2007). Tumor promoters are substances that, although they do not directly cause mutations (non-genotoxic), facilitate the growth of initiated cells, thus aiding in tumor development. Hormonal promoters mimic or disrupt hormone actions by activating receptors or engaging with signaling pathways. In contrast, non-hormonal promoters stimulate cell growth independently, often through mechanisms like the activation of protein kinase C (Fujiki *et al*., 2013; Boyland, 1985, 1985; Slaga, 1983). Ohmori and Kawamura (2009) used CTA to study the initiation and promotion potential of abietic and dehydroabietic acids from virgin and recycled paper products for food contact. While no initiation potential was observed, both compounds showed dose-dependent tumour-promoting activity at 20–60 nmol/ml.

III-Progression: Progression is marked by increased malignancy, genetic and karyotype alterations, and a tumor phenotype characterized by rapid growth, invasion, metastasis, and biochemical and morphological changes. Tumor metastasis occurs when cancer cells lose

their adhesiveness, enabling them to detach and spread to other tissues through blood and lymphatic vessels (Koya and Ibrahim, 2025; Saria, 2016; Baba and Câtoi, 2007).

IV -Invasion:

Invasion represents a crucial stage in cancer development where malignant cells penetrate the basement membrane and infiltrate surrounding tissues, enabling metastasis. This process involves multiple coordinated mechanisms: loss of cell adhesion molecules (e.g., E-cadherin) promotes tumor cell detachment; secretion of proteases (MMPs) degrades the extracellular matrix; epithelial-mesenchymal transition enhances cell motility; and stromal cell interactions remodel the tumor microenvironment. These changes collectively facilitate cancer spread and are key to tumor aggressiveness (Hanahan and Weinberg, 2011).

7.1.2.3.2.2. Cell Transformation Assay (CTA), *in vitro* bioassay

While the regulatory bioassays discussed earlier are effective and widely acknowledged for evaluating the genotoxicity of substances, they have limitations in detecting all genotoxic carcinogens and are unable to identify non-genotoxic carcinogens. To date, only a few *in vitro* bioassays are available for identifying potential carcinogenic chemicals.

In response to this challenge, the CTA has been developed to predict the potential carcinogenic properties of chemicals detecting tumor initiating and tumor promoting mode of actions. In contrast to *in vivo* carcinogenicity tests, which involve a substantial number of animals, histopathological examinations of various tissues, and high costs (OECD, 2018b), CTA is more affordable, faster, and ethically preferable alternative method for detection of a specific mode of action of potential carcinogenic chemicals. CTA is validated by the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) but is not currently used for regulatory purposes.

CTA distinguishes by its ability to identify both genotoxic (tumor-initiators) and non-genotoxic carcinogens (tumor promoters). It relies on two primary types of cells: normal diploid SHE cells and immortalized aneuploid mouse cell lines such as BALB/c 3T3, C3H/10T1/2, and Bhas 42 cells. By utilizing these cell lines, CTA becomes a valuable tool in assessing the carcinogenic potential of various substances. CTA measures the morphological transformation of cells, observed as transformed colonies in SHE cells and as foci in C3H/10T1/2, BALB/c 3T3, and Bhas 42 cells derived from a single cell (OECD, 2007).

To the best of our knowledge, CTA has never been used to test the potential carcinogenic properties of FCMs extracts or migrates. Only to assess the potential carcinogenicity of nanoparticles (Hayrapetyan *et al.*, 2023).

7.1.2.3.2.2.1. The Syrian hamster embryo cell transformation assay

The Syrian hamster embryo cell transformation assay (SHE CTA) presents numerous advantages over alternative CTAs, as emphasized by Ahmadzai *et al*. (2012). Notably, SHE CTA stands out as the only *in vitro* assay capable of modeling multistage carcinogenesis, replicating the progression from a normal diploid cell to a malignant one. This characteristic distinguishes it from CTAs utilizing aneuploid cells, which fail to accurately mimic the *in vivo* carcinogenic process. Another advantage is that SHE cells can metabolically activate carcinogens to their ultimate carcinogenic form without requiring an external activation system. Additionally, SHE cells are p53-competent primary cells.

The heterogeneous nature of SHE cell populations is a pivotal advantage, providing a diverse range of cellular targets that facilitate the detection of various carcinogens, encompassing both genotoxic and non-genotoxic types. This heterogeneity contributes to the assay's high sensitivity and specificity (92% sensitivity and 66% specificity at pH 7) (OECD, 2007). Nevertheless, it also introduces variations in sensitivity, necessitating consideration when interpreting the results (Ahmadzai *et al*., 2012).

Furthermore, SHE cells offer genetic stability and display a lower rate of spontaneous transformation, resembling human cells more closely than mouse cells, rendering them a pertinent model for studying carcinogenesis.

Nevertheless, the SHE CTA comes with certain limitations. The protocol proves time-consuming and labor-intensive, demanding large sample sizes due to the relatively low frequency of induced morphological transformation. The heterogeneity within the cell population can result in sensitivity variations when different aliquots of cells from the same culture are utilized. Furthermore, the subjective nature of scoring morphologically transformed colonies poses challenges to the method's universality (Ahmadzai *et al*., 2012).

In the course of the SHE CTA process, cells undergo treatment with tested chemicals for seven days. Subsequent to fixation and staining, the cells are visually examined for morphological transformation features and subsequently scored. Morphologically transformed SHE cells display distinct characteristics, such as a high nuclear-cytoplasmic ratio and criss-crossed,

randomly oriented, three-dimensional colony growth. These transformed cells are more prone to genetic alterations, ultimately resulting in the development of immortal cell lines (OECD, 2015 TG N° 214).

7.1.2.3.2.2.2. Bhas 42 CTA

CTA based on BALB/c 3T3 and C3H 10T1/2 cells simulate the initiation and promotion stages of the two-stage carcinogenicity process but a pre-treatment with tumor initiators is necessary to detect compounds that have tumor promotor activity (Sakai *et al*., 2010).

Sasaki *et al*. (1988) reported that BHAS 42 cells, which are derived from the transfection of BALB/c 3T3 cells with the v-Ha-ras gene, demonstrated a higher transformation frequency after exposure to the tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA). This observation indicates that Bhas 42 cells are already initiated due to the presence of the ras oncogene. Similar to BALB/c 3T3 and C3H 10T1/2 cells, Bhas 42 cells are capable of detecting both tumor-initiating and tumor-promoting activities of chemicals. However, they do not necessitate prior treatment with tumor initiators to predict the tumor-promotion activities of substances (Sasaki *et al*., 1988).

The Bhas 42 CTA, introduced by Asada *et al.* (2005), has the capability to identify Ames-negative and Ames-discordant carcinogens, primarily due to its promotion assay component. In an assessment involving 98 chemicals, the Bhas 42 assay demonstrated a concordance of 78%, with a sensitivity of 73% and specificity of 84%. The positive predictivity was found to be 86%, while the negative predictivity was 69%. Notably, the false negative rate was 27%, and the false positive rate was 16% (Sakai *et al*., 2010).

To differentiate between initiation and promotion, the initiation assay entails subjecting low-density cells, obtained one day after plating, to a 3-day treatment with a test chemical. On the other hand, the promotion assay involves treating near-confluent cells with the test chemical for a duration of 10 days (day 4–14). Following exposure to chemicals, the recording of morphologically transformed foci derived from a single cell is conducted. However, the scoring of these morphologically transformed foci remains subjective. Several typical morphological characteristics, such as spindle-shaped cells, deep basophilic staining, criss-cross patterns, piling up, and invasive growth into the monolayer of surrounding contact-inhibited cells, are observed in transformed foci. However, it should be noted that not all

transformed foci exhibit the same characteristics. The Bhas CTA was developed in both 6 and 96-well formats (OECD, 2017a).

As no studies used CTA to assess the potential carcinogenicity of FCMs extracts and migrates, the suitability of this bioassay for such complex mixtures must be investigated. Additionally, a crucial consideration is the significant dilution that occurs during testing, which poses a challenge in detecting the carcinogenic potential of NIAS present in low quantities in FCMs extracts and migrates. This underscores the importance of a pre-concentration step (applicable for all bioassays) during sample preparation.

7.1.2.3.3. DNA damage

In toxicology, it is crucial to distinguish between terms "genotoxicity" and "mutagenicity." Genotoxicity is a broad concept that describes a chemical's ability to harm a cell's genetic material. This damage can be reversible and might not always lead to permanent changes in the genetic information.

There are several DNA repair pathways in human cells, including direct reversal, base excision repair, nucleotide excision repair, mismatch repair, single-strand break repair, and double-strand break (DSB) repair. Direct reversal typically addresses pyrimidine dimers caused by UV radiation or alkylated bases. Nucleotide excision repair targets bulky adducts or DNA replication lesions that cause structural distortions in the DNA. Mismatch repair corrects mismatched base pairs in double-stranded DNA and some insertions or deletions of fewer than four nucleotides. DSB repair focuses on repairing DSB lesions (Huang and Zhou, 2021). To response to genotoxic stress, mammalian cells initiate additional mechanisms beyond DNA repair systems. These include cell cycle regulation, which allows extra time for DNA repair processes or activates apoptosis, a programmed cell death pathway that removes cells with extensive damage (Figure 1-11) (Hartwig *et al*., 2020).

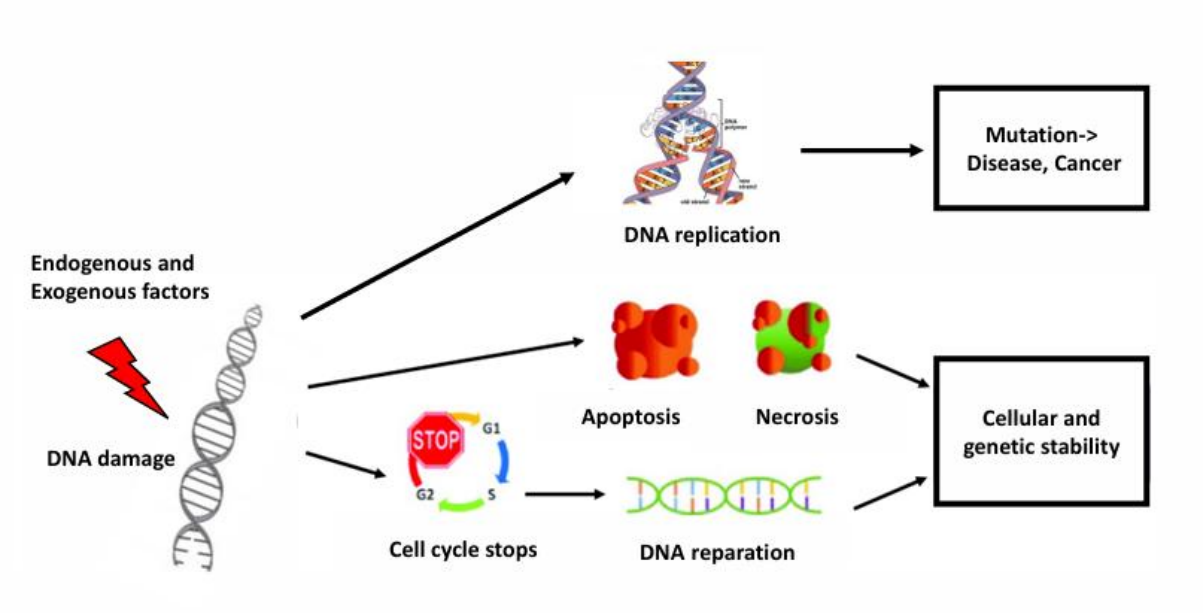


Figure 1-11. Cellular response pathways following a DNA damage (personal interpretation)

Mutagenicity, on the other hand, refers to irreversible changes in the genetic material (alterations in DNA or chromosomal structure or number) and the first step of carcinogenesis. These changes can be passed on to future cell generations if they are in germ cell and not fatal to the affected cells (OECD, 2017b).

7.1.2.3.3.1. Health effects of genotoxic chemicals

It is important to study genotoxicity of chemicals present in food as they may be associated with several health effects. Genotoxicity testing is a prerequisite step for risk assessment and requirement before authorisation of additives, flavourings, processing aids used in food applications, and FCMs additives, as these substances can cause harmful effects even at low exposure levels and may lack a safe threshold.

It is well known that somatic gene mutations are associated with several human diseases (e.g., neurofibromatosis 1 and 2, McCune-Albright syndrome, incontinentia pigmenti in males etc) (Erickson, 2010). Accumulation of DNA damage in somatic cells and organs is linked with cancer and some degenerative conditions, for example gradual functional decline and aging (Hoeijmakers, 2009) or cardiovascular diseases (De Flora and Izzotti, 2007). It has been shown that early-life somatic mutations that occur during development may cause development of some neurodegenerative diseases in later life (Frank, 2010). In addition, primary

immunodeficiencies were associated with DNA-repair disorders (EFSA, 2011; Slatter and Gennery, 2010).

Mutations in proto-oncogenes, tumor suppressor genes, and DDR genes can potentially lead to cancer. Proto-oncogenes and tumor suppressor genes are key regulators in various biological processes, particularly in controlling cell proliferation and differentiation in normal cells. When proto-oncogenes mutate, they can become oncogenes, which drive cancer development by promoting cell division. Additionally, mutations can inactivate tumor suppressor genes, which normally function as safeguards against cancer, leading to uncontrolled cell proliferation and ultimately, cancer (Kontomanolis *et al*., 2020).

Lastly, mutations in germ cells may result in infertility, difficulties during pregnancy, and hereditary diseases in the progeny (EFSA, 2011).

7.1.2.3.3.2. Genotoxicity bioassays

To assess the genotoxicity of chemicals all modes of action of genotoxic chemicals should be taken into account including DNA damage, and structural and numerical chromosomal alterations (WHO, 2022). A battery of several *in vitro* bioassays is then recommended by regulatory authorities for adequate assessment of all genotoxicity endpoints (EFSA, 2011). Commonly used *in vitro* bioassays for genotoxicity testing of FCMs are shown in Table 1-9.

Table 1-9. Regulatory bioassays for evaluating the genotoxic potential of FCMs (EFSA, 2011)

Endpoints detected	Bioassays	Relevant OECD Test Guideline (TG)
Detecting gene (point) mutation	Bacterial reverse mutation test in *Salmonella typhimurium* and *Escherichia coli*	OECD TG 471
Detecting chromosome aberrations	*In vitro* mammalian cell micronucleus test	OECD TG 487

In vivo bioassays are recommended when *in vitro* tests yield positive results (EFSA, 2011).

7.1.2.3.3.3. Bacterial Reverse Mutation or Ames test (OECD TG 471)

Bacterial reverse mutation or Ames test developed in 1975 by Ames *et al.* is one of the most used *in vitro* mutagenicity tests (Ames *et al*., 1975). The Ames test uses several histidine-dependent *Salmonella typhimurium* (TA1535, TA1537, TA97a, TA97, TA98, TA100, TA102) and

tryptophan-dependent *Escherichia coli* (WP2 uvrA, or WP2 uvrA (pKM101)) strains to detect point mutation (OECD, 2020).

These strains have different mutations in histidine or in tryptophan operon which makes them unable to synthesize these essential amino acids and inhibit their growth in the absence of them. When bacteria exposed to mutagenic chemicals, reverse mutation may occur in these operons leading to histidine or tryptophan independent strains and strains restore the ability to synthesize these amino acids and grow in the absence of them (OECD, 2020).

It is recommended to use a combination of four strains of *S. typhimurium* (TA1535; TA1537 or TA97a or TA97; TA98; and TA100) together with *E.coli* WP2 or *S. typhimurium* TA102 strains. *S. typhimurium* strains have GC base pairs at the primary reversion site and unable to detect certain oxidizing mutagens, cross-linking agents, and hydrazines. On the contrary, *E.coli* WP2 strains or *S. typhimurium* TA102 strains have an AT base pair at the primary reversion site and are able to detect these chemicals. Moreover, to increase sensitivity of these strains to genotoxic chemicals, strains possess additional mutations. For example, to increase cellular permeability for large molecules, some strains possess alteration of the lipo-polysaccharide part from the outer membrane of the bacteria (rfa mutation). Table 1-10 represents the types of mutations detected by OECD strains.

Table 1-10. The types of mutations detected by OECD strains

Strain	Type of detected mutation
S. typhimurium TA98	Frameshift
S. typhimurium TA100	Base pair substitution
S. typhimurium TA1535	Base pair substitution
S. typhimurium TA1537	Frameshift
E.coli WP2 uvrA (pKM101)	Base pair substitution

For general screening purposes, *S. typhimurium* TA98 and TA100 strains with and without metabolic activation can be used as it has been shown that these two strains together are capable of detecting up to 89% of mutagens because of the ability to detect frameshift mutations in case of TA98 and base substitutions in case of TA100 (Zeiger *et al.*, 1985).

Ames test has been used with different substance categories, such as food additives, FCMs extracts or migrates, contaminants (Landsiedel *et al.*, 2022; Rainer *et al.,* 2018; Olusegun and

Olajire, 2015; De Flora *et al.*, 1984; McCann *et al.*, 1975). However, it cannot be performed for genotoxicity testing of nanoparticles as it mostly yields negative result probably due to the penetration problems of nanomaterials through the bacterial cell wall/cell membrane (Mishra and Srivastava, 2023; Landsiedel *et al*., 2022; EFSA, 2018).

In general, Ames test has several limitations that need to be taken into account. The utilization of prokaryotic cells which have different characteristics (uptake, metabolism, chromosome structure and DNA repair processes) compared to mammalian cells is one of these limitations. Furthermore, due to the lack of enzymatic activities such as cytochromes P450 in bacteria, this test requires exogenous supplementation of the metabolic activation system S9 mix, however, it cannot imitate completely *in vivo* conditions (OECD, 2020). Also, the Ames test is not enough sensitive in order to detect all genotoxic carcinogens and cannot detect non-genotoxic carcinogens (OECD, 2020). Rainer *et al*. (2018) studied the robustness of the Ames test for detecting genotoxicity in FCMs migrates. They found that only highly potent genotoxins could theoretically be detected at 0.01 mg/kg (10ppb). Moreover, only about 10% of FCM-related genotoxic chemicals were detectable at this level. These results indicate that the Ames test lacks sufficient biological sensitivity for chemicals low-level detection in FCM migrates, highlighting the need for more sensitive alternative methods, especially for unpredictable NIAS. It has been demonstrated that 85% of mutagens can be detected at concentrations of 250 µg/plate in the Ames test (Kenyon *et al*., 2007).

7.1.2.3.3.4. Miniaturized variants of Ames test

The traditional Ames test described in OECD TG 471 guideline is difficult to implement when the number of tested samples is high or if there is a limitation in sample quantity (as it is the case with NIAS). It is time consuming, laborious, requires large amounts of test sample and exogenous metabolization system, as well as it produces a huge amount of plastic waste. Several miniaturized versions of Ames test were developed to cope above mentioned problems, however, till now none of them was recommended as an alternative for the standard OECD TG 471 bacterial reverse mutation test (OECD, 2022).

Comparison of several versions of Ames test is shown in Table 1-11. Some of these versions demonstrate similar concordance with the traditional test while reducing sample size, S9 mix requirements, and plastic waste. The choice of a miniaturized version depends on the specific

application and desired outcome. However, it is important to note that, compared to the traditional version, miniaturized versions face reproducibility issues and cannot yet fully replace the conventional Ames test.

Table 1-11. Comparison of different Ames methods

Assay name	Reference	Format	Sensitivity, Specificity, Concordance (%)	Strains	Highest concentration tested	Sample amount	Criteria for positive response judgement	Throughput level	Advantages	Limitations
Standard Ames: Plate incorporation method	OECD, 2020	Petri dishes	Sensitivity: 49.4, 58.8 %, Specificity: 73.9, 80.3 %, Concordance: 62.5, 62.9%, Positive predictivity: 76.4, 87.4%, Negative predictivity: 36.8, 55.1% (Matthews *et al*., 2006; Kirkland *et al*., 2005)	At least five, four strains of *S. typhimurium* (TA1535; TA1537 or TA97a or TA97; TA98; and TA100) and *E. coli* WP2 or TA102.	5mg/plate	100mg/mL, 50-100µL	2-fold increase	Low throughput	Regulatory genotoxicity test	Requires a high volume of sample, S9, and solutions, produces a huge amount of plastic waste, hands-on time is long, low throughput
Standard Ames-Preincubation method	OECD, 2020	Petri dishes	Sensitivity: 49.4, 58.8 %, Specificity: 73.9, 80.3 %, Concordance: 62.5, 62.9%, Positive predictivity: 76.4, 87.4%, Negative predictivity: 36.8, 55.1% Matthews *et al*., 2006; Kirkland *et al*., 2005)	At least five, four strains of *S. typhimurium* (TA1535; TA1537 or TA97a or TA97; TA98; and TA100) and *E. coli* WP2 or TA102.	5mg/plate	100mg/mL, 50-100µL	2-fold increase	Low throughput	Regulatory genotoxicity test, in comparison with plate incorporation method it is more sensitive	Requires a high volume of sample, S9, and solutions, produces a huge amount of plastic waste, hands-on time is long, low throughput
MINISCREEN (original)	Brooks, 1995	25-well plates	Good correlation with conventional Ames test	*S. typhimurium* TA98, TA100	1mg/well	25mg, 20µL	2 or 3-fold increase			
MINISCREEN (small	Burke *et al.,* 1996	25-well plates		*S. typhimurium* TA98, TA100 and		20mg, 20µL	Statistically significant			The assay is not using TA1537 and

modification of original protocol)				TA102, *E.coli* WP2(pKMI01) and WP2uv/vl(pKM10)			increase of revertant colonies in comparison with the control plates considered positive			TA1535 strains because of their low spontaneous counts
MINISCREEN assay on 6-well plates	Diehl *et al*., 2000	6-well plate	Concordance: 98–100%	*S. typhimurium* TA98, TA100, and TA102, and *E.coli* WP2uvrA2	1mg/well	21mg, 20 µl	2-fold increase	7 to 15 compounds per technician in a week	High throughput, requires less sample compared to the regular plate assay (25mg which is 5 folds less)	Handling and reading difficulties because of small well size of the 6-well plates, lower spontaneous colony number
Micro-Ames in 24-well plates	Pant *et al*., 2016; Escobar *et al.*, 2013	24-well plates	Concordance: 100%	*Salmonella typhimurium* TA98, TA100, TA1535, and TA97a	250 mg/well	8mg, 10 µL	2-fold or higher increase for TA97a and TA100; and a 3-fold or higher increase for TA98 and TA1535		Over 60-fold less compound compared to the regular plate assay (8 mg for MicroAmes vs. 500 mg for a standard Ames)	Not many publications exist on this bioassay
Micro Ames in 24-well	Proudlock and Evans, 2016; Escobar *et al*., 2013									Not many publications exist on this bioassay

Mini-Ames (mix strains)	Escobar *et al*., 2013	Petri dishes	Sensitivity: 95%, Specificity: 100%, Accuracy: 97.9%, Negative predictive power: 95.6%, Positive predictive power: 100%	Four *S. typhimurium* strains, tester strain TA100 is run as a stand-alone strain, while equal volumes of tester strains TA98, TA1535 and TA1537 are combined in a single 100-mm plate (mixed strain arm).	2mg/ plate	40mg		Twenty compounds per week can be screened in the assay	Low compound requirements, moderate throughput and high predictive value for the standard Ames assay	The potential to miss weak responders or TA 1537/ 1535-specific responses in the mixed strain arm, and the absence of *E. coli* as a 5th strain, huge plastic waste
MicroAmes assay (96-well plate)	Escobar *et al*., 2013	96-well plate, then Petri dishes	Negative and positive predictivity: 100%	Five *S. typhimurium* strains (OECD strains)	500 µg/plate	10 µL		Doubling of the testing throughput in comparison with the standard method	The lowest effective dose (per plate) is 10-fold lower than in the plate incorporation assay, close alignment to the regulatory test, low compound requirement	Huge plastic waste
Bioluminescent Ames assay	Côté *et al*., 1995	48-well microplates, then 96-well microplates	As sensitive as standard Ames test	Genetically engineered *S. typhimurium* TA98 strain expressing the lux(AB) from Vibrio harueyi		0.1mL				The assay requires dodecyl aldehyde because bacteria cannot synthesize it, bacteria have Lux AB gene, to synthesize the aldehyde they

										need luxC, lux D, and lux E
Bioluminescent Ames assay	Guadano, 1999			*S. typhimurium* TA98, TA100						
BioLuminescent Ames assay	Ackerman *et al*., 2009; Aubrecht *et al*., 2007	24-well plates	Concordance: 100% with standard Ames test	Genetically engineered *S. typhimurium* TA98, TA100 strains expressing the lux(CDABE) operon from Xenorhabdus luminescence	1.35 mg/mL	1.25m, 10 µL	2- or 2.5-fold increase of bioluminescent colonies over the untreated controls with evidence of a dose response		Reduces compound requirement, additionally, scoring is amenable to automation, which increases throughput, the use of S9 mix. The assay doesn't require addition of dodecyl aldehyde because genetically modified bacteria have Lux C, Lux D, and Lux E genes and they can synthesize the aldehyde.	
BioLuminescent Ames assay	Zwart *et al.,* 2018	96-well plate, then	Sensitivity: 81% Specificity: 38% Accuracy: 73%	Genetically engineered *S. typhimurium* TA98, TA100 strains		1 µL		Exposures of one bacteria strain with		

		384-well plates		expressing luxCDABE operon from Photorhabdus luminescens				16 samples in the presence and absence of metabolic activation		
Fluctuation Ames test	Gatehouse, 1978; Gatehouse and Delow, 1979; Green *et al*., 1977, 1976); Green and Muriel, 1976	Similar to Ames II and Ames MPF (see below)								
Ames II assay	(Gee *et al*., 1994 (development of TAMix strains), Gee *et al.,* 1998 (development of the original experiment), (Steinberg, 2013; Kamber *et al*., 2009)	24-well plate, then 384-microtiter plate	Concordance: 75-83.3%, Sensitivity: 89.5% compared to standard method	*S. typhimurium* TA98, TAMix (TA7001, TA7002, TA7003, TA7004, TA7005, TA7006)	1-5 mg/mL	5-35mg, 10µL	30 compounds per week	A clear dose-related increase over the mean number of positive wells.	Low compound requirement and the increased throughput, the ability to automate some of the steps.	A few compound classes are not reliably detected with the 2-strain testing strategy, the use of liquid medium restricts the test substances only to those which are clearly soluble in water or DMSO.
Ames MPF assay	Flückiger-Isler and	24-well plate, then	Accuracy: 78%, Specificity: 92%, Sensitivity: 70%	*S. typhimurium*: TA98, TA100 and TA1535, TA1537,		45mg, 10µL		2-fold or greater increase	A main advantage of the Ames MPF	A small bacterial sample size (low number of

	Kamber, 2014	384-microtiter plate		*E. coli* [pKM101]), WP2 uvrA)				over the baseline	over the Ames II assay is that strains recommended in the OECD guideline 471, almost four times less test chemical is needed, requires less hands-on time, it needs less plasticware, and 10 times less S9 mix, easy colorimetric readout, the Ames MPF assay is available as a kit.	mutants to start) leading to variability and a high number of repeat tests/ equivocal results and to a lack of a good cytotoxicity assessment, the use of liquid medium restricts the test substances only to those which are clearly soluble in water or DMSO.
Mini mutagenicity test (MMT)	Flamand *et al.,* 2001	6-well plates	Excellent concordance between the standard Ames test, greater sensitivity of the MMT	*S. typhimurium* TA98, TA100, TA1535, TA102, TA1538	1mg/well	300mg		3-fold increase for TA1535, TA1537, 2-fold increase for TA98, TA100, TA102		

Mutascreen	Falck *et al*., 1985	200-well plates	Similar sensitivity with the standard Ames test	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA97a, TA102, *E. coli* WP2uvrA	1mg/well					

7.1.2.3.3.5. In Vitro Mammalian Cell Micronucleus Test (OECD TG 487)

This *in vitro* micronucleus technique can identify compounds that are both clastogenic and aneugenic in mammalian cells that have undergone cell division while being exposed to the test substance or subsequently in the presence or absence of a metabolic activation system (unless metabolic competent cells were used). Following cells from human or rodent origin are commonly used in this bioassay: cultured primary human or other mammalian peripheral blood lymphocytes, TK6, CHO, V79, CHL/IU, and L5178Y cells. Other not extensively validated cell lines, such as HT29, Caco-2, HepaRG, HepG2 cells, A549, and primary Syrian Hamster Embryo (SHE) cells, are also used for *in vitro* micronucleus testing. The selection of cells depends on their ability to grow well in culture, the stability of their karyotype (including chromosome number), a stable and defined background frequency of micronucleus formation, their p53 status, DNA repair capacity, and their origin (rodent versus human) (OECD, 2023b).

After exposure of cells to genotoxic chemical, the frequency of micronuclei (MN) is recorded in the cytoplasm of mononucleated cells in interphase stage. MN is a small nuclei, separate from and additional to the main nuclei of cells and may arise due to acentric (i.e., lacking a centromere) chromosome segments or complete chromosomes that are unable to migrate to the poles during the anaphase stage of cell division. To reduce the relatively subjective assessment of MN in interphase cells, actin polymerization inhibitor cytochalasin B (cytoB) may be added before mitosis and MN are counted only in binucleated cells which undergone one mitosis is counted. In case cytoB is not applied it is important to demonstrate that cells have undergone mitosis (OECD, 2023b). Recovery time is an important parameter in this bioassay and it should be long enough to allow the completion of at least one full cell cycle post-treatment. This duration varies depending on the specific cell type used in the assay, as different cell lines have different cycle lengths.

It is important to note that chromosome aberrations seen in metaphase cells may not be transmitted, while MN in interphase cells represents damage that has been passed on to daughter cells. Figure 1-12 demonstrates events happening during *In Vitro* Mammalian Cell Micronucleus Test.

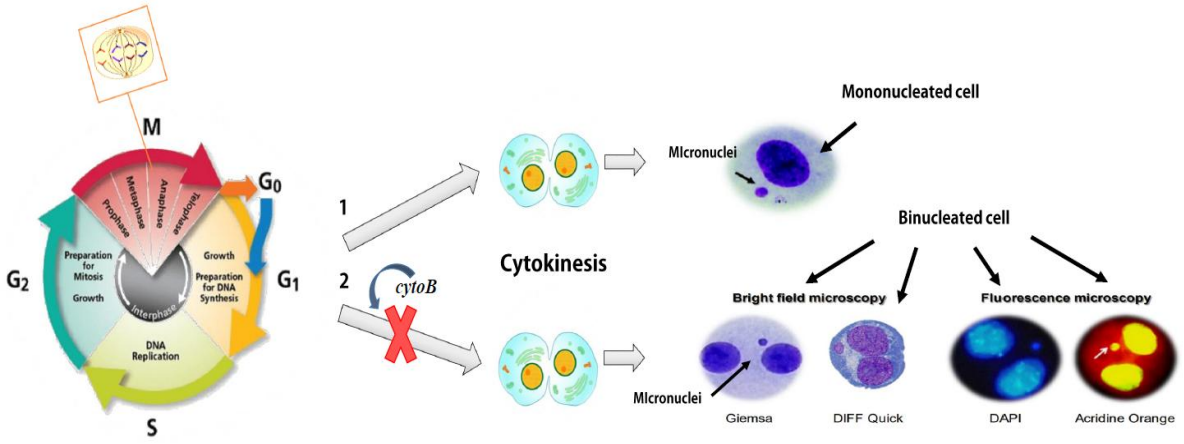


Figure 1-12. Illustration of events happening in cells during *In Vitro* Mammalian Cell Micronucleus Test (1: without addition of cytochalasin B (cytoB), 2: with addition of cytoB) (personal interpretation)

This test alone cannot differentiate clastogen or aneugen chemicals, however, if required additional immunochemical labeling of kinetochores, or hybridization with centromeric/telomeric probes fluorescence *in situ* hybridization may be used to determine the mode of action of genotoxic chemicals (OECD, 2023b).

The bioassay has several limitations, such as the use of an exogenous metabolic system (unless the cells are metabolically competent), possible artifactual positive results due to several experimental conditions, such as changes in pH or osmolality, interaction with the cell culture medium, or excessive levels of cytotoxicity, as well as the obligation of scoring MN in cells which have undergone mitosis during or after treatment with the test chemical (OECD, 2023b). Table 1-12 shows the performance of *in vitro* micronucleus test in detecting rodent carcinogens.

Table 1-12. Performance of *in vitro* micronucleus test in detecting rodent carcinogens

	Reference : Kirkland *et al*. (2005)	Reference : *Matthews et al*. (2006)
No. of substances	**115**	**97**
Sensitivity %	78.7	87.3
Specificity %	30.8	23.1
Concordance %	67.8	70.1
Positive predictivity %	79.5	75.6
Negative predictivity %	29.6	40.0

7.1.2.3.3.6. *In vitro* MicroFlow

In vitro MicroFlow method developed by Litron Laboratories (Rochester, NY 14623, United States) is one of the miniaturized variants of the *in vitro* micronucleus assay. It can distinguish true MN from apoptotic and necrotic chromatin *via* successive labelling, providing accurate MN readings even in the presence of significant numbers of dead cells. The method was developed using mouse and human lymphoblastoid cell lines (L5178Y and TK6) and provides automated scoring using flow cytometry (Bryce *et al*., 2007; Avlasevich *et al*., 2006). To distinguish between the chromatin of living cells and those that are of dead/dying cells, ethidium monoazide is added which traverses the damaged outer membrane of these cells and covalently bonds to DNA by photoactivation. To release nuclei and MN, the cytoplasmatic membrane and RNA are then digested. Another nucleic acid dye, called SYTOX Green, is introduced during the lysis step which labels all chromatin (Avlasevich *et al*., 2006).

7.1.2.3.3.7. Other *in vitro* commonly used genotoxicity bioassays

Except above mentioned regulatory *in vitro* genotoxicity bioassays, other *in vitro* genotoxicity bioassays were also developed and are widely used to determine the genotoxicity of chemicals. Some of them will be described in this thesis.

7.1.2.3.3.7.1. BlueScreen HC

Previously mentioned regulatory bioassays are time-consuming, laborious and required large amount of sample, so there is a need for development of mammalian based high-throughput screening assays which can screen large number of materials using small amount of sample during short period of time.

BlueScreen HC is a high-throughput *in vitro* genotoxicity assay developed by Gentronix Ltd which can detect direct-acting mutagens and clastogens, as well as compounds that act indirectly such as aneugens, and topoisomerase and polymerase inhibitors. The assay may perform with and without a metabolic activation system to screen large scale of genotoxic chemicals. It uses a human-derived, p53-competent TK6 cell line which is transfected with a luminescence-based reporter system that represents luciferase from the marine copepod Gaussia that exploits the

proper regulation of the growth arrest and DNA damage-inducible alpha (GADD45a) gene (Hughes *et al*., 2012). It shows 80% sensitivity and 100% specificity (Hughes *et al*., 2012).

GADD45a gene is located mostly in cell nucleus and considered as stress sensor gene. In mammalian cells, it is involved in different signaling responses of cells to physiological or environmental stresses resulting to cell cycle arrest, DNA repair, cell survival, senescence, or apoptosis. It mediates its activity *via* interaction with other cellular proteins (e.g., proliferating cell nuclear antigen, p21, cdc2/cyclinB1 complex etc) responsible for cells cycle regulation and responses of cells to stress (Figure 1-13) (Cretu *et al*., 2009).

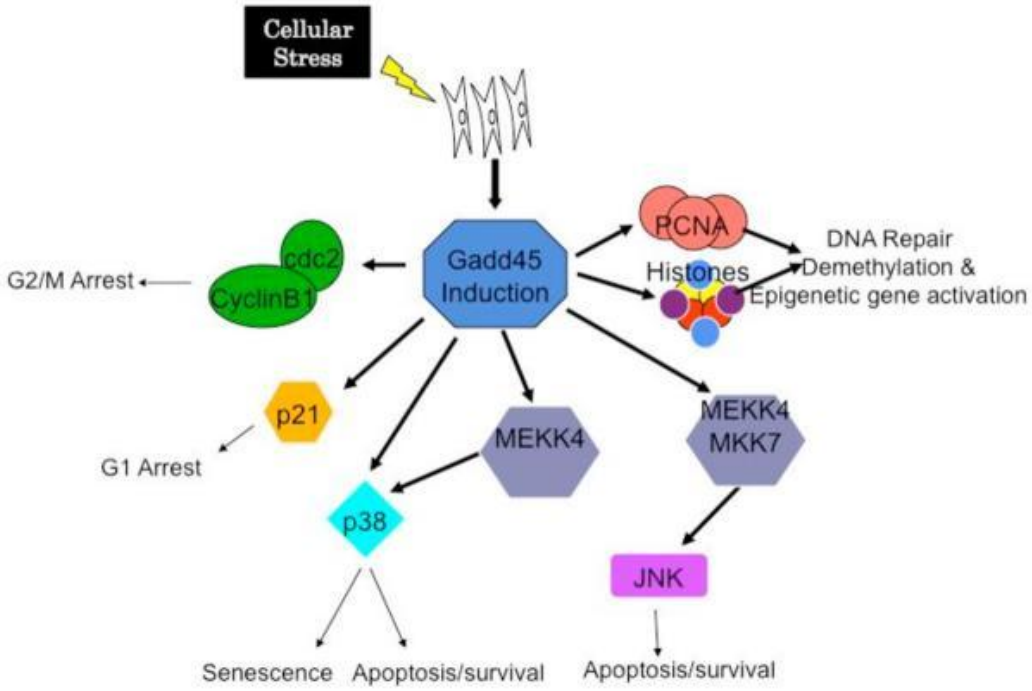


Figure 1-13. Main targets of the Gadd45 gene when the cell is under stress from Cretu *et al*. (2009)

Several malignant hemopathies and a variety of solid tumors have been shown to express Gadd45 aberrantly (Cretu *et al*., 2009).

In response to exposure of cells to a genotoxic substance, GADD45a expression increases which results in a dose-dependent increase in luciferase from the GADD45a reporter. To measure the amount of luciferase exported naturally from the cells, a solution of the substrate coelenterazine is injected into the medium which produces easily measurable a brief flash of luminescence.

Illustration of events which occur in cells during BlueScreen HC assay are shown in Figure 1-14.

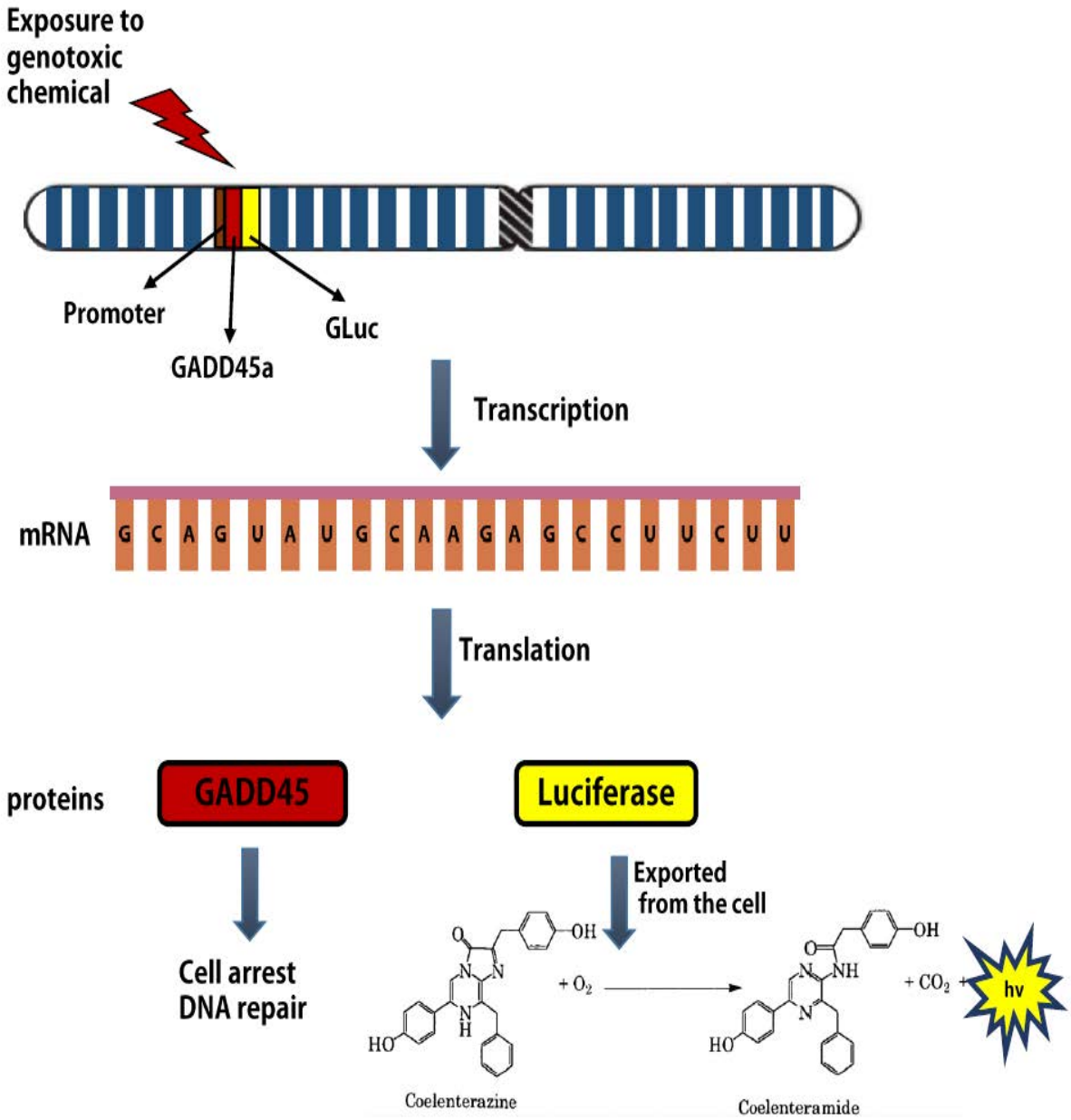


Figure 1-14. Illustration of events happening in cells during BlueScreen HC assay (personal interpretation)

7*.1.2.3.3.7.2.* DNA Damage Response test

Another high-throughput *in vitro* genotoxicity test, namely the DDR test capable of detecting both DNA-reactive and non-DNA-reactive chemicals using TK6 human lymphoblastoid cells was developed by Litron Laboratories (Bryce *et al*., 2016).

High sensitivity (92.1 %) and specificity (96.2 %) for the DDR test were reported as it measures the induction of several DDR biomarkers: γH2AX which is a biomarker of clastogenicity sensitive to DNA DSB (Matsuzaki *et al*., 2010; Kinner *et al*., 2008; Rogakou *et al*., 1999), phospho-histone H3 which is a biomarker of aneugenicity (Wilde *et al*., 2020), and p53 which is a biomarker of genotoxicity (Bhaskaran *et al*., 1999). This bioassay covers all modes of actions of genotoxic chemicals (clastogenicity, aneugenicity, and genotoxicity) and allows fast reading of the results after 24 hours of treatment by flow cytometry (Bryce *et al*., 2017).

TK6 human lymphoblastoid cells are treated with test chemicals in the presence and absence of metabolic activation system. After exposure treatment the induction of above-mentioned DDR biomarkers are measured *using* fluorescent labelled antibodies against p53, gH2AX, and phospho-histone H3 *via* flow cytometry (Bryce *et al*., 2016).

Other genotoxicity bioassays have previously been performed for FCM testing, such as the chromosomal aberration test, the sister chromatid exchange assay, the comet assay, and the Rec-assay (Groh and Muncke, 2017; Severin *et al.*, 2017).

7.1.2.3.3.7.3. GENOMARK

The GENOMARK assay employs metabolically competent human HepaRG cells and is built on transcriptomic data generated using 12 reference compounds with confirmed *in vivo* genotoxicity and 12 reference compounds shown to be non-genotoxic *in vivo*. The genotoxic substances were chosen to represent a broad range of mechanisms, including bulky DNA adduct formation, DNA alkylation and cross-linking, free radical–mediated DNA strand breaks, inhibition of tubulin polymerisation, and the action of base analogues (Ates *et al.*, 2018).

The GENOMARK biomarker panel consists of 84 target genes, together with five housekeeping genes, whose expression is measured using a quantitative PCR array. In brief, HepaRG cells are cultured for seven days and then exposed for 72 hours to the test substance at the IC10 concentration, as determined by an MTT cytotoxicity assay based on formazan production.

Following exposure, total RNA is extracted, reverse-transcribed into complementary DNA, and analysed using a pre-spotted quantitative PCR plate to assess the expression of the 84 GENOMARK genes (Ates *et al*., 2018).

GENOMARK has been shown to enable rapid and reliable identification of genotoxic hazards, while also providing insight into the relative potency of the tested substances (Thienpont *et al*., 2023b, 2023a).

7.1.2.3.3.7.4. TGx-DDI biomarker

The TGx-DDI biomarker was established as a toxicogenomic gene expression signature designed to detect chemicals capable of inducing DNA damage in cultured human cells. It was developed to overcome key shortcomings of conventional *in vitro* genotoxicity assays, particularly their limited specificity. In addition, TGx-DDI has demonstrated value in clarifying ambiguous outcomes from standard genotoxicity tests and in offering mechanistic insight into cellular DNA damage responses (Li *et al*., 2019). The development of this biomarker was a collaborative initiative involving the Health and Environmental Sciences Institute (HESI) Genomics Committee, Health Canada, Georgetown University, and the U.S. National Toxicology Program.

TGx-DDI was derived using a toxicogenomics strategy applied to expression data from 28 reference compounds tested in p53-competent TK6 human lymphoblastoid cells. These compounds included directly DNA-reactive genotoxic agents, indirect-acting agents that induce DNA damage through mechanisms such as topoisomerase inhibition or disruption of DNA synthesis, and chemicals that are non-DNA-reactive and negative in conventional genotoxicity assays (Li *et al*., 2015). From this dataset, a 64-gene expression panel was identified that reliably discriminates between DNA damage–inducing (DDI) and non-DDI chemicals (Li *et al*., 2017, 2015). The likelihood that a test compound induces DNA damage is determined by comparing its gene expression profile with those of the reference chemicals. It is important to note that TGx-DDI is not designed to detect aneugenic mechanisms of genotoxicity.

Since its introduction, the TGx-DDI biomarker has undergone extensive validation in multiple independent laboratories, across different human cell systems, and under conditions both with and without metabolic activation using liver S9 fractions. Its performance has also been

confirmed across a wide range of transcriptomic platforms, including Agilent and Affymetrix microarrays, NanoString nCounter, RT-qPCR, RNA sequencing, and TempO-Seq (Fortin *et al*., 2023; Allemang *et al*., 2021; Buick *et al*., 2021, 2020, 2017, 2015; Chen, 2021; Cho *et al*., 2019; Li *et al*., 2017; Yauk *et al*., 2016).

The HESI Emerging Systems Toxicology in the Assessment of Risk Committee is currently pursuing formal qualification of TGx-DDI through the U.S. Food and Drug Administration's Biomarker Qualification Program. This effort focuses on evaluating the biomarker for a clearly defined regulatory context of use, with particular emphasis on reproducibility and reliability.

To support its qualification as a robust and reproducible tool for enhancing genotoxicity hazard assessment, a multi-site ring trial was conducted involving four laboratories. Fourteen blinded test chemicals were evaluated using harmonized experimental protocols. Under these validated conditions, TGx-DDI achieved a sensitivity of 100%, a specificity of 86%, and an overall accuracy of 91% in discriminating DDI compounds from non-DDI agents. Strong agreement across laboratories was observed, with transcriptomic profiles demonstrating high cross-site correlation (Wang *et al.*, 2025).

7.1.2.3.3.7.5. ToxTracker

ToxTracker is a stem cell-based reporter assay used to detect genotoxic substances. It integrates six distinct fluorescent reporter genes, each responsive to specific cellular signaling pathways. Two of these pathways reflect direct DNA damage, while the remaining stress-related responses indicate cytotoxic mechanisms such as oxidative stress or protein misfolding, which can indirectly contribute to genotoxic effects (Hendriks *et al*., 2016, 2012). The cellular signaling responses utilized in ToxTracker are shown in Figure 1-15.

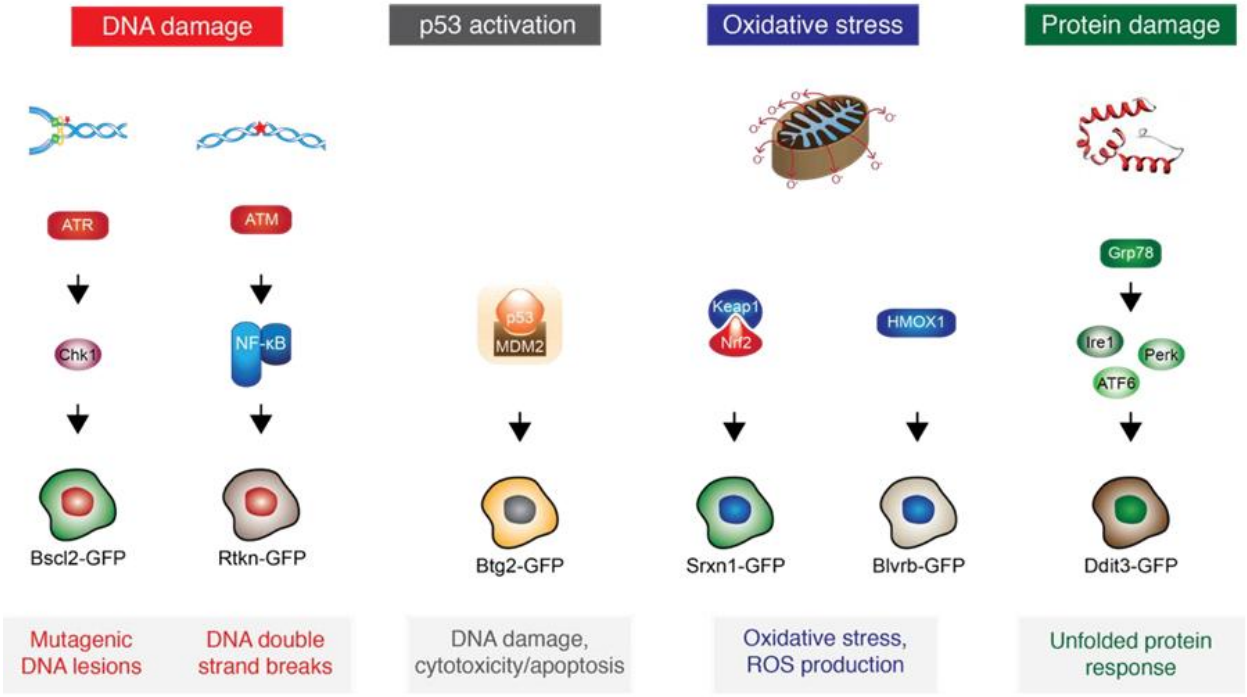


Figure 1-15. Overview of the cellular signaling pathways that activate the different ToxTracker reporter genes (Hendriks *et al.,* 2012; OECD, 2025)

The biomarker genes used in the ToxTracker assay were identified through toxicogenomics studies in which mouse embryonic stem cells (mESCs) were exposed to a range of genotoxic and non-genotoxic carcinogenic compounds (Hendriks *et al*., 2011).

Six distinct mESC reporter cell lines were generated, each harboring a single green fluorescent protein-tagged reporter gene linked to either direct or indirect genotoxic mechanisms (Hendriks *et al*., 2012). Activation of the green fluorescent protein reporters in the ToxTracker assay is quantified by comparing exposed cultures with unexposed controls.

Interlaboratory validation studies confirmed the transferability and accuracy of ToxTracker in identifying *in vivo* genotoxic agents, achieving a sensitivity of 87% and a specificity of 90% (Hendriks *et al*., 2024). ToxTracker can be incorporated as a key component of an *in vitro* genotoxicity testing battery and is particularly useful as a follow-up tool when standard *in vitro* genotoxicity assays yield equivocal results.

The OECD has released a draft Test Guideline for the ToxTracker assay for public consultation (OECD, 2025).

7.1.2.3.4. Endocrine disruption

The endocrine system plays a vital role in maintaining the biological equilibrium of organisms by regulating cell growth, development, reproduction, and overall homeostasis. ED are external substances or mixtures that alter the functioning of the endocrine system, thereby causing adverse health effects in an intact organism, its offspring, or specific populations (IPCS, 2002). To effectively identify EDs, conducting bioassays on whole organisms is necessary, while it is feasible to assess potential EDs with properties and specially their mode of action that may lead to endocrine disruption in an intact organism using *in vitro* methods (ECHA/EFSA/JRC, 2018).

7.1.2.3.4.1. Health effects of endocrine disruptors

There is a noticeable increase in the prevalence of various diseases and disorders linked to the endocrine system. For example the rate of following endocrine-related diseases or disorders has been increased over past decades: low semen quality in man resulting in a decreased capability to father children, genital malformations, such as non-descending testes (cryptorchidism) and penile malformations (hypospadias) in baby boys, adverse pregnancy outcomes, such as preterm birth and low birth weight, neuro-behavioural disorders associated with thyroid disruption, endocrine-related cancers (breast, endometrial, ovarian, prostate, testicular and thyroid), earlier onset of breast development in young girls, obesity and type 2 diabetes (UNEP/WHO, 2013).

It has been shown that EDs may show non-monotonic dose-response (NMDR) effect, which raises questions about the safety thresholds established by regulatory authorities (Vandenberg *et al*., 2012). NMDR describes a situation where the body's response to increasing doses of a chemical compound does not follow a straightforward linear pattern. This is in contrast to a linear relationship where higher doses consistently lead to stronger responses. NMDR contradicts the conventional principles of toxicology, challenging the notion that the magnitude of the effect is solely determined by the dose (Vandenberg *et al*., 2012). It suggests that at specific low doses, significant effects can occur, making responses less predictable and risk assessment challenging.

Research has demonstrated that a multi-component mixture containing various xenoestrogens at concentrations below their individual no observed effect levels may still produce a notable effect due to cumulative interactions (Silva *et al*., 2002). Hence, mixture risk assessment is challenging but essential for a better protection of human health and the environment, and regulatory action is necessary to advance in this area (Kortenkamp and Faust, 2018).

The characterization of an EDs hinges on three distinct criteria: its endocrine activity, the adversity of effects, and the establishment of a credible link between its endocrine activity and the observed adverse effects (ECHA/EFSA/JRC, 2018). EDs can exert their effects through various mechanisms, including direct ones (genomic or non-genomic) or indirect ones, such as interfering with hormone synthesis (they can also disrupt the enzymes responsible for hormone synthesis), hormone transport and metabolism, and binding to hormone receptors (UNEP/WHO, 2013). Information for assessing endocrine activity can be collected at five different levels, as outlined in Table 1-13 (ECHA/ EFSA/JRC, 2018; OECD, 2018c).

Table 1-13. Different levels of information to assess endocrine disrupting activity of chemicals (ECHA/ EFSA/JRC, 2018; OECD, 2018c)

Level	Example
Level 1 Existing data and non-test information	• Physical and chemical properties, e.g., molecular weight, reactivity, volatility, biodegradability • All available (eco)toxicological data from standardized or non-standardized tests. • Read across, chemical categories, QSARs and other *in silico* predictions, and ADME model predictions
Level 2 *In vitro* experiments providing data about selected endocrine mechanism(s)/pathways (mammalian and non-mammalian methods)	• Estrogen receptor transcriptional activation (TG 455, TG 457) • Androgen receptor transcriptional activation (TG 458) • Thyroid transcriptional activation (if/when TGs are available) • Steroidogenesis *in vitro* (TG 456) • MCF-7 cell proliferation assays (ER ant/agonist) • Other assays as appropriate
Level 3/4/5 *In vivo* screening assays	• Uterotrophic Bioassay in Rodents (TG 440) • Hershberger Bioassay in Rats (TG 441) • Fish Short Term Reproduction Assay (TG 229) • Amphibian Metamorphosis Assay (TG 231)

Level 1 encompasses both existing data and new non-test information. This phase involves gathering details on the physical and chemical properties of chemicals, as well as

(eco)toxicological data from both standardized and non-standardized tests. It also includes read-across data, chemical categorization, (Q)SAR and other *in silico* predictions, and predictions related to absorption, distribution, metabolism, and excretion models (OECD, 2018c). Level 2 comprises *in vitro* assays that provide data on specific endocrine mechanisms or pathways, utilizing both mammalian and non-mammalian methods (OECD, 2018c). Some of Level 2 *in vitro* bioassays will be discussed within the context of this manuscript.

A comprehensive evaluation of adversity and endocrine activity requires the integration of all available information from *in silico*, *in vitro*, and *in vivo* sources, including epidemiological, clinical studies and well-documented case reports and observations. This assessment should be carried out through a weight-of-evidence approach, which involves a thorough consideration of this diverse array of data to arrive at well- rounded conclusions (ECHA/EFSA/JRC, 2018).

It is important to conduct bioassays that have received validation from either the OECD or US EPA for various endocrine receptor-mediated modes, including those involving the Estrogen Receptor (ER), AR, thyroid disrupting activity and Steroidogenesis interference. However, for thyroid hormone disrupting activity, level 2 OECD validated bioassays as well as specific *in vivo* (Level 3) mechanistic tests on mammals are not yet available (ECHA/EFSA/JRC, 2018).

7.1.2.3.4.2. ED and FCMs/FCAs

The presence of chemicals, even at low exposure levels, can disrupt the endocrine system, as is the case of NIAS found in mixtures of FCMs/FCAs. Therefore, it is crucial to assess the potential endocrine-disrupting properties of NIAS when exposed to low levels. However, the toxicity of NIAS can only be assessed when their chemical identity is identified, and they are available in pure form, which is challenging. As a result, testing the overall toxicity of migrates or extracts from FCMs and FCAs is one approach to fulfill this regulatory requirement.

The endocrine disrupting activity of extracts or migrates of FCMs and FCAs was evaluated using various *in vitro* bioassays conducted by different researchers. Several extracts from paper and paperboard demonstrated estrogenic and AhR activity in different bioassays. Some plastic FCMs extracts exhibited estrogenic, anti-estrogenic, androgenic, and anti-androgenic activities, as reported by various studies. Conversely, other studies indicated a negative response for the estrogenic activity of coating extracts (Groh and Muncke, 2017). The endocrine-disrupting

activity of 42 food contact silicone products from the Chinese market was evaluated after migration testing in EtOH 95% (a food simulant) at 70 °C for 2 hours under accelerated conditions. Among the 31 kitchenware items tested, 84% exhibited hormonal activities, including estrogenic (64%), anti-estrogenic (19%), androgenic (42%), and anti-androgenic (39%) effects, as determined by the Dual-Luciferase reporter gene assay (Feng *et al.*, 2023).

The SIN List ("Substitute It Now!"), developed by ChemSec, includes chemicals identified as SVHC according to the criteria set by the REACH regulation (**https://sinlist.chemsec.org/**). There is also TEDX list for potential ED (**https://www.endocrinedisruption.org/interactive-tools/tedx-list-of-potential-endocrine-disruptors/search-the-tedx-list**).

European Chemicals Agency (ECHA) has established ED assessment list which includes the substances undergoing an ED assessment that have been brought for discussion to ECHA's ED Expert Group (**https://www.echa.europa.eu/ed-assessment**).

The Database on Migrating and Extractable Food Contact Chemicals (FCCmigex), compiled by researchers from the Food Packaging Forum in collaboration with eight academic institutions, includes approximately 5,300 food contact chemicals and over 35,500 entries (**https://foodpackagingforum.org/resources/databases/fccmigex**).

The Food Contact Chemicals database (FCCdb) provides a comprehensive list of intentionally added food contact chemicals, along with available hazard and regulatory information. Covering a global scope, its current version (5.0) identifies 12,285 unique food contact chemicals potentially used in the production of FCMs and FCAs (**https://foodpackagingforum.org/resources/databases/fccdb**).

PEPPER, founded in 2019, is a French non-profit association (under the French 1901 law) that brings together public and private stakeholders to speed up the identification of endocrine-disrupting chemicals by enhancing regulatory science. It is supported by the French "Programme of Investments for the Future" (PIA), government departments and industry associations. Its primary focus is on the pre-validation of toxicology and ecotoxicology testing methods used to evaluate substances for endocrine-disrupting effects.

The platform selects test methods that are scientifically sound and relevant, coordinates inter-laboratory studies to verify their reproducibility and reliability, and assists developers in

preparing validation dossiers for submission to international bodies such as the OECD and ECVAM. By addressing the stage before formal regulatory validation, PEPPER bridges the important gap between research and regulation (https://ed-pepper.eu/en/).

7.1.2.3.4.3. Transactivation *in vitro* assays to detect endocrine disrupting activity

In vitro Transactivation Assays (TA) rely on a direct or indirect interaction between the substances and a specific receptor. This receptor plays a crucial role in modulating the transcription of a reporter gene, leading to the production of a measurable gene product.

7.1.2.3.4.3.1. Estrogen receptor transactivation assays (OECD TG 455)

Chemicals exhibiting estrogenic properties can potentially interact with ERs, leading to changes in the transcription of genes regulated by estrogen. Consequently, this can result in the activation or suppression of critical cellular processes, including those essential for cell proliferation, normal fetal development, and reproductive function (Escande *et al.*, 2006; Kuiper *et al*., 1998).

Therefore, the proper functioning of the estrogen system is vital for normal development (ontogenesis), reproductive health, and the integrity of the reproductive system.

There are two major subtypes of nuclear ERs: ER-α and ER-β. These receptors are encoded by distinct genes, and respective proteins serve specific biological functions within organisms. These receptors exhibit different affinities for ligand binding and are distributed across various tissues in a manner that depends on the receptor type (Paterni *et al*., 2014). The current models primarily focus on assessing the activation or inhibition of ERα, as it plays a central role in mediating the classic estrogenic response.

Two fully validated (OECD TG 455) reference test methods for detection of ERs agonists and antagonists are:

- The stably transfected transactivation (STTA) assay using the (h) ERα-HeLa-9903 cell line; and
- The VM7Luc ER TA assay using the VM7Luc4E2 cell line which predominately expresses hERα with some contribution from hERβ (OECD, 2021).

Although ER TA assays are valuable for screening and prioritizing potential ER active substances, they do come with limitations that should be acknowledged. Firstly, these assays are conducted *in vitro*, and it is essential to avoid directly extrapolating their results to the complex signaling and regulation of the intact endocrine system *in vivo*. Secondly, as mentioned earlier, ED can result from various mechanisms, including TA mediated by the ERs, which is a fundamental mechanism of ED. ER TA assays cannot detect all the possible modes of action of ED chemicals. Lastly, certain chemicals referred to as selective ER modulators may exhibit both agonist and antagonist activities in a cell-type-specific manner, and a negative response in ER TA assays is not sufficient evidence to rule out a chemical's agonist or antagonist activity (OECD, 2021).

7.1.2.3.4.3.2. Androgen receptor transactivation assays (OECD TG 458)

The principle of AR TA bioassays described by OECD TG 458 is similar to the above-mentioned ER TA assays validated by OECD, but they measure signals originating from ligand binding to AR.

The interaction between androgen agonists and antagonists with AR can activate or inhibit the transcription of genes responsive to androgens, which may trigger adverse health effects by disrupting systems regulated by androgens. Such disruptions may influence fundamental mechanisms such as cellular proliferation, the normal development of the fetus, functioning of the reproductive system.

Protocol of fully validated reference test methods to test AR TA is described by OECD, TG 458 (OECD, 2023c).

7.1.2.3.4.3.3. Thyroid receptor transactivation assay

Freitas *et al.* (2011) developed and validated a stable *in vitro* luciferase reporter gene assay for the rapid and specific quantification of thyroid hormone receptor-mediated activity. This bioassay enables the detection of hormones such as triiodothyronine (T3) and thyroxine (T4), their structural analogs, as well as environmentally relevant contaminants. The assay was established using the GH3 rat pituitary tumor cell line, which naturally expresses both isoforms of the thyroid hormone receptor. To measure a receptor-specific endpoint in a high-throughput format, the GH3 cells were stably transfected with a luciferase reporter gene under the control of a thyroid hormone response element. This configuration allows the detection of T3 at

picomolar concentrations within just 24 hours of exposure. The underlying principle of the assay is comparable to that of previously described transactivation bioassays.

7.1.2.3.4.4. H295R Steroidogenesis Assay (OECD TG 456)

The goal of the H295R Steroidogenesis Assay is to identify substances that have the potential to influence the production of 17β-estradiol (E2) and testosterone (T). This involves both the induction and inhibition of their synthesis through various mechanisms including modulation of the expression, synthesis or function of enzymes involved in the production, transformation, or elimination of steroid hormones (OECD, 2023d).

Inhibition of hormone production may result from various direct and indirect factors, for example, direct competitive enzyme binding, influence on co-factor (e.g., nicotinamide adenine dinucleotide phosphate (NADPH) and cyclic adenosine monophosphate (cAMP)), increased steroid metabolism, or gene expression suppression of specific enzymes within the steroidogenesis pathway. Induction of hormone production typically takes an indirect route, often involving the influence of co-factors like NADPH and cAMP, reduction of steroid metabolism, or enhancing the expression of steroidogenic genes (OECD, 2023d).

The steroidogenic pathway is a complex intracellular biochemical process that initiates with a series of reactions converting cholesterol into various steroid hormones, including sex hormones like E2 and/or T.

The bioassay utilizes the human H295R adreno-carcinoma cell line which contains genes responsible for encoding essential enzymes implicated in steroidogenesis. This enables the detection of effects on both corticosteroid synthesis and the production of sex steroid hormones, such as androgens, and estrogens. Despite the fact that the assay's validation focuses on T and E2 detection, it potentially holds broader applications.

Following exposure of cells to the test item, hormone concentrations in the medium are measured using a variety of methods, e.g., ELISA, RIA, LC-MS, LC-MS/MS (OECD, 2023d).

7.1.2.3.4.5. T-screen assay

The "T-screen" is a cell proliferation assay designed for the *in vitro* detection of thyroid receptor agonists and antagonists. This assay utilizes the GH3 cell line, derived from a rat pituitary tumor, which proliferates in response to thyroid hormone T3 in a serum-free medium. GH3 cells exhibit a growth response to T3 due to their high levels of thyroid receptor expression. Following exposure to T3 cell proliferation is measured. Compounds that act as agonists will promote cell proliferation by mimicking the effects of T3. Conversely, antagonists will inhibit T3-induced cell growth by interfering with the hormone's action on the receptors. To quantify cell proliferation, the T-screen employs the dye resazurine, which is a reliable indicator of metabolic activity. Resazurine is initially in an oxidized, blue state. Within the mitochondria of viable GH3 cells, enzymes reduce resazurine to resorufin, a compound that is highly fluorescent. The fluorescence intensity of resorufin correlates directly with the number of viable cells present in the assay. After cell treatment, the fluorescence is measured and data is then compared to the maximum effect induced by T3 alone, which is set as the 100% benchmark. The proliferation results are expressed as a mean percentage of this maximum T3-induced effect, allowing for a clear interpretation of the agonistic or antagonistic properties of the tested compounds (OECD, 2012; Gutleb *et al*., 2005).

7.1.2.3.4.6. Iodide uptake assay for sodium iodide symporter (NIS1) function

The thyroid gland plays a significant role in regulating the endocrine system. The initial stage of thyroid hormone production T3 and T4 in thyroid gland involves the uptake of iodide from the bloodstream into thyroid follicular cells. This process is mediated by the sodium iodide symporter (NIS1), which plays a critical role in various thyroid disorders such as thyroid cancer, autoimmune conditions, and congenital hypothyroidism (Figure 1-16) (Heppel-Holden, 2023).

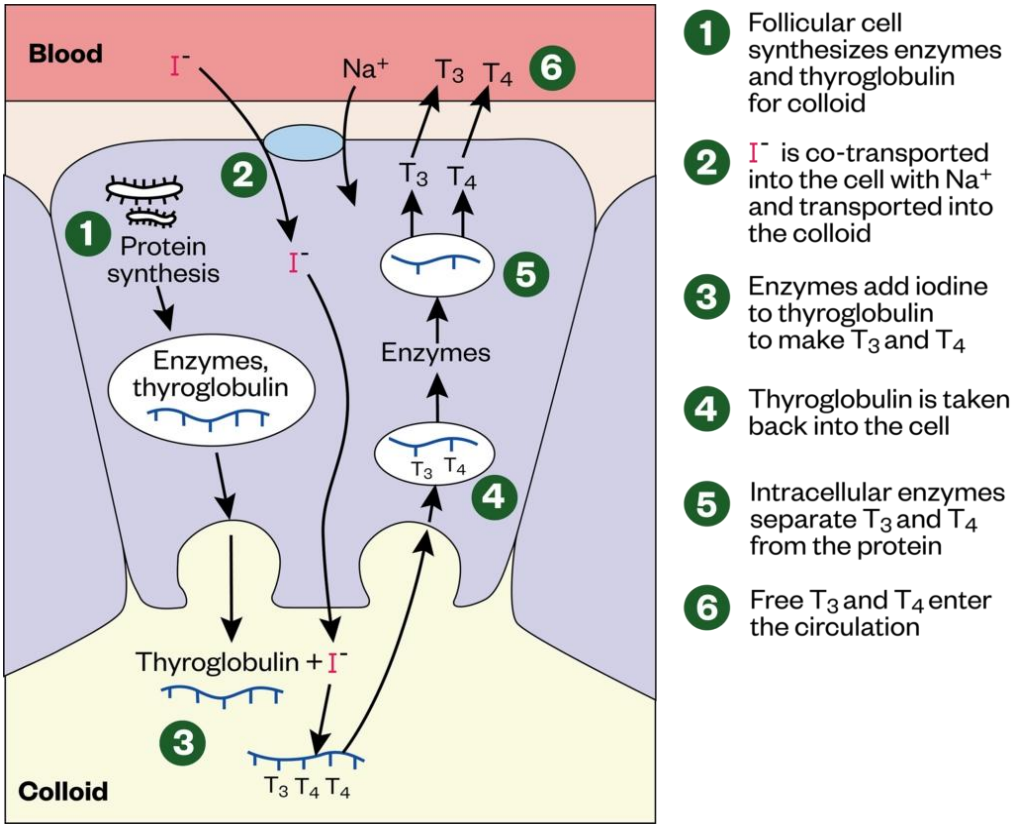


Figure 1-16. Thyroid hormone synthesis (Heppel-Holden, 2023)

Additionally, NIS1 facilitates active transport of iodide in other body tissues, including salivary glands, gastric mucosa, and lactating mammary glands. In these tissues, it aids in transferring iodide into milk for the biosynthesis of thyroid hormones in nursing infants (Darrouzet *et al*., 2014; Riesco-Eizaguirre and Santisteban, 2006; Dohán *et al*., 2003).

The classical method for assessing NIS function involves measuring radioiodide uptake in NIS-expressing cells using $Na^{125}I$, as described by Hallinger *et al.* (2017), Lecat-Guillet *et al*. (2007). However, safety concerns have limited its widespread use. To address these issues, an alternative non-radioactive assay called iodide uptake experiments using Fisher rat thyroid low-serum 5 (FRTL5) cells was developed by Waltz *et al.* (2010) in a 96-well plate format. This assay is based on the iodide-catalyzed Sandell-Kolthoff reaction between arsenic (III) and cerium (IV), enabling spectrophotometric measurement of iodide trapped within cells. The reaction involves the slow reduction of yellow cerium (Ce)(IV) to colorless Ce (III) by As(III), which is greatly accelerated by traces of iodide (catalytic effect). The rate of this reaction is directly proportional to the

concentration of iodide present. Cells were treated with test chemicals and a NaI solution of known concentration. Afterward, solutions of ammonium cerium (IV) sulfate and sodium arsenite (III) were introduced into each well and the reduction in iodide intake caused by a test chemical is measured.

7.1.2.3.4.7. Thyroid peroxidase inhibition assay

Thyroid peroxidase (TPO) is an essential protein involved in thyroid hormone biosynthesis, facilitating the organification of iodide and the iodination of tyrosyl residues of thyroglobulin. TPO-inhibition is one of the mechanisms of thyroid disruption. *In vitro* assay utilizing human recombinant TPO (hrTPO), which was stably transfected into the human follicular thyroid carcinoma cell line FTC-238 to measure TPO activity. hrTPO is obtained by extracting the cell membranes from FTC-238/TPO cells using digitonin and assessing the peroxidase activity *in vitro* with the guaiacol oxidation assay. TPO activity is quantified as micromoles of H_2O_2 reduced per minute per milligram of protein. The TPO inactivation assay involves preincubating the protein extracts with selected compounds, followed by evaluating the peroxidase activity using the guaiacol assay (OECD, 2012; Schmutzler *et al.*, 2007).

A high-throughput assay was developed to screen hundreds to thousands of chemicals for potential effects on TPO activity. This assay is based on the TPO-mediated conversion of the fluorescent horseradish-peroxidase substrate Amplex UltraRed to a fluorescent product, using rat thyroid gland microsomes as the source of TPO activity (Paul *et al*., 2014).

7.1.2.4. Selection of *in vitro* bioassays performed in this thesis

It is assumed that there is no 'safe dose' for some genotoxic chemicals, they can exert effects even at very low concentrations without thresholds such as DNA reactive substances. This precautionary approach is based on a mechanistic model, considering the theoretical possibility that a single molecule could cause a DNA lesion, potentially leading to a mutation (EFSA, 2011). Endocrine-disrupting chemicals can also exert effects at very low concentrations. A NMDR effect, where the response to increasing doses of a chemical is not linear, was observed for some

endocrine-disrupting chemicals. This indicates that significant effects can occur at certain low doses, making responses less predictable (Vandenberg *et al*., 2012).

Therefore, it is crucial to assess the genotoxicity and endocrine-disrupting properties of NIAS able to be released from FCMs.

- Selected bioassays to test identified NIAS with a commercial standard

EFSA recommends employing a combination of two regulatory bioassays, namely the bacterial Ames test for gene mutation and the *in vitro* micronucleus assay for chromosome aberration and number change, as the initial step in testing the mutagenicity of chemicals. These two bioassays together cover all modes of action of mutagenic chemicals (EFSA, 2011). For identified NIAS with a commercial standard, it was decided to follow EFSA's recommendations and assess the mutagenicity using the standard Ames test and the *in vitro* micronucleus assay, as there is no limitation regarding the quantity of identified NIAS. However, for the *in vitro* micronucleus assay, it was decided to use flow cytometry-based micronucleus assay to simplify the reading process, as standard method is known to be time-consuming and laborious.

Furthermore, to validate the *in silico* positive alert regarding sodium/iodide symporter (NIS1) activity for the identified NIAS with commercial standard, an iodide intake assay for sodium iodide symporter (NIS1) function was conducted based on the iodide-catalyzed Sandell-Kolthoff reaction between arsenic (III) and cerium (IV) according to Waltz *et al*. (2010).

- Selected bioassays for testing of FCMs extracts and migrates

As previously mentioned, while the standard Ames test is recommended by EFSA for assessing the mutagenicity of chemicals, it may not be the most suitable option for testing the mutagenicity of extracts or migrates from FCMs. Standard Ames test typically requires larger quantities of test chemicals, a key issue when dealing with FCMs where NIAS occur at low concentrations. Conversely, miniaturized versions are more suitable for mutagenicity testing of FCMs extracts/migrats (Rainer *et al*., 2019). Several miniaturized variants of the Ames test, which demonstrate similar concordance to the traditional method, have been developed (Flamand *et al*., 2001; Diehl *et al*., 2000; Pant *et al*., 2016; Flückiger-Isler and Kamber, 2014). For instance, Flamand *et al*. (2001) introduced the MMT, a miniaturized bacterial reverse mutation test conducted using 6-well plates (OECD, 2022).

In this thesis, it was decided to use the MMT over other miniaturized versions of the Ames test to assess the mutagenicity of FCMs. The MMT operates on the same principles as the traditional Ames test but the quantities of reagents are reduced by a factor of five. Similar to standard Ames test MMT effectively identifies point mutations but not structural and numerical chromosome aberrations.

Hence, adhering to EFSA's recommendation of using battery of bioassays to cover all modes of action of mutagenic chemicals (EFSA, 2011), DDR test was also selected to analyze FCMs extracts and migrates, as this bioassay exhibits remarkable sensitivity (92.1%) and specificity (96.2%). It not only identifies clastogenic (γH2AX) (Matsuzaki *et al*., 2010; Kinner *et al*., 2008; Rogakou *et al.,* 1999) and aneugenic (phospho-histone H3) (Wilde *et al*., 2020) biomarkers but also measures the induction of genotoxicity biomarkers such as p53 (Bhaskaran *et al*., 1999), thus providing comprehensive insights compared to the *in vitro* micronucleus assay.

To evaluate the matrix effect, FCMs extracts and migrates were tested in the MMT and DDR assays, with or without spiking with known positive references substances.

Additionally, to assess the potential carcinogenicity of the FCMs extracts and migrates, CTA was performed. This assay can detect chemicals that act as tumor initiators and promoters (Sasaki *et al*., 1988). The potential carcinogenicity of these mixtures was assessed comprehensively to verify *in silico* alerts for carcinogenicity of several chemicals identified within these mixtures.

7.2. Exposure assessment of identified and unidentified non-intentionally added substances

To assess exposure to NIAS migrating from FCMs, it is crucial to gather data on daily food consumption and the presence or absence of specific NIAS in each food products individuals consume (ILSI, 2007).

Once the 'safe exposure level' for a particular NIAS has been determined, the next step is to evaluate exposure. Initially, a conservative screening approach is used, which relies on the concentration of the substance and food consumption data. If this calculated exposure exceeds the established 'safe exposure level,' a stepwise-tiered approach is employed to refine the exposure estimate. This approach includes deterministic, refined deterministic, and probabilistic

methods. Cumulative exposure is calculated by summing exposure data for each type of packaged food item and comparing it to an appropriate threshold, while also considering uncertainties in the process (ILSI, 2015).

Conducting migration tests provides a means to quantify both predictable and unpredictable NIAS migrating from FCMs (EU, 2011).

For the estimation of the migration of predictable NIAS, there are two distinct approaches available:

- The worst-case scenario calculates the total mass transfer from the material or article to the foodstuff. This approach assumes 100% transfer and, as a result, tends to overestimate the actual migration levels, presenting the most extreme migration scenario (ILSI, 2023).
- Simulation using mathematical models that consider various factors such as material properties, diffusion rates, contact conditions, and other relevant parameters to replicate the migration process. This method offers a more sophisticated and realistic prediction of migration compared to the worst-case scenario, yielding a more accurate representation of the real-world migration levels (ILSI, 2023).

For plastic FCMs, it is commonly assumed that 6 dm^2 of packaging material approximately equals 1 kg of packaged food. This ratio allows for straightforward calculation of exposure, which can then be expressed as kg bw per day. However, it is important to recognize that this standard ratio may not always apply, particularly in cases like pizza boxes where the actual ratio of packaging material to food mass can differ significantly. In such situations, a detailed assessment of the surface-to-volume ratio is crucial (EU, 2011). Exposure should be also calculated for children if canned food is intended for this population group as children are the most sensitive population group with the lowest bw.

By using the migration data from the packaging, it is possible to calculate the exposure using the following equation:

Exposure [µg/person/day] = migration [µg/dm^2] × surface area [dm^2/person/day] (ILSI, 2015).

The toxicological data required for NIAS depends on the level of exposure. The EFSA has proposed a tiered approach to toxicity testing for substances migrating from FCMs. Genotoxicity testing is mandatory, even when migration results in low exposure levels. Additionally, three thresholds of human exposure—1.5, 30, and 80 µg/kg bw per day—are suggested as triggers for requiring further toxicity data (Table 1-14) (EFSA, 2016a).

Table 1-14. A tiered approach to toxicity testing proposed by EFSA (EFSA, 2016a)

Tier number	Specification of tier	Required toxicity data
Tier 1	If human exposure is up to 1.5 µg/kg bw per day or if the substance is classified as Cramer class I and exposure is less than 30 µg/kg bw per day.	Genotoxicity studies and available information including an appropriate literature search is required.
Tier 2	If human exposure is from 1.5 to 80 µg/kg bw per day.	In addition to toxicity data mentioned for Tired 1 extended 90-day oral toxicity study in rodents is required.
Tier 3	If human exposure is higher than 80 µg/kg bw per day.	In addition to data mentioned in Tired 1 and 2 studies on ADME, reproduction and developmental toxicity, long-term toxicity/carcinogenicity is required.

Consumer exposure estimates for FCMs have been updated using EFSA's Comprehensive European Food Consumption Database, replacing older assumptions with more accurate, age-specific data. This new model, which sets higher food consumption values for infants and toddlers across four food categories, offers greater protection than the previous SCF default model (EFSA, 2016a).

7.3. Risk assessment of non-intentionally added substances

7.3.1. Risk assessment of identified non-intentionally added substances

Identifying hazards associated with NIAS requires a comprehensive review of scientific data obtained from databases, literature, and research studies. The process prioritizes human studies, followed by animal studies and *in vitro* bioassays, with read-across and (Q)SAR analysis used as a last resort. Hazard characterization involves linking the dose to toxicity to establish safe exposure

levels. Toxicological studies might be unnecessary if the properties of the NIAS are already well-known (ILSI, 2015).

Figure 1-17 shows common methods of NIAS identification and characterization. For IAS required toxicological information is associated with the level of IAS migration, with higher migration levels requiring more comprehensive data. Regardless of the migration level, a genotoxicity assessment is necessary (EFSA, 2008). A similar approach can be applied for NIAS from FCMs (EFSA, 2016a). In cases where such data is lacking, the TTC approach can be employed if the structure of the NIAS is known and consumer exposure can be measured (Kroes *et al.*, 2004).

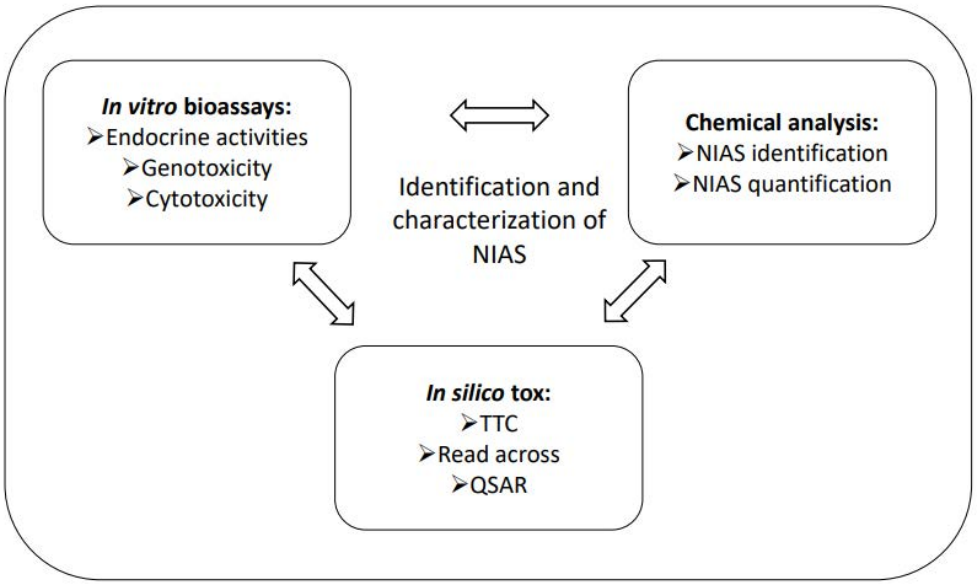


Figure 1-17. Common methods for NIAS identification and characterization

In 2008, EFSA published guidelines for assessing the safety of IAS in plastic FCMs (EFSA, 2008). However, no equivalent guidance exists for NIAS, making their risk assessment particularly challenging. Conventional assessment methods are often inapplicable due to limited information on NIAS formation, sources, presence, and toxicity.

A major obstacle is the lack of standardized analytical methods and the limited sensitivity of current techniques, which are often inadequate for detecting trace levels of NIAS in complex matrices (ILSI, 2015). Risk assessments are typically triggered when NIAS concentrations exceed the regulatory threshold of 0.01 mg/kg, as set by Commission Regulation (EU) 10/2011 (EU, 2011). This threshold is based on the analytical limit of detection (LOD), not on toxicological data.

While the LOD reflects the minimum amount detectable by an instrument, it does not correlate with health risk.

Comprehensively identifying and evaluating all NIAS is often unfeasible. These substances frequently occur in complex mixtures at low concentrations, and many pose minimal risk.

Additionally, the lack of commercial standards and the costly, labor-intensive process of synthesizing these substances for identification, quantification, and toxicological assessment further complicates the task. As a result, the focus has shifted to non-targeted screening approaches, combining advanced analytical techniques, *in vitro* bioassays, and *in silico* toxicological models (ILSI, 2015).

For certain NIAS, toxicological assessments conducted by national or European authorities provide TDI values, which are crucial for hazard characterization during risk assessment. However, when these official toxicological evaluations are not available, an alternative approach is to seek toxicological data from existing literature (ILSI, 2015). Several databases exist allowing research of information regarding the target substances from FCMs, such as Chemsec's SIN List (**https://sinlist.chemsec.org/**), List of SVHC of REACH or databases developed by Food Packaging Forum, such as Food Contact Chemicals database (FCCdb) and database on Migrating and Extractable Food Contact Chemicals (FCCmigex) (**https://foodpackagingforum.org/resources#databases**).

7.3.1.1. Threshold of toxicological concern approach

As previously mentioned, NIAS include impurities, contaminants, and degradation products that are often found in food at trace amounts. These substances frequently lack sufficient toxicological data in the literature, making it difficult for toxicologists to assess the associated risks. In some cases, the amount of NIAS migrating from FCMs is substantial enough to produce numerous chromatographic peaks using analytical instruments. The synthesis and toxicity testing of chemicals through various bioassays is a labor-intensive and costly process, especially when dealing with a large number of chemicals. Therefore, a practical solution is needed, one that does not require extensive toxicological data but still allows for the determination of "safe exposure levels" for chemicals with known structures. To address this issue, scientists have introduced the

concept of the TTC approach. Initially proposed by Cramer, Ford, and Hall. The TTC concept serves as a preliminary assessment tool for substances present at low levels in food, helping to decide whether a comprehensive risk assessment or additional toxicological testing is necessary (EFSA, 2019b; Kroes *et al*., 2004; Cramer *et al*., 1976). The TTC principle involves establishing a generic exposure level for chemicals, below which potential health risks are considered minimal. This entails evaluating actual or anticipated exposure levels against this threshold to assess the acceptability of associated risks. If the estimated exposure exceeds the applicable TTC threshold, a non-TTC approach is necessary to ascertain potential adverse health effects (EFSA, 2019b; Munro *et al*., 2008; Kroes *et al*., 2005).

When implementing the TTC approach, it is imperative to assess first the potential for DNA-reactive mutagenicity and/or carcinogenicity. This evaluation should rely on the weight of evidence. In cases where experimental data is lacking, evidence can be derived from read-across from chemicals with similar structures, the utilization of structural alerts, or employing (Q)SAR models (EFSA, 2019b).

To enhance the reliability of predictive tools, it is advisable to employ a minimum of two independent (Q)SAR models, each based on distinct training sets or algorithms. This dual approach, encompassing both knowledge-based and statistically based models, enhances the sensitivity and specificity of predictions (EFSA, 2016b). Distinct TTC values are assigned to potential DNA-reactive mutagens and/or carcinogens (0.0025 µg/kg bw per day), as well as organophosphates or carbamates (0.3 µg/kg bw per day) due to their neurotoxic effect (Table 1-15). These assigned values are lower compared to chemicals classified under various Cramer classes (EFSA, 2019b).

Cramer's decision tree employs a classification system based on a set of 33 questions (with yes/no responses) related to chemical structure, as well as occurrences in both food and the body. This allows the categorization of substances into three distinct classes: Class I with a low order of toxicity, Class II with a moderate order of toxicity, and Class III with a high order of toxicity (Table 1-16). TTC values for each Cramer Class are presented in Table 1-15 and were calculated based on a daily food consumption of 1 kg per person weighing 60 kg.

In the present day, the implementation of the Cramer classification system has evolved to encompass computer-based applications such as Toxtree (current version v3.1.0, as of May 2018) and the OECD QSAR Toolbox (current version v4.2, as of February 2018).

Table 1-15. TTC values based on classification of substances according to guidance of EFSA (2019b) (bw: body weight, values are calculated for an adult with bw of 60kg)

Classification	TTC value in µg/person per day	TTC value in µg/kg bw per day
Potential DNA-reactive mutagens and/or carcinogens	0.15	0.0025
Organophosphates and carbamates	18	0.3
Cramer Class III	90	1.5
Cramer Class II	540	9.0
Cramer Class I	1,800	30

Table 1-16. Description of Cramer classes (Cramer *et al.*, 1976)

Cramer class	Description of Cramer class	Structures included in the Cramer class
Class I	Substances characterized by simple chemical structures and effective metabolic pathways, implying minimal oral toxicity.	normal constituents of the body (excluding hormones); simply-branched, acyclic aliphatic hydrocarbons; common carbohydrates; common terpenes; substances that are sulfonate or sulfamate salts, without any free primary amines
Class II	Substances characterized by less innocuous structures than Class I, but lacking toxic structural features like Class III.	common components of food; substances containing no functional groups other than alcohol, aldehyde, side-chain ketone, acid, ester, or sodium, potassium or calcium sulfonate or sulfamate, or acyclic acetal or ketal and are either a monocycloalkanone or a bicyclic substance with or without a ring ketone
Class III	Substances characterized by structures lacking an initial presumption of safety, exhibiting potential toxicity, or possessing reactive functional groups.	structures that contain elements other than carbon, hydrogen, oxygen, nitrogen or divalent sulfur; certain benzene derivatives; certain heterocyclic substances; aliphatic substances containing more than three types of functional groups

Prior to applying the TTC approach in risk assessment, it is crucial to understand that this method is not suitable for substances with established toxicity data. In such cases, the risk assessment should rely on these data rather than on the TTC approach. Therefore, the initial step involves searching for toxicity data related to the substance (or a similar compound) and assessing data availability for a substance-specific risk assessment, including any relevant read-across considerations (EFSA, 2019b).

TTC approach should not be applied in several cases listed in Table 1-17.

Table 1-17. List of substances to which the TTC approach is not applicable (EFSA, 2019b)

Substance	Description
Substance for which there are well-established toxicity data	
Substance falls within the scope of EU food/feed legislation requiring toxicity data submission	Examples include, food and feed additives, novel food, pesticides, food packaging additives or monomers
Substances not represented in the database or outside the domain of applicability	- Inorganic substances, - Proteins, - Nanomaterials, - Radioactive substances, - Organosilicon substances, - Metals in elemental, ionic, or organic form (however, TTC can be applied for organic salts with essential metal counter ions (e.g., sodium)).
Substances with special properties	- High potency mutagenic carcinogens (referred to as the “cohort of concern”, comprises aflatoxin-like-, N-nitroso-, and alkyl-azoxy compounds.) - Steroids - Substances with a potential for bioaccumulation (e.g., substances like polyhalogenated-dibenzodioxins, -dibenzofuransand -biphenyls)

Regarding NIAS, TTC approach can be applied as long as NIAS can be identified and their structure elucidated. In the recent opinion of EFSA it was suggested to use challenge tests with surrogate contaminants to evaluate decontamination capability of recycling processes regarding recycled polyethylene terephthalate (PET). A reference contamination level of 3 mg/kg PET is set for post-consumer PET. Residual concentrations of surrogates in recycled PET are compared to modeled concentrations, ensuring migration does not exceed a dietary exposure of 0.0025 µg/kg bw/day

which is the lowest TTC value for potential genotoxicity. Recycling process with effective decontamination capability will allow to use recycled PET with unknown NIAS (EFSA, 2024).

7.3.1.1.1. Application of the threshold of toxicological concern approach to not fully defined chemical mixtures

To apply the TTC approach for partially characterized mixtures, such as FCM's extracts/migrates, it is crucial to provide information or analysis to confirm the absence of substances falling under exclusion categories. In such cases, unidentified mixture components may be assumed DNA-reactive, invoking a TTC value of 0.0025 µg/kg bw for their cumulative sum. In the event that an evaluation establishes the absence of DNA reactivity concerns and verifies the non-inclusion of organophosphates or carbamates within the mixture, the mixture may be considered directly Cramer Class III (EFSA, 2019b).

Considering that FCMs are complex mixtures with many unknown and unpredictable substances (NIAS), making complete identification impractical as most NIAS are present at very low concentrations, often posing minimal safety concerns, a pragmatic, science-based approach is needed to prioritize and assess unidentified NIAS based on exposure and toxicological relevance.

7.3.2. Risk assessment of unidentified non-intentionally added substances

For NIAS that are detected but remain unidentified, analytical chemistry testing is essential for structure elucidation. When full identification of these NIAS is not possible, partial characterization can be used to classify them into specific chemical groups. If partial identification fails to provide sufficient information for risk assessment, an alternative approach is to apply the CoMSAS method (see below), as proposed by Koster *et al*. (2014). The application of this method allows for the prioritization of unknown NIAS that may pose potential health concerns within the risk assessment framework (ILSI, 2015).

7.3.2.1. Complex mixture safety assessment strategy for unknown (unidentified) non-intentionally added substances

FCMs are usually complex matrixes consisting of various starting materials. Additionally, the potential for contamination and impurities in these materials, along with the formation of

breakdown products during the entire supply chain, adds to their complexity. Research indicates that analyzing chromatograms of FCMs often reveals an intricate pattern of numerous peaks, resembling a 'forest of peaks' in chromatography (Figure 1-18) (Schilter *et al.*, 2019; Grob *et al*., 2010).

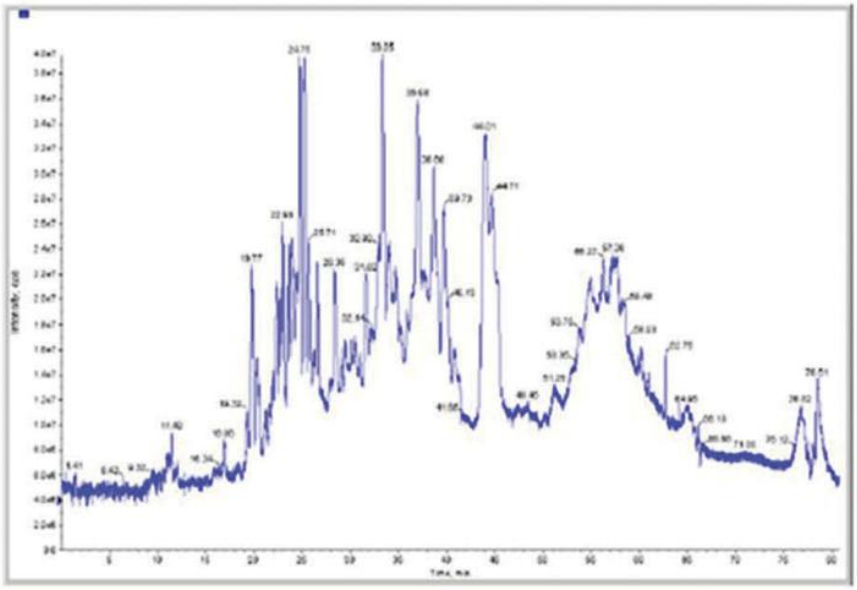


Figure 1-18. Illustrative examples of forests of peaks (Schilter *et al*., 2019)

When the number of detected NIAS is high, it becomes challenging to provide sufficient evidence that these substances in FCMs do not pose a safety issue. It is evident that achieving a complete identification of all detected NIAS is not practical due to its time-consuming nature. Also, for most NIAS reference standards are lacking making it even more difficult for the identification of these molecules. Moreover, it is undesirable to identify all NIAS since many of these substances exist at low concentrations, and their exposure levels may be minimal, posing negligible safety concerns for consumers.

Therefore, there is a crucial need for a scientifically based approach that enables a pragmatic safety assessment of unknown (unidentified) NIAS present at low exposure levels in food contact extracts.

The complex mixture safety assessment strategy (CoMSAS) described by Koster *et al*. (2014) was developed to overcome this problem. Within the risk assessment framework, it enables the prioritization of unidentified NIAS that might raise health concerns. By categorizing NIAS into

those with potential toxicological importance and those of comparatively lesser significance, determined by their individual exposure levels, the workload required for identifying, characterizing, and evaluating unfamiliar compounds present at low concentrations can be significantly minimized.

CoMSAS allows estimation of exposure to unknown NIAS, if the exposure of a substance in question is below certain thresholds, it may be considered not of health relevance in case specific classes of substances are excluded.

CoMSAS is a stepwise approach using targeted analytical techniques to exclude specific groups of compounds following the TTC decision tree of Kroes *et al*. (2004) and modifications as proposed by Munro *et al*. (2008) to point out NIAS for which further substances specific risk assessment is necessary (Koster *et al*., 2014; Rennen *et al*., 2011).

From an analytical perspective, the process demands less effort for identification as it is not using the conventionally used LOD (10 µg kg^{-1} food for substances migrating from the FCMs) but an exposure threshold set at 90 µg day^{-1}. This methodology covers certain high toxic substances (genotoxic, mutagenic, carcinogenic, reprotoxic chemicals). The exposure threshold employed within this approach aligns with the threshold set for substances falling under Cramer class III, as proposed by Kroes *et al*. (2005). Nevertheless, this exposure threshold is applicable exclusively under circumstances where there is substantiated evidence indicating that the unidentified NIAS does not belong to chemical groups characterized by high toxicity or substances excluded by the TTC approach (Koster *et al*., 2014; Rennen *et al*., 2011; Kroes *et al*., 2005).

CoMSAS consists of five steps which are shown in Figure 1-19. If the exposure of substances exceeds the exposure threshold of 90 µg day^{-1} (step 1) they should be identified and submitted to a substance-specific risk assessment.

The exclusion or detection of highly toxic substances or chemicals falling within the exclusion category according to TTC approach can be achieved through the utilization of targeted analytical techniques. Nevertheless, it becomes challenging to eliminate substances with structural alerts for genotoxicity, particularly when their number is high knowing that exist 39 recognized structural alerts that are associated with this specific endpoint (Benigni *et al*., 2013). Koster *et al*. (2014) propose a practical strategy involving the utilization of genotoxicity bioassays to

effectively rule out the presence of substances with genotoxic potential within a mixture. Particularly they recommend the use of the BlueScreen HC assay, a sensitive test for gene mutations, clastogenicity and aneugenicity which covers all modes of action of genotoxic chemicals.

Upon receiving a positive response from the bioassay, the key priority is to pinpoint the chemical responsible for the positive effect. If direct identification is unachievable, the focus shifts toward separation of the substance from the mixture. It can be carried out by fractionation of FCMs mixture with preparative size-exclusion chromatography. Subsequently, different fractions of the mixture undergo genotoxicity bioassay testing, enabling the identification of the migrate(s) responsible for triggering the genotoxic response. It is important to keep in mind that 'matrix' effect may also affect the overall toxicity of the mixture and negative response is possible for all tested fraction if the positive effect was due to cumulative/ matrix effect (Koster *et al.*, 2014).

If the potential existence of NIAS with genotoxic capabilities cannot be ruled out, an exposure threshold of 0.15 µg/person per day (0.0025 µg/kg bw per day), as stipulated by the TTC, is considered. This threshold designates the point below which no significant health risks are presumed to arise from a substance. Substances exhibiting potential genotoxicity and being present at exposure levels exceeding 0.15 µg/person per day (0.0025 µg/kg bw per day) necessitate a thorough substance-specific risk assessment for comprehensive evaluation (Koster *et al.*, 2014).

The final step involves excluding allergenic effects using literature data and/or targeted methods for known allergens. It is important to note that proteins, which can cause sensitization and allergic reactions, are excluded from the TTC approach. Known allergenic proteins (e.g., soy, casein, peanut) and the sensitizing potential of novel proteins must be evaluated when relevant. In addition, allergenicity of FCMs extracts may be linked to the use of biobased materials (Mossburger and Scherf, 2024; Koster *et al*., 2014).

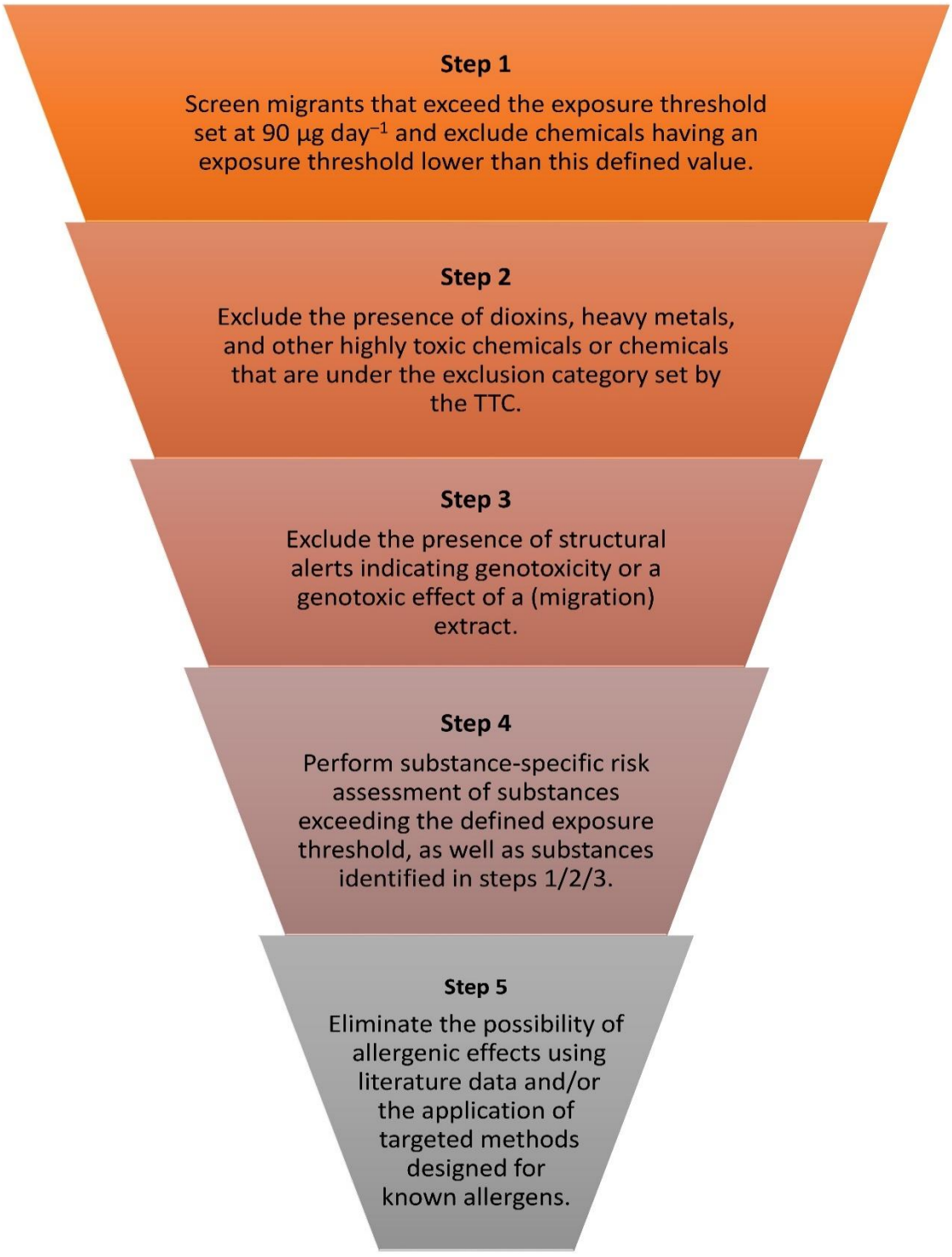


Figure 1-19. Representation of complex mixtures safety assessment strategy (CoMSAS) described by Koster *et al*. (2014)

8. Overview of validation and international acceptance of new or updated test methods for hazard assessment

Current regulatory *in vitro* genotoxicity assays show low sensitivity for detecting FCMs genotoxins. The Ames test (OECD TG 471), for instance, is difficult to apply with large sample numbers or limited sample quantities, as seen with NIAS, and often fails to detect low-concentration genotoxins. Although miniaturized Ames versions have been developed, none are yet accepted as alternatives to the standard test. Chapter 6 of this thesis presents the pre-validation of the NanoAmes bioassay, a miniaturized Ames test for detecting low-level genotoxic chemicals. Bioassays were conducted by Packtox and GenEvolution, testing genotoxic and non-genotoxic reference chemicals with and without S9 mix using S*. typhimurium* strains TA98 and TA100, to evaluate the assay's repeatability and reproducibility. *S. typhimurium* strains TA98 and TA100 with or without metabolic activation were used for interlaboratory validation study as these strains can detect up to 89% of mutagens (Zeiger *et al.*, 1985).

Before proceeding to an interlaboratory validation study, it is crucial to understand the requirements of the organization for economic co-operation and development (OECD) for bioassay validation and the international acceptance process for new or updated test methods. This thesis provides a brief overview of these validation principles.

In 2005, the OECD published guidance document on validation and international acceptance of new or updated test methods for hazard assessment (OECD, 2005). The guidance outlines key validation principles, focusing on test reliability, relevance, and regulatory approval when implementing new testing methods. It incorporates insights from past OECD workshops and is designed to support regulatory agencies, industry experts, and researchers in implementing new testing methods. Test method validation is essential for ensuring the consistency and accuracy of hazard assessment procedures (OECD, 2005). OECD outlines key principles for validation of new or updated test methods for hazard assessment, regardless of whether they are *in vivo* or *in vitro* tests, or whether they assess impacts on human health or the environment, known as the 'Solna Principles' which include:

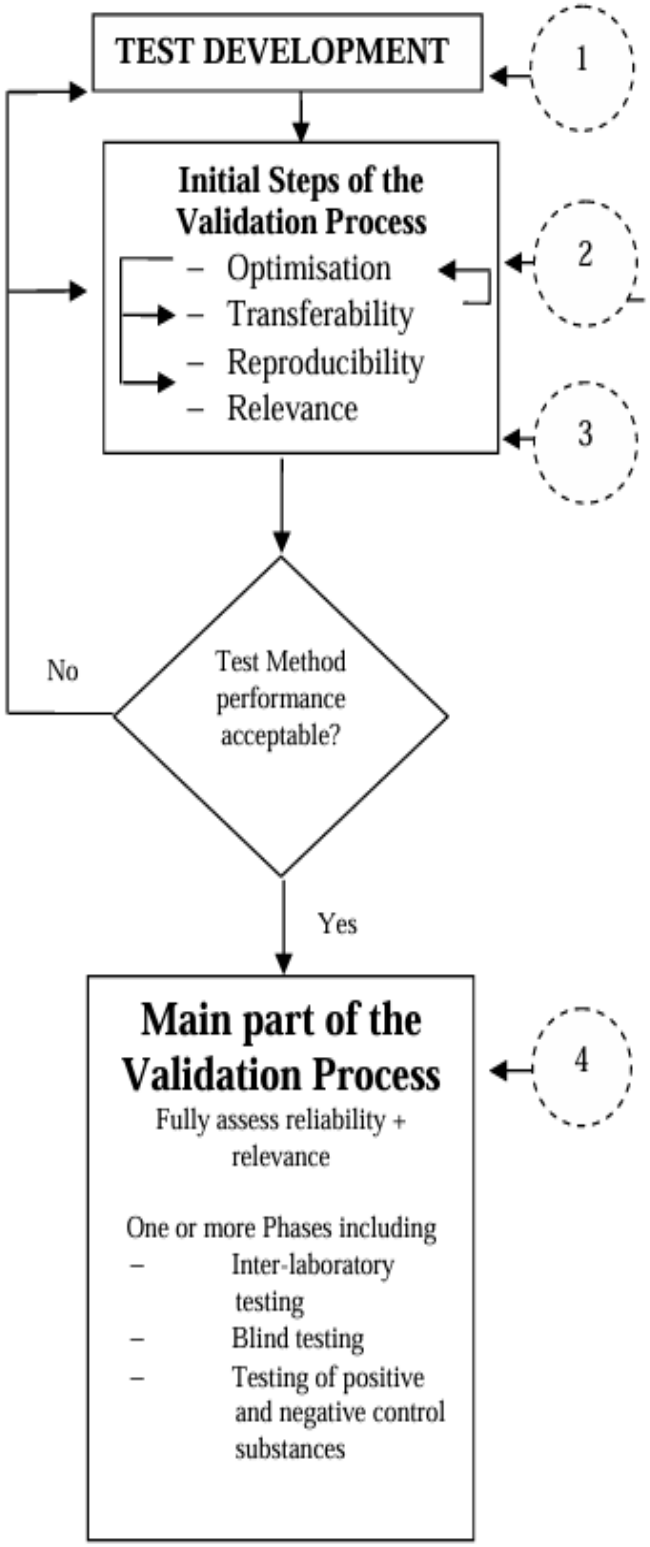
1) The test method should have a clear scientific and regulatory rationale.
2) Its endpoint(s) should relate to *in vivo* effects and toxicity, with limitations noted.

3) A detailed, publicly available protocol must include data analysis and decision criteria, with peer-reviewed results.
4) Intra- and inter-laboratory variability, repeatability, and reproducibility should be demonstrated.
5) Performance should be validated using preferably coded reference chemicals.
6) The method should be evaluated against existing toxicity data and target species information.
7) All validation data must be available for review.
8) Data should comply with OECD Principles of GLP principles (OECD, 2005).

A validated test may be recommended as an OECD TG if it meets key criteria of Solna Workshop. The following criteria are also important to consider:

1) Predicts the end-point of interest demonstrating either a linkage between (i) the new test and an existing test method or (ii) the new test and effects in the target species.
2) Provides risk assessment data as good as or better than existing methods.
3) Has sufficient data for relevant chemicals and products.
4) Is robust, transferable, allows standardization and avoids reliance on unique or patented equipment.
5) Is cost-effective and widely usable.
6) Justifies its use scientifically, ethically, and economically, considering animal welfare and the 3Rs (OECD, 2005).

Figure 1-20 and 1-21 illustrates the various stages and essential factors involved, from test development to validation and regulatory approval, for new and updated toxicology testing methods as outlined by the OECD (OECD, 2005).



PREVALIDATION

Point 1

Each element of a test (for example chemical exposure time, histopathology, clinical chemistry, etc.,) needs to be carefully explored and evaluated to determine the optimum conditions/details.

Point 2

At least one laboratory independent from the test method developing laboratory will conduct the assay for an initial assessment and review of its inter-laboratory transferability and reproducibility. In case the test method fails to provide sufficient reproducibility, depending on the degree of failure, it may or may not be considered for further optimisation (33, 34).

Point 3

Test methods may fail to show acceptable performance in terms of relevance rather than a lack of reproducibility. This could be the result of a weakness of the test method protocol. If further test optimisation does not solve the performance issue, redesign of parts of the test method may be in order or a rejection of the proposed test method should be considered.

INTER-LABORATORY VALIDATION

Point 4

In certain cases where the result of the prevalidation was not sufficient to justify further validation, but was nevertheless promising, a test method could, after refinement, proceed to the main part of the validation study, provided this phase is properly designed to deal with the imperfections. In this case it is recommended to perform the main validation study in more than one phase.

Figure 1-20. Steps from test development to validation and key steps for optimizing test method according to OECD (OECD, 2005)

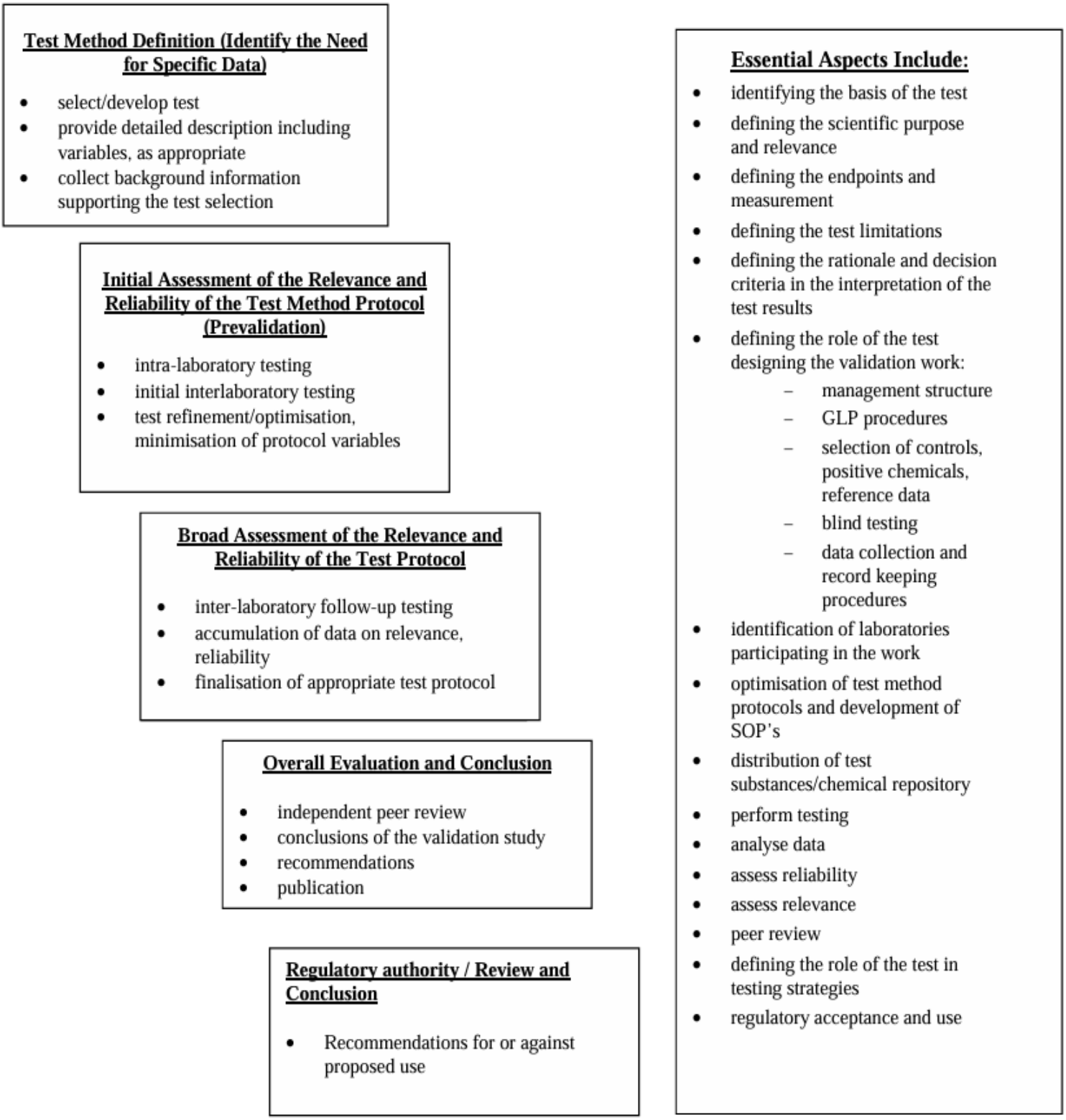


Figure 1-21. Important factors for the validation and regulatory approval of new and revised toxicology testing methods according to OECD (OECD, 2005)

Gourmelon and Delrue (2016) emphasized that while OECD validation principles continue to be relevant, adaptations are needed to keep pace with technological advancements in testing methods. A major challenge in chemical safety assessment is the need for integrated approaches to testing and assessment for complex endpoints. Alternative methods alone may not be adequate for evaluating intricate endpoints like reproductive toxicity and carcinogenicity. Developing effective test strategies requires a deep understanding of mechanisms of action and toxicity pathways. Additionally, regulatory acceptance depends on clearly defined validation objectives and carefully selected reference chemicals. Another challenge is the validation of high-throughput screening assays. These automated techniques are increasingly used in regulatory screening, but their validation may require modifications that prioritize technical reliability over inter-laboratory reproducibility.

To ensure regulatory approval, it is essential to establish a well-defined list of reference chemicals and maintain precise calibration of testing equipment (Gourmelon and Delrue, 2016).

The EURL ECVAM is a key institution in the EU, committed to advancing non-animal testing alternatives in scientific research, product safety assessments, and regulatory practices. Operating as part of the European Commission's Joint Research Centre, EURL ECVAM focuses on the development, validation, and promotion of scientifically robust alternatives, including *in vitro* methods, computational models, and cutting-edge technologies. Through collaboration with international regulatory bodies, research institutions, and industry partners, the laboratory ensures these alternative approaches are not only reliable and reproducible but also integrated into regulatory frameworks, driving their broader adoption (European Commission Joint Research Centre. EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM).

It is important to highlight that EURL ECVAM provides a list of chemicals suitable for assessing the sensitivity and specificity of new or modified mammalian cell genotoxicity tests, specifically to prevent misleading positive results. This list categorizes chemicals into three groups, as shown in Table 1-18 (Kirkland *et al*., 2016).

Table 1-18. Different group of chemicals recommended to be tested to evaluate sensitivity and specificity of new or modified mammalian cell genotoxicity tests (Kirkland *et al.,* 2016)

ECVAM group	Description
Group 1: chemicals that should be detected as positive in *in vitro* mammalian cell genotoxicity tests.	They are *in vivo* genotoxins (DNA-reactive or not) and often known mutagenic carcinogens, with some classified as probable aneugens.
Group 2: chemicals that should give negative results in *in vitro* mammalian cell genotoxicity tests.	They are usually non-genotoxic *in vivo*, often non-carcinogenic or rodent carcinogens with a non-mutagenic mechanism.
Group 3: chemicals that should give negative results in *in vitro* mammalian cell genotoxicity tests, but have been reported to induce gene mutations in mouse lymphoma cells, chromosomal aberrations or micronuclei, often at high concentrations or at high levels of cytotoxicity.	They are generally non-genotoxic *in vivo*, Ames test-negative, and either non-carcinogens or rodent carcinogens with a non-mutagenic mode of action.

In addition, the Guidance Document on Good In Vitro Method Practices which was published under a project coordinated by the EURL ECVAM provides best practices for developing and implementing *in vitro* methods in regulatory human safety assessments, aiming to prevent reproducibility issues in *in vitro* toxicological science. It offers guidance on both method development for chemical safety assessment and the laboratory environment for data generation (OECD, 2018a).

II. RESEARCH STRATEGY

This thesis is structured into 6 chapters each addressing critical aspects of the identification, characterization, and toxicity evaluation of NIAS from polyester coatings.

The overall research strategy involved the identification and characterization of volatile, semi-volatile, and non-volatile NIAS from polyester coatings. Extraction and migration tests were conducted using food simulants or extraction solvents, followed by the identification of migrating NIAS using various analytical tools. Hazard assessment of the identified substances was performed first through *in silico* predictive models and then relevant *in vitro* bioassays was performed for identified NIAS with available commercial standard to address specific alerts predicted *in silico*.

Beyond chemical analysis, extracts and migrates of polyester coatings were also evaluated for genotoxicity and potential carcinogenicity using *in vitro* bioassays.

Additionally, the fate of non-volatile NIAS in the gastrointestinal tract was studied using a new ACN extraction method on a polyester-coated tin plate. Chemical analysis was performed to detect parent NIAS, and the samples were subjected to simulated *in vitro* intestinal digestion to monitor both the parent compounds and their transformation products.

The last part of the thesis focuses on the pre-validation of the NanoAmes bioassay, a miniaturized version of the regulatory Ames test designed to detect genotoxic substances at very low concentrations. Genotoxic and non-genotoxic reference chemicals were tested, both with and without metabolic activation (S9 mix), using *Salmonella typhimurium* strains TA98 and TA100 at Packtox and GenEvolution laboratories. Results from both laboratories were compared to assess the reliability, repeatability, and relevance of the NanoAmes bioassay.

Chapters 1 to 4 (Figure 2-1) focus on the **identification and characterization of volatile, semi-volatile, and non-volatile NIAS from polyester coatings,** as well as t**heir toxicity assessment**. Extractions and migration tests were conducted using ACN (24 hours at 40°C) and EtOH 95% (10 days at 60°C) in migration cells. These tests involved a single polyester-coated tin plate (from five batches) and one uncoated plate to investigate the migration of NIAS from the polyester coatings. After the extraction or migration tests, samples were concentrated and analyzed using non-targeted approaches, including LC-HRMS and GC-HRMS.

For the identified NIAS (fenozan and four cyclic oligoesters), an *in silico* hazard assessment was performed using various *in silico* predictive tools. Relevant *in vitro* bioassays were conducted on identified NIAS for which a commercial standard was available (fenozan), to verify specific *in silico* alerts. These included:

- Ames test: To evaluate point mutations.
- Micronucleus assay: To detect clastogenic and aneugenic mutagenicity.
- Iodide uptake assay: To investigate *in silico* alerts for sodium/iodide symporter (NIS1) activity.

In addition to chemical analysis, the extracts and migrates from FCMs were tested for genotoxicity using *in vitro* methods such as the MMT and the DDR test. Furthermore, an *in vitro* CTA was employed to assess the potential carcinogenicity of FCMs extracts and migrates.

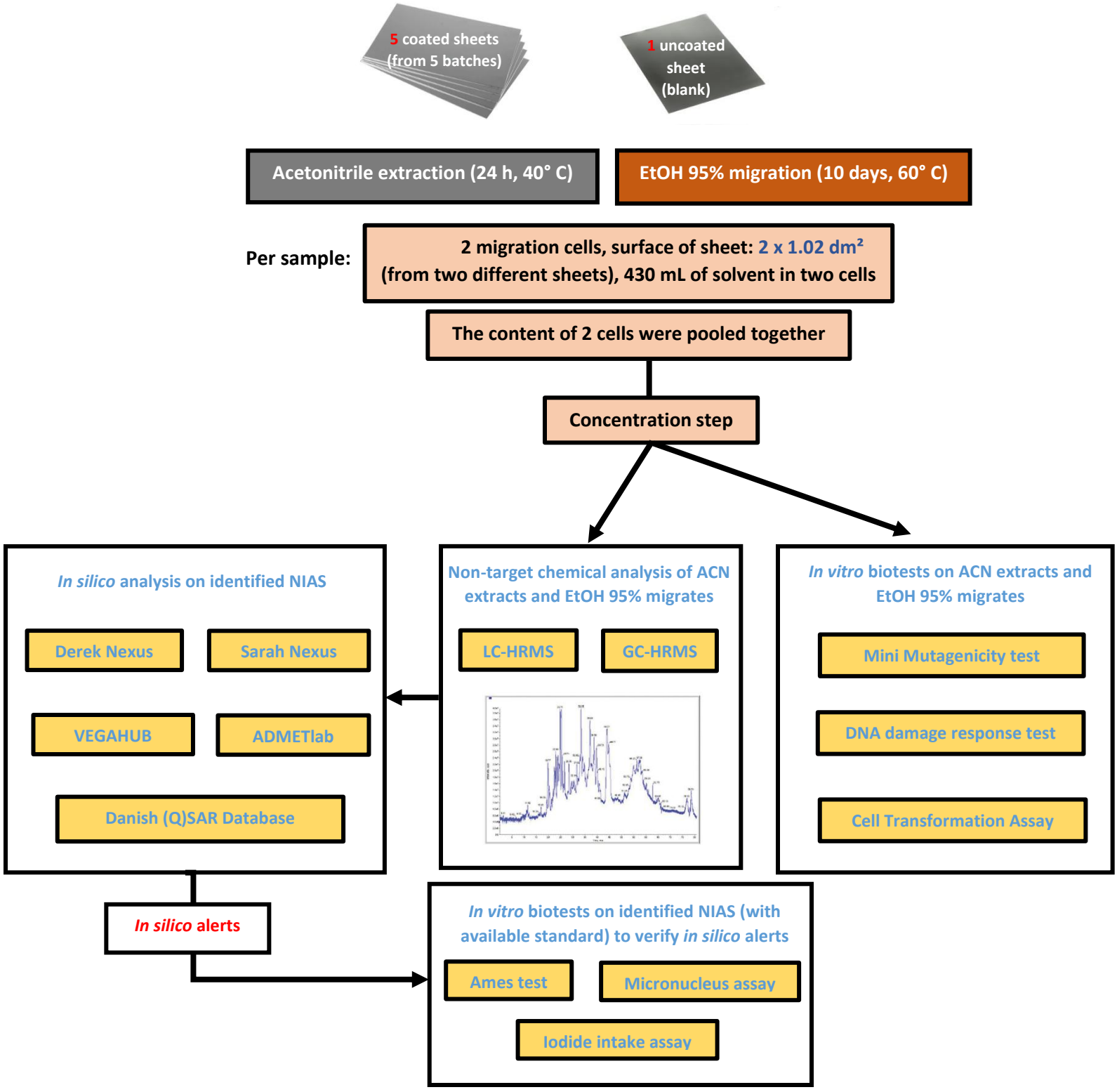


Figure 2 - 1. Illustration of the research strategy involved in the Chapters 1-4

Chapter 5 focuses on **identifying non-volatile NIAS originating from polyester coatings and examining their fate in the gastrointestinal tract** (Figure 2-2). A new ACN extraction was performed on a single polyester-coated tin plate (from five batches) by grating each coating and immersing it in the solvent for 24 hours at 40°C to obtain a more concentrated extract. After incubation, the samples were pooled and concentrated five -fold (CA_{mix} from now on). A chemical analysis of the CA_{mix} (50-fold concentrated) was conducted using LC-HRMS to detect as many parent NIAS as possible. Furthermore, the CA_{mix} was subjected to simulated *in vitro* intestinal digestion, and both the parent compounds and their anticipated transformation products were monitored using LC-HRMS.

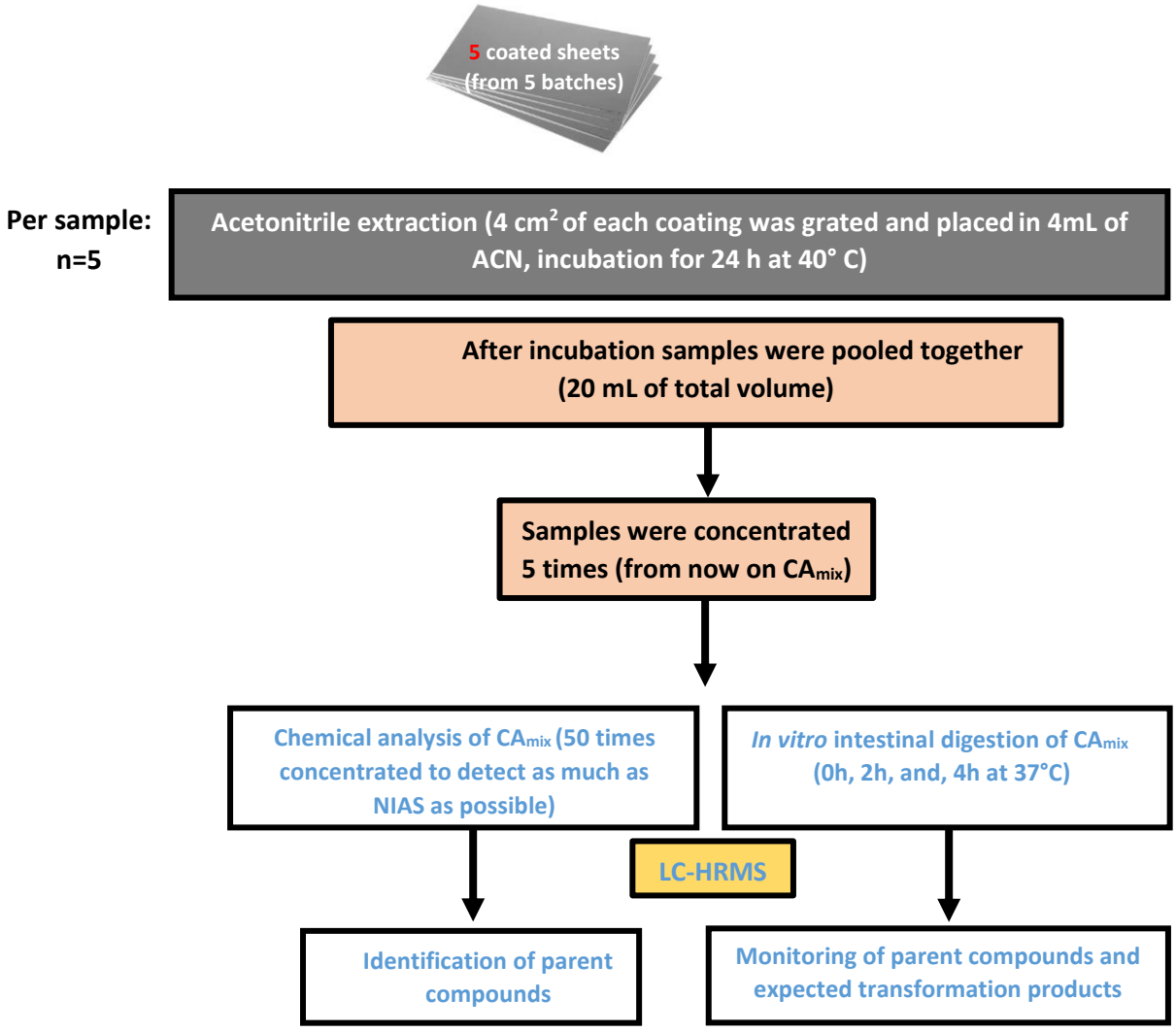


Figure 2 - 2. Illustration of the research strategy involved in the Chapters 5

Chapter 6 (Figure 2-3) focuses on a pre-validation of the NanoAmes bioassay, developed by the GenEvolution laboratory to detect genotoxic chemicals at very low concentrations. The bioassay was conducted by Packtox and GenEvolution laboratories using 14 reference chemicals as part of the initial validation process. This process involved testing a range of genotoxic and non-genotoxic reference chemicals, along with their respective negative solvent controls, both with and without a metabolic activation system (S9 mix), using *S. typhimurium* strains TA98 and TA100. The results from both laboratories were compared to assess the reliability and relevance of the NanoAmes bioassay and to evaluate its repeatability and reproducibility.

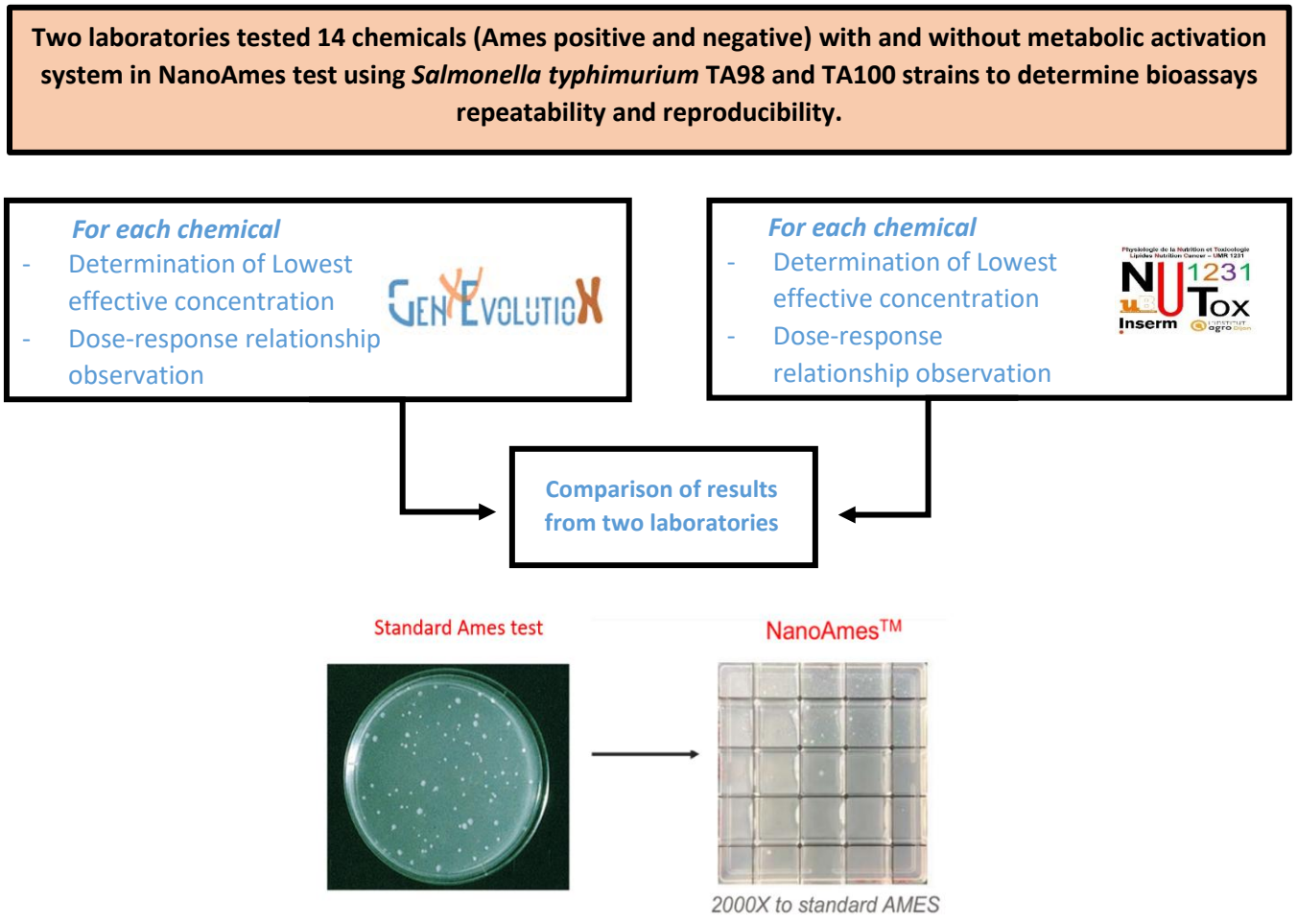


Figure 2 - 3. Illustration of the research strategy involved in the Chapters 6

III. RESULTS

Chapter 1

In silico hazard assessment of identified NIAS (fenozan and four cyclic oligoesters)

Summary

Introduction

The safety of polyester coatings in food cans remains a concern due to the possible migration of NIAS into food. Given the high number of NIAS able to migrate from FCMs and the lack of reference standards, comprehensive toxicological testing is not feasible, posing a major challenge for risk assessment using conventional *in vitro* and *in vivo* testing methods.

In this study, we identified fenozan (see Chapter 3) and four cyclic oligoesters (see Chapter 2) composed of BD, tPA, and TCDDM monomers —c(2TCDDM+2tPA), c(2BD+TCDDM+3tPA), c(BD+2TCDDM+3tPA), and c(3TCDDM+3tPA)—in ACN extracts and EtOH 95% migrates from polyester-coated metal plates. Hydrolysis is required to confirm the presence of specific monomer isomers, as LC-HRMS cannot distinguish them. Using Freelance Chamespider software, we generated all possible structural isomers of the identified oligoesters for toxicity assessment. The objective of this study was to evaluate the potential toxicity of identified NIAS—fenozan and four cyclic oligoesters—using i*n silico* QSAR tools, this assessment will provide a first-tier screening to prioritize compounds of concern, guiding future bioassays to confirm specific toxicological risks. Various QSAR tools—Derek Nexus v6.1, Sarah Nexus v6.1, VEGAHUB v1.1.5, the Danish (Q)SAR Database, and ADMETlab 3.0—were employed to predict genotoxicity, carcinogenicity, endocrine disruption, developmental toxicity, organ toxicity, and Log $k_{o/w}$ values for these NIAS.

Results

In silico hazard assessment of fenozan

Mutagenicity: VEGAHUB reported *in vitro* micronucleus activity with moderate reliability. The Danish (Q)SAR Database indicated DNA binding (OECD alert for alkyl phenols), frameshift Ames mutagens, mutations in thymidine kinase locus in mouse lymphoma *in vitro* cells, MN in mouse

erythrocytes *in vivo* (micronucleus test), dominant lethal mutations in rodents *in vivo*, and DNA breaks in the comet assay in mouse *in vivo*.

Carcinogenicity: The Danish (Q)SAR Database predicted carcinogenicity in mice and structural similarity to phenol-type compounds alert.

Organ Toxicity: VEGAHUB classified fenozan as hepatotoxic with low reliability. Derek Nexus also identified it as a plausible hepatotoxicant and gave an equivocal prediction for thyroid toxicity.

Endocrine Disruption: VEGAHUB and the Danish (Q)SAR Database predicted weak ER α binding. The latter also suggested potential disruption via thyroperoxidase inhibition, sodium/iodide symporter interference, and binding to pregnane X and constitutive androstane receptors.

Developmental Toxicity: VEGAHUB software predicted developmental toxicity with moderate reliability.

In silico hazard assessment of oligoesters

In total, 147 isomers were designed: 7 isomers for c(2TCDDM+2PA), 12 isomers for c(3TCDDM+3PA), 56 isomers for c(BD+2TCDDM+3PA), 72 isomers for c(2BD+TCDDM+3PA). LogP values of all four oligoesters are higher than 10.

Mutagenicity: All *in silico* tools predicted no mutagenic potential for any isomer. However, ADMETlab detected 3 alerts for genotoxic carcinogenicity toxicophore rule for some isomers of four identified oligoesters.

VEGAHUB classified all isomers as Cramer Class III, indicating a higher likelihood of toxicity.

Carcinogenicity: VEGAHUB predicted carcinogenicity for all four oligoesters. ADMETlab gave positive predictions only for isomers of two oligoesters—c(BD+2TCDDM+3PA) and c(2BD+TCDDM+3PA)—both containing the BD monomer.

Endocrine Disruption: Derek predicted no endocrine activity, while VEGAHUB indicated low-reliability ER binding for 27 isomers of c(2BD+TCDDM+3PA). Additionally, various isomers from all four oligoesters showed potential activity toward aromatase and ER binding domains with varying reliability.

Developmental Toxicity: VEGAHUB and Derek Nexus both showed potential developmental toxicity. VEGAHUB classified several isomers as active with low reliability, while Derek suggested plausible teratogenicity for multiple isomers.

Discussion

Fenozan

***Mutagenicity*:** Fenozan showed positive *in silico* predictions for mutagenicity in both VEGAHUB and the Danish (Q)SAR Database, mainly linked to alkylphenol-related DNA binding alerts. These compounds are known to form reactive quinone methides that can damage DNA OECD, 2010).

Endocrine Disruption: Low-reliability alerts for ERα binding and activation were observed in VEGAHUB and Danish (Q)SAR tools. Danish (Q)SAR also predicted potential interference with thyroid-related targets (thyroperoxidase, sodium/iodide symporter) and nuclear receptors (pregnane X and constitutive androstane receptors). These effects can be further assessed using validated assays such as STTA, iodide uptake inhibition assay, thyroperoxidase inhibition assay, human and mouse pregnane X receptor reporter gene assay and human constitutive androstane receptor-3 (CAR-3) assay (OECD, 2021; Paul *et al*., 2014; Waltz *et al*., 2010; Vignati *et al*., 2004).

Organ Toxicity: VEGAHUB and Derek Nexus predicted hepatotoxicity for fenozan with low to plausible reliability. Alerts were linked to structural similarity with 2-arylacetic acids, 3-arylpropionic acid and alkylphenols. Arylacetic and arylpropionic acids both associated with hepatotoxicity (Dong *et al*., 2005; Bailey and Dickinson, 2003; Zimmerman, 1999). Human hepatotoxicity data are few for alkylphenol or its derivatives, however, some studies demonstrated a small number of liver injuries as liver necrosis (Kamijo *et al*., 2003; Green, 1975). Derek Nexus also gave an equivocal alert for thyroid toxicity, based on similarity to 2,6-di-tert-butylphenol derivatives, which have shown thyroid effects in rodent studies (ANSES, 2016a).

Developmental Toxicity: VEGAHUB predicted fenozan as a developmental toxicant with moderate reliability. Its structure aligns with chemical groups known for developmental toxicity, such aromatic compounds containing alkyl chain with alcohol, aldehyde, acid functional groups; poly-Cl aryloxy derived acids and esters (Wu *et al*., 2013). Confirmation would require *in vivo* studies, such as OECD TG 416 two-generation tests (OECD, 2001).

*O*ligoesters

Four cyclic oligoesters identified in this study have molecular weights under 1000 Da meaning that they may be absorbed by human body (EFSA, 2008). LogP values of 10.94–12 suggest their bioaccumulation potential (EFSA, 2008).

Mutagenicity & Carcinogenicity: All isomers tested negative for mutagenicity but showed positive carcinogenicity alerts in VEGAHUB and ADMETlab, especially for those containing BD monomers. Structural alerts included polycyclic aromatics and phthalate- phthalate diesters and monoesters- for cyclics, suggesting possible tumor-promoting mechanisms. Literature results showed that genotoxicity and carcinogenicity are associated with aromatic hydrocarbons with three or more aromatic rings (EFSA, 2023a). CTA can be used to test pure chemicals for potential carcinogenicity.

Endocrine Disruption: Several isomers, particularly of c(2BD+TCDDM+3PA), showed potential ER binding and aromatase interaction, though reliability was variable. These findings can be further explored using STTA, or aromatase inhibition assays (Heidary *et al.*, 2021; OECD, 2021; Ji *et al.*, 2014). Ubeda *et al*. (2020) found mild AR antagonism in similar oligoesters, though with different monomeric origins.

Developmental Toxicity: VEGAHUB and Derek Nexus flagged all four oligoesters for potential developmental toxicity, linking some predictions to phthalate ester structures. This alert describes the teratogenicity of phthalates (EFSA, 2019).

Finally, the four cyclic oligoesters were classified as Cramer class III substances aligning with literature data, where cyclic oligoesters identified by various authors were similarly classified as Cramer class III substances (Eckardt *et al*., 2020, 2019; Lestido-Cardama *et al*., 2022b).

While QSAR is valuable for early hazard screening, its reliability varies by endpoint and model. It cannot predict toxicity of unknown mixtures and performs best when multiple tools and expert judgment are combined. Performance is particularly limited for endpoints beyond the Ames test and for substances outside a model's applicability domain (Benigni *et al*., 2019; Van Bossuyt *et al*., 2018; Cherkasov *et al*., 2014). These findings provide initial insights into potential toxicological hazard that require further risk assessment.

ARTICLE

1. **Introduction**

The safety of can's polyester coatings is in doubt regarding the migration of volatile and non-volatile NIAS from packaging to food as to date scarce toxicological data are available for these chemicals. In general, 'forest of picks' is obtained after chemical analysis of FCM's extracts and migrates (Grob *et al*., 2010). Thorough toxicological analysis of all identified NIAS is not practical as their number in FCMs is high and very few reference standards are available allowing their toxicity testing in different *in vitro* or *in vivo* bioassays.

Once the NIAS structure is identified, predictive *in silico* QSAR tools can prioritize substances with the highest concern. Substances predicted positively by these tools can subsequently undergo testing in various *in vitro* or *in vivo* bioassays to validate predictive outcomes.

In silico QSAR models utilize various computational resources, such as databases that store data on the physicochemical and toxicological properties of molecules, software capable of generating molecular descriptors, statistical modelling packages, expert systems, toxicity prediction modelling methods, and simulation and visualization tools (Raies and Bajic, 2016).

In the frame of this manuscript, we identified fenozan (refer to Chapter 3) and 4 cyclic oligoesters (refer to Chapter 2) composed of BD, tPA, and TCDDM monomers ((c(2TCDDM+2tPA), c(2BD+TCDDM+3tPA), c(BD+2TCDDM+3tPA), c(3TCDDM+3tPA)) in polyester-coated metal plates' ACN extracts and EtOH 95% migrates.

Different QSAR tools were used to predict the toxicity and Log $k_{o/w}$ (octanol-water partition coefficient) value of fenozan and the different isomers of four cyclic oligoesters as scarce information was available for these chemicals in the literature. Two complementary QSAR prediction methodologies are recommended to be applied to predict mutagenicity of chemicals: one should be expert rule-based, and the other should be statistically based (Benigni *et al*., 2019). In addition, combined use of several QSAR tools are demonstrated to improve performance of the prediction (Benigni *et al*., 2019).

The Log $k_{o/w}$ is an important parameter in toxicology and chemical safety assessment. It serves as a key indicator of a substance's lipophilicity, directly affecting its bioavailability, bioaccumulation potential, toxicological behavior, and environmental fate. According to EFSA, if Log $k_{o/w}$ value is below 3 the substance has no potential for accumulation in the mammalian body, however, if Log $k_{o/w}$ value is 3 or higher, it does not necessarily indicate accumulation in the body,

and additional investigation is required to confirm the potential for accumulation, hence, prediction for Log $k_{o/w}$ value remains important parameter to investigate potential toxic effect of chemicals (EFSA, 2008). QSAR analysis were performed using Derek Nexus v 6.1 (Lhasa Limited, Leeds, UK), Sarah Nexus v 6.1 (Lhasa Limited, Leeds, UK), VEGAHUB 1.1.5 software and Danish (Q)SAR Database (2015; **http://qsar.food.dtu.dk/**) and ADMETlab 3.0 (**https://admetmesh.scbdd.com/**) online tools. CAS registry number or simplified molecular input line-entry system (SMILES) formulas of each molecule were introduced in each QSAR tool for the analysis. Toxicological endpoints predicted were genotoxicity, carcinogenicity, endocrine disrupting activity, developmental toxicity, and organ toxicity.

- **Cyclic oligoesters**

No information regarding their toxicities was found in the literature for the four identified cyclic oligoesters. To confirm the presence of various isomers of monomers involved in the composition of the oligoesters, hydrolysis is necessary since LC-HRMS cannot differentiate them. The coating manufacturer mentioned tPA and BD, but not phthalic acid and 1,3-butanediol, as they lack SMLs. Furthermore, many structural isomers can be formed from combinations of different isomers of BD (e.g., 1,3-butanediol), tPA (e.g., phthalic acid), and the way TCDDM and 1,3-butanediol are branched with other isomers. This study designed all possible structural isomers of the identified oligoesters in order to assess their toxicity using *in silico* predictive tools.

- **Fenozan**

Regarding the presence of fenozan, some data were available, it is already a well-known FCM NIAS(Ji *et al*., 2024; Hu *et al*., 2021; Vera *et al*., 2018; Bertoldo and Ciardelli, 2004; Haider and Karlsson, 2002), literature data showed that its Log $k_{o/w}$ value is equal to 4.7 (National Center for Biotechnology Information, 2024), and its bioavailability is equal to 46.8 - 57.8% in rats. Fenozan may be rapidly absorbed by the human body and slowly eliminated from it, underscoring the significance of safety assessment of this antioxidant's degradation product (Prokopov *et al*., 2006a, Prokopov *et al*., 2006b).

On the ECHA website, fenozan is a pre-registered substance with REACH EC Number 243-556-1 and listed in Annex III which is the criteria for 1-10 tonnes registered substances (European Chemicals Agency). According to the classification provided by companies to ECHA in CLP

notifications this substance is harmful if swallowed. The findings of De Abrew *et al*. (2022) did not associate fenozan, with substances exhibiting estrogenic, androgenic, thyroid, or steroidogenic activities *in vitro* using toxicogenomic analysis.

Interestingly, an anti-carcinogenic effect of fenozan was demonstrated by different authors *in vivo* (Erokhin *et al*., 2007).

The objective of this study was then to assess toxicities of identified NIAS (fenozan and four cyclic oligoesters) originating from polyester coatings. Toxicity prediction of identified NIAS *via* different *in silico* QSAR tools will serve as an initial approach to screen large set of compounds and prioritize them according to their safety concern. If prediction of hazard occurs, bioassays can be used later on to verify *in silico* alerts regarding specific toxicological endpoint.

2. Results

2.1. *In silico* hazard assessment of fenozan

The results of *in silico* analysis are presented in Table 3-1.

Mutagenicity

Prediction for *in vitro* micronucleus activity was positive with moderate reliability in VEGAHUB software. All three alerts were alkylphenols with two of them being long-chain alkylphenols (Figure 3-1).

Table 3 - 1. Results of *in silico* QSAR tools for fenozan toxicity (CAS RN: 20170-32-5)

Toxicological endpoints	Derek Nexus	Sarah Nexus	Danish (Q)SAR Database	VEGAHUB
Mutagenicity	Negative	Negative	• DNA binding by OECD, alerts in alkyl phenols • frameshift Ames mutagens (models: CASE Ultra-POS_OUT, SciQSAR-POS_IN) • potent Ames mutagens, reversions ≥ 10 times controls (models: Battery, Leadscope/SciQSAR-POS_IN, CASE Ultra -POS_OUT) • mutations in thymidine kinase locus in mouse lymphoma/*in vitro* cells (model: CASE Ultra -POS_OUT) • micronucleus test in mouse erythrocytes *in vivo* (models: CASE Ultra -POS-OUT) • dominant lethal mutations in rodents *in vivo* (model: SciQSAR-POS_IN) • comet assay in mouse *in vivo* (models: Battery/CASE Ultra/SciQSAR-POS_IN)	*In vitro* Micronucleus activity (IRFMN/VERMEER) 1.0.0 model-**Active ****
Carcinogenicity	Negative	–	• FDA RCA cancer in male mouse (models: Leadscope -POS_IN, CASE Ultra- POS_OUT) • oncologic primary classification, alerts in phenol type compounds	Negative
Developmental toxicity	Negative	–	Negative	• Developmental Toxicity model (CAESAR) 2.1.7- **Toxicant****, • Developmental/Reproductive Toxicity library (PG) 1.1.0- **Toxicant****
Endocrine-disrupting activity	Negative	–	• ER α binding, balanced training set/ human *in vitro* (model: SciQSAR-POS_IN) • ER activation, CERAPP data *in vitro* (model: Leadscope-POS_OUT) • Thyroperoxidase inhibition QSAR1 and QSAR2/rat *in vitro* (model: Leadscope -POS_IN)	ER Relative Binding Affinity model (IRFMN)-**Active ***

			• Sodium/iodide symporter (NIS1), higher sensitivity and specificity (model: Leadscope - POS_IN), • • Pregnane X receptor binding/human *in vitro* (models: Battery, Leadscope, SciQSAR-POS_IN, CASE Ultra-POS_OUT), • Constitutive androstane receptor (model : Leadscope-POS_IN)	
Organ toxicity	• Hepatotoxicity/mammal-**Plausible** • Thyroid toxicity/mammal-**Equivocal**	–	–	Hepatotoxicity model (IRFMN) 1.0.0-**Toxic ***

POS: positive; IN: inside applicability domain; OUT: outside of applicability domain; _: the endpoint is not applicable with the *in silico* QSAR tool; *: specifies the reliability of the prediction (***: the most reliable; *: the less reliable).

Danish (Q)SAR Database showed positive prediction for DNA binding (by OECD) associated with alkyl phenols alert, frameshift Ames mutagens, mutations in thymidine kinase locus in mouse lymphoma *in vitro* cells, MN in mouse erythrocytes *in vivo* (micronucleus test), dominant lethal mutations in rodents *in vivo*, and DNA breaks in the comet assay in mouse *in vivo*.

Carcinogenicity

Regarding carcinogenicity, Danish (Q)SAR Database gave positive prediction for cancer in mouse. It also detected similarities between fenozan structure with phenol-type compounds alert.

Organ toxicity

Hepatotoxicity IRFMN model 1.0.0 of VEGAHUB software classified fenozan as a hepatotoxic chemical with a low reliability. A fragment of fenozan was found to match with hepatotoxic alert N°17 (Figure 3-1). Moreover, fenozan was classified as a plausible (predictions for toxicity decrease in confidence in the following order: certain>probable>plausible>equivocal) hepatotoxic chemical in mammals using Derek Nexus software due to the similarities in its structure with Alert N° 620: 2-arylacetic or 3-arylpropionic acid, and Alert N° 666: para-alkylphenol or derivative.

Lastly, equivocal prediction for thyroid toxicity in mammals was obtained using Derek Nexus software due to its similarities with Alert-Rapid Prototype147:2.6-di-tert-butylphenol derivative (Figure 3-2).

Endocrine disrupting activities

Positive results with low reliability for ER α binding by VEGAHUB software using ER Relative Binding Affinity IRFMN model were obtained. Similar results were observed using Danish (Q)SAR Database which also showed positive prediction for ER α binding and ER activation. In addition, Danish (Q)SAR Database showed positive prediction for other molecular initiating event linked to thyroid disruption such as thyroperoxidase inhibition, sodium/iodide symporter, pregnane X receptor binding, as well as constitutive androstane receptor binding.

Developmental toxicity

Fenozan was classified as a developmental toxicant using VEGAHUB software as it showed positive prediction with moderate reliability for developmental toxicity in CAESAR 2.1.7 model. In addition, the molecule matches with 9a category (with moderate reliability) of

developmental/reproductive toxicity library (PG) 1.1.0 which is described by aromatic compounds containing alkyl chain with alcohol, aldehyde, acid functional groups; poly-Cl aryloxy derived acids and esters (Figure 3-1).

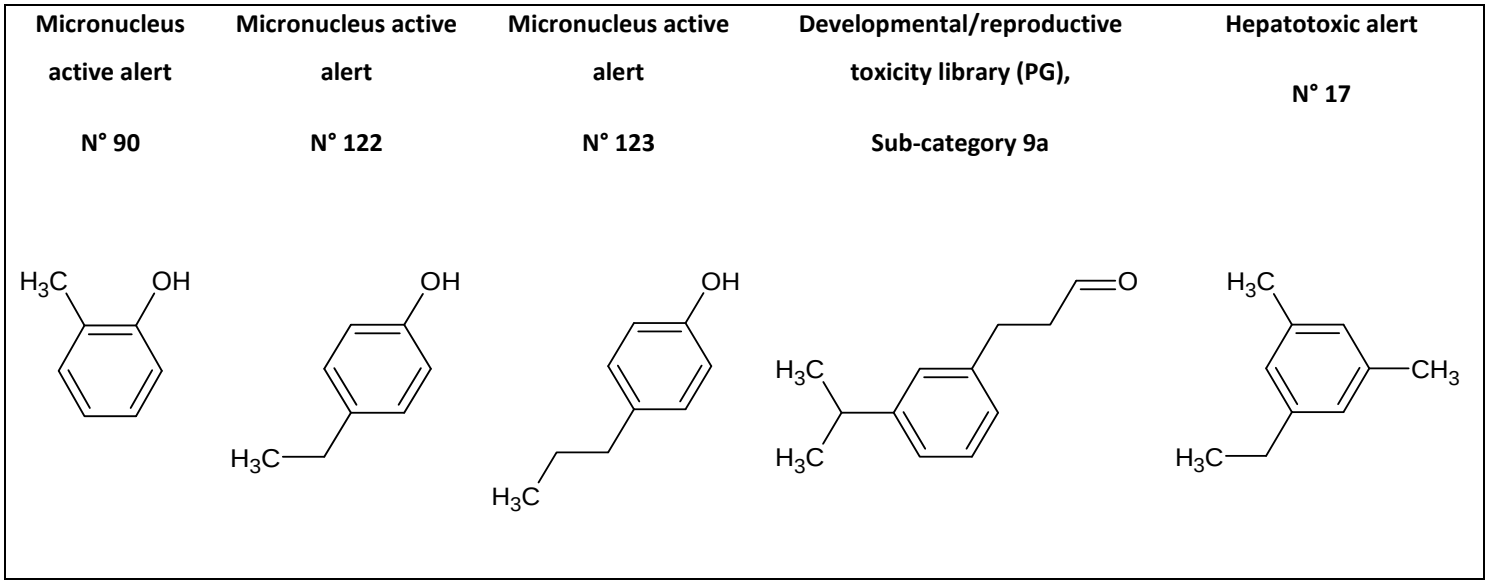


Figure 3 - 1. Structural alerts for different toxicological endpoints identified by VEGAHUB software

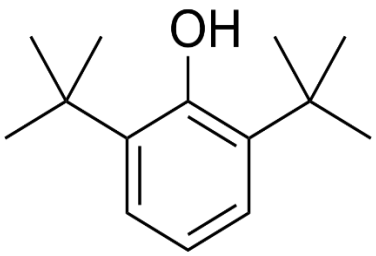


Figure 3 - 2. The structure of alert-rapid prototype147:2.6-di-tert-butylphenol derivative (Derek Nexus software)

2.2. In silico hazard assessment of oligoesters

Freelance Chamespider software was used to design isomers of the four previously identified oligoesters. Only monomers mentioned in the coating formulation (BD and tPA) and their isomers

having no SMLs (1,3-butanediol and phthalic acid) were used to design the structure of identified oligoesters (EU, 2011).

In total, 147 isomers were designed: 7 isomers for c(2TCDDM+2PA), 12 isomers for c(3TCDDM+3PA), 56 isomers for c(BD+2TCDDM+3PA), 72 isomers for c(2BD+TCDDM+3PA). The structures of different isomers of four identified oligoesters are shown in Figures 3-3, 3-4, 3-5, and 3-6.

Predicted LogP values for four oligoesters obtained by Derek Nexus software are shown in Table 3-2. LogP values of all four oligoesters are higher than 10.

Table 3 - 2. Prediction for LogP values of different isomers of four identified oligoesters using Derek Nexus software

Oligoester	Derek
c(2TCDDM+2PA) (7 isomers)	11.19
c(3TCDDM+3PA) (12 isomers)	12
c(BD+2TCDDM+3PA) (56 isomers)	12
c(2BD+TCDDM+3PA) (72 isomers)	10.94-11.02

Mutagenicity

A negative prediction for mutagenicity was obtained with all *in silico* tools and for all isomers. In addition, ADMETlab detected 3 alerts for genotoxic carcinogenicity toxicophore rule (substructure responsible for DNA reactivity) for some isomers of four identified oligoesters (Table 3-3).

Cramer class for all isomers of four identified oligoesters was predicted using VEGAHUB software. All isomers of identified oligoesters were classified as Cramer class III chemicals with the probability of showing higher toxicity.

Carcinogenicity

For the carcinogenicity, VEGAHUB *in silico* tool showed positive prediction for all four identified oligoesters. It gave alerts for carcinogenicity in different carcinogenicity models used (Table 3-4). Two structural alerts were identified in carcinogenicity model (ISS) 1.0.2: alert SA18, which represents polycyclic aromatic hydrocarbons (three or more fused rings, not hetero-aromatic) and alert SA42 which corresponds to phthalate diesters and monoesters- for cyclics (Table 3-4).

Structural alerts obtained for carcinogenicity from carcinogenicity model (IRFMN/Antares) 1.0.0 and (IRFMN/ISSCAN-CGX) 1.0.0 are shown in Table 3-4.

In addition, ADMETlab showed positive prediction only for all isomers of two identified oligoesters: c(BD+2TCDDM+3PA), c(2BD+TCDDM+3PA), having BD monomer in their structure (Table 3-3).

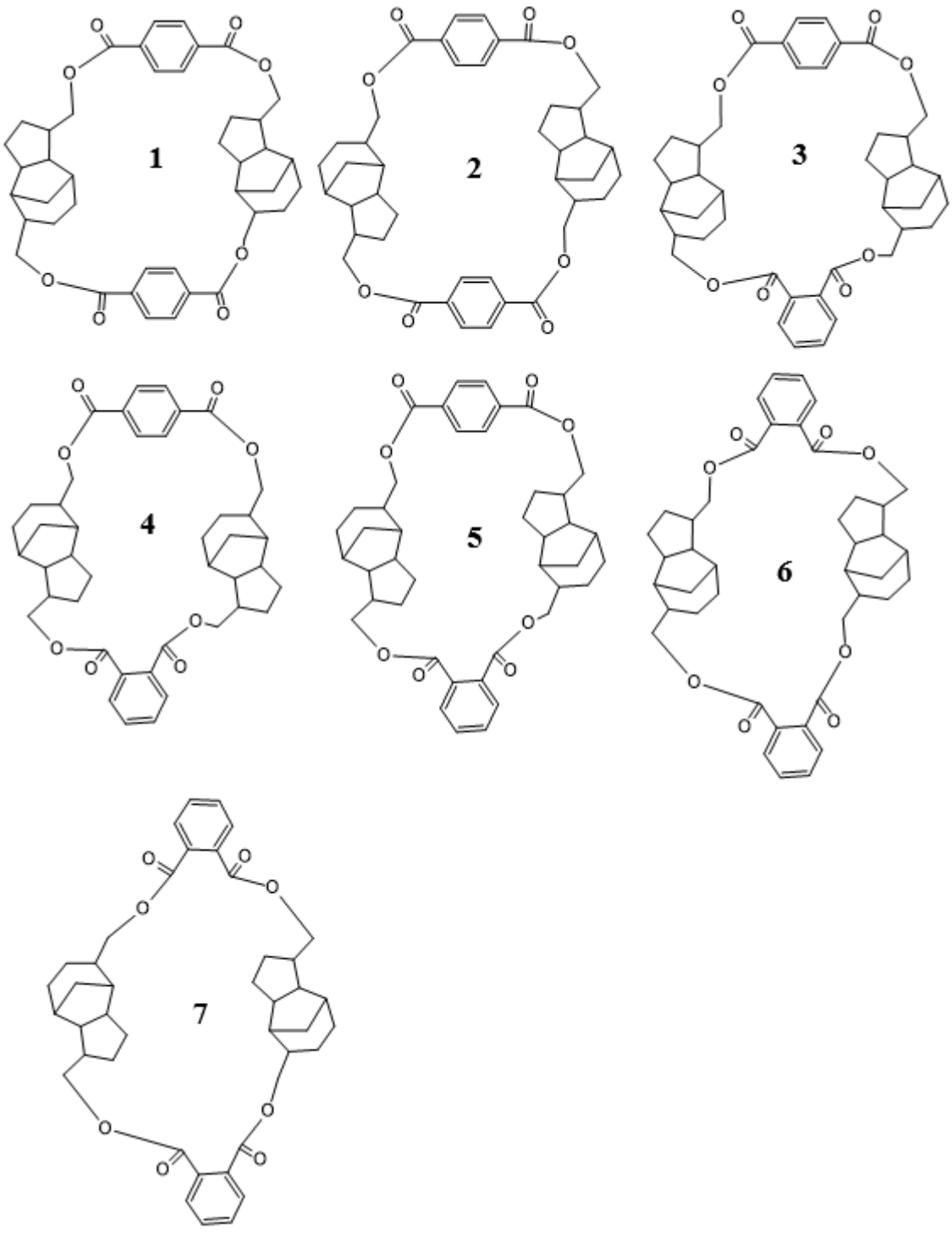


Figure 3 - 3. Structures of 7 isomers of c(2TCDDM+2PA)

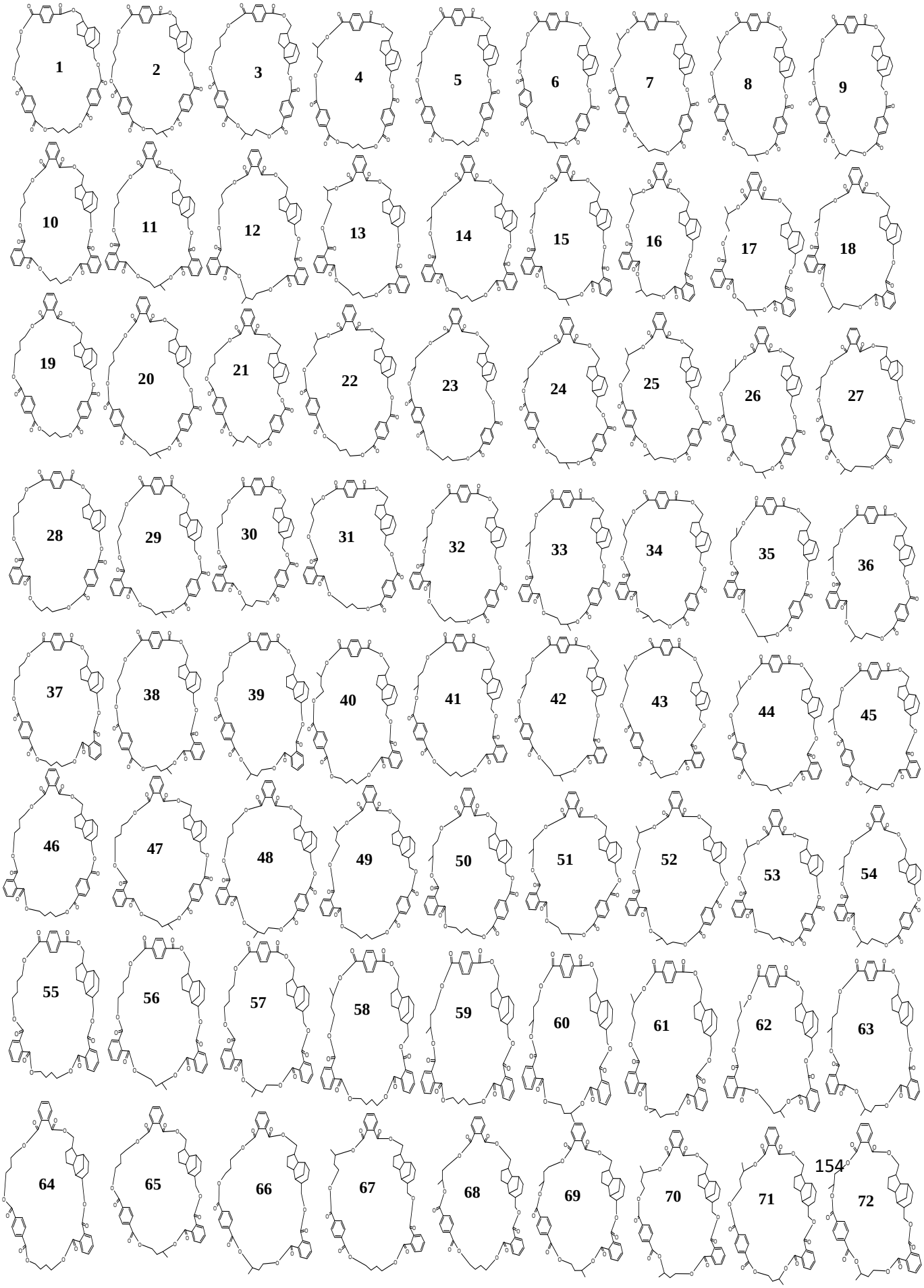

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Figure 3 - 4. Structures of 72 isomers of c(2BD+TCDDM+3PA)

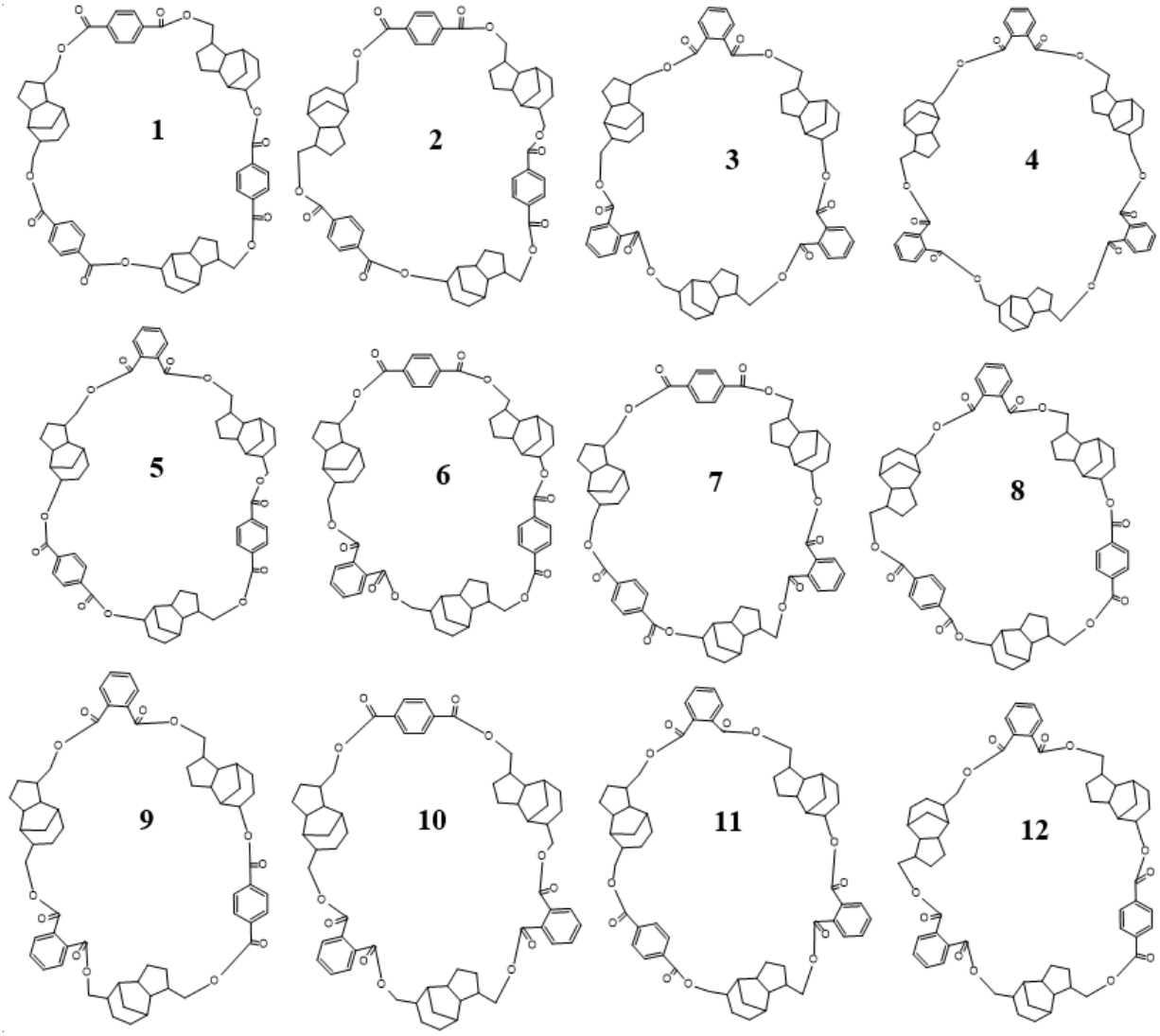

Figure 3 - 5. Structures of 12 isomers of c(3TCDDM+3PA)

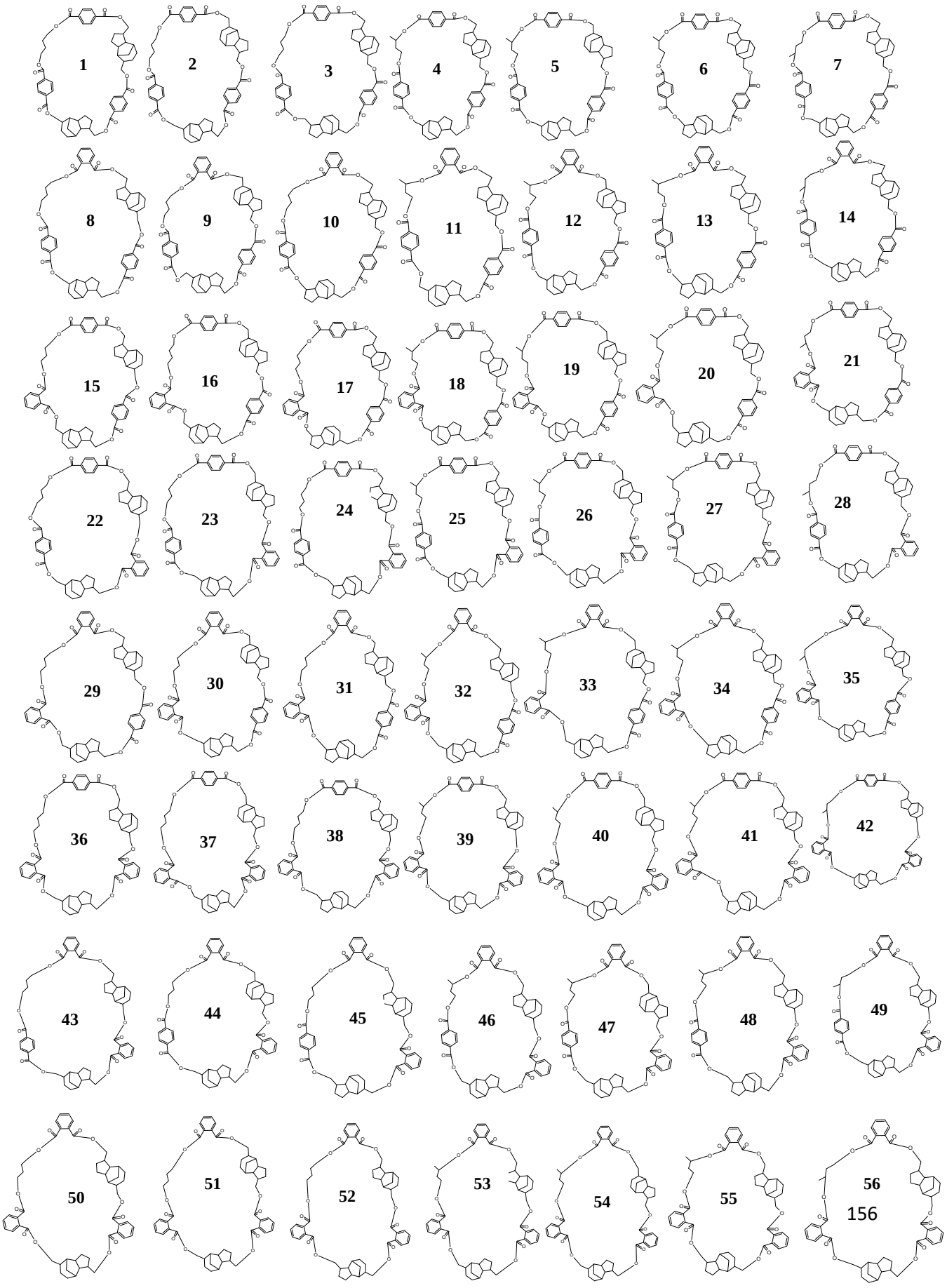
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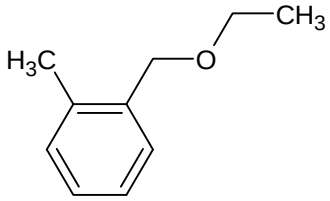
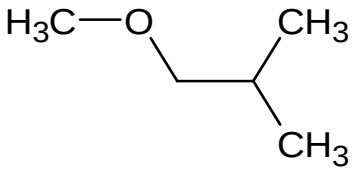
Figure 3 - 6. Structures of 56 isomers of c(BD+2TCDDM+3PA)

Table 3 - 3. Mutagenicity and carcinogenicity prediction for different structural isomers of identified four oligoesters by different *in silico* tools

Structure	Derek Nexus	Sarah Nexus	ADMETlab	VEGAHUB
c(2TCDDM+2PA) **7 isomers**	Non-mutagenic Non-carcinogenic	Non-mutagenic -	Non-mutagenic Non-carcinogenic Genotoxic carcinogenicity toxicophore rule: 3 alert(s) (5 isomers: 1- 5)	Non-mutagenic Carcinogen ** in ISS 1.0.2 model (5 isomers: 3- 7, alert SA42) Carcinogen ** in IRFMN/Antares 1.0.0 model (5 isomers: 3- 7) Carcinogen ** in IRFMN/ISSCAN-CGX 1.0.0 model (7 isomers)
c(3TCDDM+3PA) **12 isomers**	Non-mutagenic Non-carcinogenic	Non-mutagenic -	Non-mutagenic Non-carcinogenic Genotoxic carcinogenicity toxicophore rule: 3 alert(s) (8 isomers: 5- 12)	Non-mutagenic Carcinogen ** in ISS 1.0.2 model (2 isomers: 1, 2, with alert SA18, 10 isomers: 3-12, with alert SA18 and SA42) Carcinogen * in IRFMN/Antares 1.0.0 model (10 isomers: 3-12, with alert 88) Carcinogen ** in IRFMN/ISSCAN-CGX 1.0.0 model (12 isomers)
c(BD+2TCDDM+3PA) **56 isomers**	Non-mutagenic Non-carcinogenic	Non-mutagenic -	Non-mutagenic Carcinogen * (56 isomers) Genotoxic carcinogenicity toxicophore rule: 3 alert(s) (42 isomers: 8- 49)	Non-mutagenic Carcinogen * or ** in CAESAR 2.1.9 model (56 isomers) Carcinogen ** in ISS 1.0.2 model 56 isomers (7 isomers: 1-7, with alert SA18, 49 isomers: 8-56, with alert SA 18 and SA42) Carcinogen* in IRFMN/Antares 1.0.0 model (49 isomers: 8-56) Carcinogen ** in IRFMN/ISSCAN-CGX 1.0.0 model (56 isomers)
c(2BD+TCDDM+3PA) **72 isomers**	Non-mutagenic Non-carcinogenic	Non-mutagenic -	Non-mutagenic Carcinogen * (72 isomers) Genotoxic carcinogenicity toxicophore rule: 3 alert(s) (54 isomers: 19-72)	Non-mutagenic Carcinogen* in CAESAR 2.1.9 model (9 isomers: 1- 9) Carcinogen ** in ISS 1.0.2 model, 72 isomers (9 isomers: 1- 9, with alert SA18, 63 isomers: 10-72, with alert SA18 and SA42) Carcinogen * in IRFMN/Antares 1.0.0 model (63 isomers :10-72) Carcinogen ** in IRFMN/ISSCAN-CGX 1.0.0 model (72 isomers)

*: specifies the reliability of the prediction (***: the most reliable; *: the less reliable).

Table 3 - 4. Structural alerts for carcinogenicity detected by VEGAHUB software using different carcinogenicity models

Carcinogenicity model (ISS) 1.0.2	Carcinogenicity model (IRFMN/Antares) 1.0.0	Carcinogenicity model (IRFMN/ISSCAN-CGX) 1.0.0
Alert SA18: polycyclic aromatic hydrocarbons (three or more fused rings, not heteroaromatic)	**Alert no. 88** 	**Alert no. 29** 
Alert SA42: phthalate diesters and monoesters-for cyclics		

Endocrine-disrupting activity

For endocrine-disrupting activity, a negative prediction was obtained using Derek *in silico* tool, however, VEGAHUB software gave positive prediction with low reliability for 27 isomers of c(2BD+TCDDM+3PA) oligoesters using ER relative binding affinity IRMN model (Table 3-5). Furthermore, several isomers of all four identified oligomers were classified as active for aromatase and ER/ ER ligand-binding domain with low, middle, and high reliability (Table 3-5).

Developmental Toxicity

Among all used *in silico* tools only VEGAHUB and Derek Nexus allow prediction for developmental toxicity. Both software detected an alert for this endpoint (Table 3-6). Several isomers of the four identified oligoesters were classified active with low reliability for developmental toxicity in VEGAHUB software using developmental toxicity CAESAR 2.1.7 model, however, software did not give the structural alert responsible for positive prediction. In addition, plausible prediction for teratogenicity was obtained using Derek software for several isomers of the four identified oligomers. Alert N° 600, which represents phthalate mono-or di-ester, was associated to plausible prediction for teratogenicity.

Table 3 - 5. Prediction of endocrine-disrupting activity for different isomers of four identified oligoesters using different *in silico* tools

Structure	Derek Nexus	Sarah Nexus	ADMETlab	VEGAHUB
c(2TCDDM+2PA) 7 isomers	Negative	-	Active * or ** for aromatase (7 isomers) Active * for ER (5 isomers: 1- 5) Active *** for ER ligand-binding domain (7 isomers)	Negative
c(3TCDDM+3PA) 12 isomers	Negative	-	Active * or ** for aromatase (10 isomers: 1, 2, 5- 12) Active * or ** for ER (10 isomers: 1, 2, 5- 12) Active *** for ER ligand-binding domain (12 isomers)	Negative
c(BD+2TCDDM+3PA) 56 isomers	Negative	-	Active * * for aromatase (49 isomers: 1- 49) Active * for ER (49 isomers: 1-49) Active *** for ER ligand-binding domain (56 isomers)	Negative
c(2BD+TCDDM+3PA) 72 isomers	Negative	-	Active ** for aromatase (60 isomers: 1- 9, 19-45, 47-63, 65- 72) Active * for ER (63 isomers: 1- 9, 19- 72) Active *** for ER ligand-binding domain (72 isomers)	Active * in ER relative binding affinity IRFMN model (27 isomers:15- 18, 24- 27, 33- 36, 42- 45, 51-54, 60- 63, 69-72)

*: specifies the reliability of the prediction (***: the most reliable; *: the less reliable); _: the endpoint is not applicable with the *in silico* QSAR tool.

Table 3 - 6. Prediction of developmental toxicity/teratogenicity for different isomers of four identified oligoesters using different *in silico* tools

Structure	Derek Nexus	Sarah Nexus	ADMETlab	VEGAHUB
c(2TCDDM+2PA) 7 isomers	Plausible for teratogenicity in mammal (5 isomers: 3-7, Alert N° **600**: Phthalate mono-or di-ester)	-	-	Toxicant * in developmental toxicity CAESAR 2.1.7 model (4 isomers: 1, 2, 4, 5)
c(3TCDDM+3PA) 12 isomers	Plausible for teratogenicity in mammal (10 isomers: 3- 12, Alert N° **600:** Phthalate mono-or di-ester)	-	-	Toxicant * in developmental toxicity CAESAR 2.1.7 model (6 isomers: 1, 2, 5- 8)
c(BD+2TCDDM+3PA) 56 isomers	Plausible for teratogenicity in mammal (49 isomers: 8- 56, Alert N° **600**: Phthalate mono-or di-ester)	-	-	Toxicant * in developmental toxicity CAESAR 2.1.7 model (31 isomers: 1-7, 11- 14, 18- 21, 25- 28, 32- 35, 39- 42, 46- 49)
c(2BD+TCDDM+3PA) 72 isomers	Plausible for teratogenicity in mammal (63 isomers: 10-72, Alert N° **600**: Phthalate mono-or di-ester)	-	-	Toxicant * in developmental toxicity CAESAR 2.1.7 model (44 isomers: 2- 9, 20-27, 29- 36, 38- 45, 51- 54, 60- 63, 69- 72)

*: specifies the reliability of the prediction (***: the most reliable; *: the less reliable); _: the endpoint is not applicable with the *in silico* QSAR tool.

3. Discussion

In this study, toxicities of previously identified NIAS (fenozan and four cyclic oligoesters) from polyester coatings were predicted *via* different QSAR tools.

3.1. Fenozan

Fenozan was reported in the literature as a degradation product of different sterically hindered phenolic antioxidants, such as Irganox 1010, and Irganox 1024 (Ji *et al*., 2024; Liu and Mabury, 2021; Bertoldo and Ciardelli, 2004; Haider and Karlsson, 2002).

To the best of our knowledge, the toxicity of fenozan has not been fully evaluated, with available data being proprietary and inaccessible to the public. Log $k_{o/w}$ value of fenozan is equal to 4.7 (National Center for Biotechnology Information, 2024), although values above 3 suggest potential for accumulation, further investigation is required to confirm any bioaccumulation (EFSA, 2008). Literature data showed that bioavailability of fenozan ranges from 46.8 -57.8%. Fenozan was rapidly absorbed by rabbit and the blood maximal concentration reached its peak within 12 min after delivery. Additionally, fenozan was slowly eliminated from the rabbit's organism during 11.3 and 11.8 h (Prokopov *et al*., 2006a**,** Prokopov *et al*., 2006b).

- *Mutagenicity*

VEGAHUB software gave positive prediction for *in vitro* micronucleus activity linked to alkylphenols alerts. In addition, Danish (Q)SAR Database showed positive prediction for DNA binding (by OECD) associated with alkylphenols alert, frameshift Ames mutagens, mutations in thymidine kinase locus in mouse lymphoma *in vitro* cells, MN in mouse erythrocytes *in vivo* (micronucleus test), dominant lethal mutations in rodents *in vivo*, and DNA breaks in the comet assay in mouse *in vivo*.

Both *in silico* tools showed alerts related to alkyl phenols. The primary route of DNA binding of alkyl phenols is their oxidation by cytochrome P450 to a quinone methide followed by Michael addition (Figure 3-7, OECD, 2016).

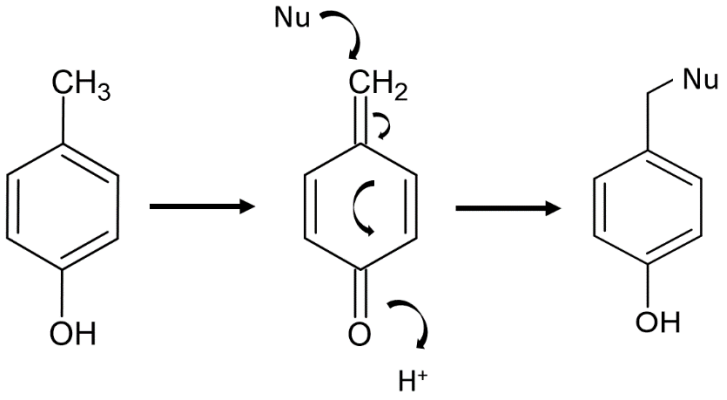


Figure 3 - 7. The scheme of alkyl phenols oxidation to a quinone methide followed by Michael addition (Nu: biological nucleophile) (OECD, 2016)

Endocrine disrupting activities

Regarding endocrine disrupting activity, positive prediction with low reliability for ER α binding was obtained by VEGAHUB software using ER Relative Binding Affinity IRFMN model. Similar results were observed using Danish (Q)SAR Database which also showed positive prediction for ER α binding and ER activation. *In silico* alerts regarding ER α binding and ER activation can be verified by testing fenozan on the STTA assay using the (h) ERα-HeLa-9903 cell line and the VM7Luc ER TA assay using the VM7Luc4E2 cell line which predominately expresses hERα with some contribution from hERβ (OECD, 2021).

Positive prediction for thyroperoxidase inhibition, sodium/iodide symporter, pregnane X receptor binding, and constitutive androstane receptor binding was also obtained by Danish (Q)SAR Database.

Sodium/iodide symporter (NIS1) is involved in iodide (I^-) transport to the thyroid follicular cells and plays a pivotal role in thyroid hormone synthesis. Fenozan can be tested on iodide uptake assay using rat thyroid FRTL-5 cells to verify *in silico* positive alert for sodium/iodide symporter (for data refer to Chapter 4).

Thyroperoxidase is an enzyme involved in thyroid hormone biosynthesis and catalyses both the iodination of tyrosine residues to form monoiodotyrosine and diiodotyrosine residues and the coupling of iodotyrosine residues in Tg, resulting in the formation of T3 and T4. Inhibition of its function may affect thyroid function and lead to hypothyroidism (Ruf and Carayon, 2006). To verify *in silico* alert for this toxicological endpoint, thyroperoxidase inhibition assay can be conducted on fenozan (Paul *et al*., 2014).

Pregnane X receptor and constitutive androstane receptor are sensors for endobiotic and xenobiotic substances and are involved in their detoxification process (Wada *et al*., 2009). Human and mouse pregnane X receptor reporter gene assay and human CAR-3 assay can be conducted to verify positive alerts obtained for these endpoints (Vignati *et al*., 2004).

Organ toxicity

Hepatotoxicity IRFMN model 1.0.0 of VEGAHUB software classified fenozan as a hepatotoxic chemical with a low reliability. A fragment of fenozan was found to match with hepatotoxic alert N°17. Moreover, fenozan was classified as a plausible hepatotoxic chemical in mammals using

Derek Nexus software due to the similarities in its structure with Alert N° 620: 2-arylacetic or 3-arylpropionic acid, and Alert N° 666: para-alkylphenol or derivative.

Arylacetic and arylpropionic acids as a common class of nonsteroidal anti-inflammatory medications that have been linked to hepatotoxicity (Dong *et al*., 2005; Bailey and Dickinson, 2003; Zimmerman, 1999).

For alert of alkylphenol or its derivatives, human hepatotoxicity data are few. After acute chemical poisoning, patients manifest a small number of liver injuries as liver necrosis (Kamijo *et al*., 2003; Green, 1975). In experimental animals with low glutathione levels, centrilobular necrosis, and cholestasis have been shown to occur in response to high doses of para-alkylphenols (Mizutani *et al*., 1991; Powell and Connolly, 1991). The metabolic activation of these chemicals to quinone methide reactive intermediates, which can react with cellular macromolecules and cause changes in normal cell function, has been hypothesized as the mechanism of toxicity (Monks and Jones, 2002; Thompson *et al*., 1996).

Lastly, equivocal prediction for thyroid toxicity in mammals was obtained using Derek Nexus software due to its similarities with Alert-Rapid Prototype147:2.6-di-tert-butylphenol derivative (Figure 3-2). This alert was created utilizing a private data collection of 1467 compounds that were categorized based on whether thyroid histopathologic lesions were present or absent in studies employing repeat doses in rats that lasted an average of 28 days. This rapid prototype alert was generated by six substances in the data set, five of which were thyroid-toxic.

In addition, French Agency for Food, Environmental, and Occupational Health and Safety hypothesized that there is a concern for thyroid effect due to the exposure of 2,6-di-tert-butyl-4-methylphenol (BHT), an analogue substance with rapid prototype alert as several rat studies showed changes to thyroid physiology due to exposure to this chemical (ANSES, 2016a).

- *Developmental Toxicity*

Fenozan was classified by VEGAHUB software as a developmental toxicant as it showed positive prediction with moderate reliability for this toxicological endpoint in CAESAR 2.1.7 model. Fenozan's structure matches with 9a category (with moderate reliability) of developmental/reproductive toxicity library (PG) 1.1.0 which is described by aromatic compounds containing alkyl chain with alcohol, aldehyde, acid functional groups; poly-Cl aryloxy

derived acids and esters. These results derive from the paper of Wu *et al*. (2013) where an empirically based decision tree based on 716 chemicals (664 positive, 16 negative, and 36 with insufficient data) was developed to assess whether or not a chemical has structural features that are consistent with chemical structures known to be toxic to development and reproduction. In this paper, all 716 substances were grouped into 25 categories including five receptor-binding and 20 chemical–structure–related categories. Aromatic compounds containing alkyl chain with alcohol, aldehyde, acid functional groups; poly-Cl aryloxy-derived acids, and esters were categorized into one of the chemical-structure–related category (number 9) as several studies showed their developmental and reprotoxic effect. It is important to mention that only *in vivo* experiments will permit to verify *in silico* alerts for developmental and reproductive toxicity, e.g., a two-generation reproduction toxicity study (OECD TG 416) (OECD, 2001), can be performed to verify *in silico* alerts for developmental and reproduction toxicity.

3.2. *O*ligoesters

Previously one cyclic tetramer and three cyclic hexamers were identified in polyester coatings ACN extracts and EtOH 95% migrates.

To identify the constituent monomer isomers of oligoesters, they should be hydrolyzed. Various isomers are expected due to the different isomers of starting monomers (the ones mentioned by the manufacturer for the coating's formulation and their possible isomers having no SMLs in ResAP(2004)).

With a molecular weight below 1000 Da, these oligoesters may be absorbed by human body (EFSA, 2008). As they are not commercially available, evaluating their toxicity requires synthesizing oligoesters first, a process that is both expensive and time-consuming.

In our study, all possible structural isomers of four identified oligoesters were designed, and their toxicity was assessed using QSAR tools as a first step to assess hazard.

LogP value of four identified oligoesters varies from 10.94 to 12 suggesting a potential for accumulation in the mammalian body (EFSA, 2008).

Mutagenicity and potential carcinogenicity

A negative prediction for mutagenicity was obtained with all *in silico* tools for all isomers. For the carcinogenicity, VEGAHUB software showed alerts for all four identified oligoesters in different carcinogenicity models. Two structural alerts were identified in carcinogenicity model (ISS) 1.0.2: alert SA18, which represents polycyclic aromatic hydrocarbons (three or more fused rings, not hetero-aromatic) and alert SA42 which corresponds to phthalate diesters and monoesters- for cyclics.

Literature results showed that genotoxicity and carcinogenicity are associated with aromatic hydrocarbons with three or more aromatic rings (EFSA, 2023a).

Moreover, ADMETlab showed positive prediction for carcinogenicity only for all isomers of two identified oligoesters: c(BD+2TCDDM+3PA), c(2BD+TCDDM+3PA), having BD monomer in their structure. It was assumed that positive alert was related to the presence of BD monomer.

Negative prediction for mutagenicity and positive prediction for carcinogenicity for four oligoesters allow to suppose that these oligomers could have other mode of action, e.g., tumor-promoting properties. CTA can be used to test pure chemicals for potential carcinogenicity.

Endocrine-disrupting activity

Regarding endocrine-disrupting activity, positive prediction with low reliability was obtained for 27 isomers of c(2BD+TCDDM+3PA) oligoesters using ER relative binding affinity IRMN model in VEGAHUB software. Furthermore, several isomers of all four identified oligomers were classified as active for aromatase and ER/ ER ligand-binding domain with low, middle, and high reliability. Aromatase converts androgen (such as T) to estrogen (such as E2) in human body (Chan *et al*., 2016). Several aromatase inhibition assays can be performed to verify *in silico* alert for aromatase activity, such as homogeneous time-resolved fluorescence aromatase assay or a fluorometric CYP19A1 (aromatase) activity assay in live cells (Heidary *et al*., 2021; Ji *et al.*, 2014).

In silico alerts regarding ER/ER relative binding affinity can be verified by testing oligoesters on the STTA assay using the (h) ERα-HeLa-9903 cell line and the VM7Luc ER TA assay using the VM7Luc4E2 cell line which predominately expresses hERα with some contribution from hERβ (OECD, 2021). Ubeda *et al*. (2020) assessed endocrine activity of two cyclic oligoesters migrating from multilayer packaging through *in vitro* assays. The findings revealed a mild AR antagonism activity, with no observed arylhydrocarbon receptor activity or binding to the thyroid hormone

transport protein. It is important to note that although these oligoesters share a cyclic structure with our compounds, they are derived from different monomers.

Developmental toxicity

Alerts for developmental toxicity were obtained by VEGAHUB and Derek Nexus software for all four oligoesters. VEGAHUB software did not provide structural alerts responsible for positive prediction. Alert N° 600, which represents phthalate mono-or di-ester, was associated to plausible prediction for teratogenicity obtained for four oligoesters by Derek Nexus software. This alert describes the teratogenicity of phthalates (EFSA, 2019).

Finally, the four cyclic oligoesters were classified as Cramer class III substances. These findings align with literature data, where cyclic oligoesters identified by various authors were similarly classified as Cramer class III substances (Lestido-Cardama *et al.*, 2022b; Eckardt *et al.*, 2020, 2019).

Although *in silico* QSAR tools are powerful methods for predicting the toxicity of chemicals, they cannot fully replace *in vitro* and *in vivo* bioassays. QSAR tools can be used as a first screening approach to detect alerts regarding different toxicological endpoints. However, these tools have several drawbacks. Firstly, the structure of the chemicals must be known to predict their toxicity. Once the structure is disclosed, the toxicity of these chemicals can be predicted. Furthermore, QSAR methods cannot be applied to mixtures, e. g. FCMs extracts containing unknown chemicals, unlike bioassays.

Another drawback of QSAR methodologies is the lack of knowledge regarding the applicability domain of the model (Van Bossuyt *et al*., 2018; Cherkasov *et al*., 2014).

It has been shown that the performance of predictions is lower when only one tool is used for the analysis. EFSA recommends combining several QSAR tools to increase the reliability and confidence of mutagenicity assessments of chemicals. They demonstrated that the combination of different QSAR tools increases the sensitivity but decreases the specificity of the analysis. Additionally, both sensitivity and specificity were improved when the application of various QSAR tools were combined with expert judgment (Benigni *et al*., 2019). In addition, the performance of QSAR tools were also improved for carcinogenicity prediction when battery of tools was used (European Commission. Joint Research Centre. Institute for Health and Consumer Protection,

2010)**.** Another drawback is that QSAR tools are not sufficiently reliable to predict different toxicological endpoints. For example, Benigni *et al*. (2019) showed that while QSAR tools demonstrated sufficient reliability for the Ames test, their performance needs improvement for assessing other mutagenicity endpoints.

Van Bossuyt *et al.* (2018) investigated the performance of different QSAR tools for mutagenicity prediction of FCMs. They demonstrated that performance varies among different QSAR tools but improves when combining several tools together, particularly for paper and board FCMs. Additionally, the importance of the applicability domain was illustrated by the limited performance of the tools in predicting mutagenic substances that are new to the model (e.g., not included in the training set).

4. Conclusion

This first study was performed to predict the toxicity of previously identified NIAS (fenozan and four cyclic oligoesters) from polyester coatings ACN extracts and EtOH 95% migrates using *in silico* QSAR tools due to limited available toxicity data.

Predictive hazard assessment of fenozan, showed positive alerts for mutagenicity (VEGAHUB, Danish (Q)SAR Database), carcinogenicity (Danish (Q)SAR Database), developmental toxicity (VEGAHUB), endocrine disrupting activity (VEGAHUB, Danish (Q)SAR Database), and organ toxicity (Derek Nexus, VEGAHUB).

For oligoesters, negative prediction for mutagenicity was observed whatever the software. In contrast, positive prediction for carcinogenicity was obtained for the four oligoesters using VEGAHUB and ADMEETlab tools suggesting that these oligomers may have other mode of action than a DNA direct reactivity, for example, tumor-promoting mode of action. In addition, positive prediction was obtained for developmental toxicity/ teratogenicity (Derek Nexus, VEGAHUB), and endocrine-disrupting activity (ADMET lab, VEGAHUB) for several isomers of the four oligoesters.

These findings provide initial insights into potential toxicological hazard that require further risk assessment.

Chapter 2-Article 1

Identification of non-volatile non-intentionally added substances from polyester food contact coatings and genotoxicity assessment of polyester coating's migrates

Summary

Introduction

FCMs, such as metal cans, often require internal coatings to prevent corrosion and maintain food safety. While epoxy-phenolic coatings have traditionally dominated the industry, growing concerns about BPA migration have led to a shift toward polyester-based alternatives. Although regulations mostly target IAS, NIAS can still migrate from these coatings into food. This study aims to identify non-volatile NIAS and evaluate their potential genotoxicity *in vitro*, using samples from five batches of a single polyester-coated tinplate, to support consumer safety in relation to canned food consumption.

Materials and methods

Five batches of polyester-coated tin plates, along with one uncoated tin plate, were subjected to migration testing and extraction. Two circular samples (1.02 dm^2 each) from each material were hermetically sealed in Calipack migration cells (CEN - EN 1186 standard). Each cell was filled with 430 mL of either ACN or EtOH 95% and incubated at 40 °C for 1 day for ACN samples and 60 °C for 10 days for EtOH, covering both uncoated and coated plates. ACN, a mid-polarity solvent, was used to simulate a worst-case extraction scenario, while EtOH 95% served as a substitute for lipophilic food simulants like vegetable oil, in line with EU Regulation 10/2011 (EU, 2011). After incubation, extracts and migrates were pooled by solvent type, concentrated 53.75-fold using rotary evaporation followed by nitrogen drying at 40 °C. Solvent blanks were prepared similarly. Following migration or extraction, samples were concentrated and analyzed using LC-HRMS for non-targeted identification. Identified oligoesters were semi-quantified using deuterium-labeled IS. The Toxtree software (v3.1.0, Ideaconsult Ltd., Sofia, Bulgaria) was employed to assign Cramer

classes to the identified compounds, while predictive LogP values were estimated using the Meylan/Kowwin model in VEGA HUB software (v1.1.5).

The genotoxic potential of the extracts and migrates was assessed using two *in vitro* assays: the MMT with multiple *Salmonella typhimurium* strains via direct incorporation (TA100, TA98, TA102, TA1537) and pre-incubation (TA100, TA98) methods, and the DDR assay using TK6 human lymphoblastoid cells. To rule out false-negative results due to matrix effects, migration samples were spiked with corresponding positive controls and tested in parallel with the MMT and DDR assays.

Results

Non-targeted LC-HRMS analysis revealed the presence of four cyclic oligoesters composed of BD, tPA, and TCDDM monomers—specifically, c(2TCDDM+2tPA), c(2BD+TCDDM+3tPA), c(BD+2TCDDM+3tPA), and c(3TCDDM+3tPA)—in both ACN extracts and EtOH 95% migrates of polyester-coated metal plates. These oligoesters were identified with a confidence level of 2, with molecular weights below 1000 Da, and showed high predicted LogP values (>11). Semi-quantitative analysis indicated that the concentrations of c(BD+2TCDDM+3tPA), c(3TCDDM+3tPA), and c(2TCDDM+2tPA) ranged from 45.4 to 85.0 µg/dm² (equivalent to 272.4–510 µg/kg food), while c(2BD+TCDDM+3tPA) was present at lower levels, between 3.1 and 6.1 µg/dm² (18.6–36.6 µg/kg food).

Both ACN extracts and EtOH 95% migrates tested negative for genotoxicity in the MMT across all bacterial strains and experimental conditions. Similarly, the DDR assay showed no evidence of clastogenic, aneugenic, or genotoxic effects in any of the tested samples. Spiking experiments confirmed the absence of matrix effects under the conditions used.

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Identification of non-volatile non-intentionally added substances from polyester food contact coatings and genotoxicity assessment of polyester coating's migrates

Ruzanna Hayrapetyan [a], Ronan Cariou [b], Anne Platel [c], Julie Santos [c], Ludovic Huot [c], Véronique Monneraye [d], Marie-Christine Chagnon [a], Isabelle Séverin [a,*]

[a] Université de Bourgogne Franche-Comté, LNC UMR1231, Nutrition Physiology and Toxicology Team (NUTox), F-21000, Dijon, France
[b] Oniris, INRAE, LABERCA, F-44307, Nantes, France
[c] Univ. Lille, CHU Lille, Institut Pasteur de Lille, ULR 4483, IMPECS - IMPact de l'Environnement Chimique sur la Santé Humaine, F-59000, Lille, France
[d] MASSILLY Holding, F-71000, Macon, France

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ABSTRACT

Can's polyester coatings are intended to replace epoxy-phenolic ones due to rising safety concern regarding the potential release of bisphenol A under increased regulations and consumer pressure. In this study, hazard linked to the migration of non-intentionally added substances from a single polyester-coated tin plate (5 batches) to canned food has been studied. Migration tests were performed using acetonitrile (ACN) and ethanol (EtOH) 95 %. Non-targeted analyses by liquid chromatography–high-resolution mass spectrometry revealed the presence of four cyclic oligoesters classified as Cramer class III substances with an estimated exposure (calculated for French population only) below the threshold of toxicological concern value of 1.5 μg/kg b.w./day, suggesting a no safety concern. Moreover, migrates were tested using *in vitro* genotoxicity DNA damage response (DDR) test and mini mutagenicity test (MMT) with different strains of *S. Typhimurium* using direct incorporation (TA100, TA98, TA102, TA1537) and pre-incubation (TA100, TA98) methods. Samples were negative in both bioassays suggesting the absence of genotoxicity/mutagenicity of the mixtures. To verify any false negative response due to matrix effect, migrates were spiked with corresponding positive controls in parallel with the MMT and the DDR test. No matrix effect was observed in these experimental conditions.

1. Introduction

Food packaging has an irreplaceable role worldwide protecting the food against chemical contamination while making easier food transportation and preventing aromatic changes or quality decreases. It is useful for conveniently preparing and serving foodstuff, also providing informational support to consumers, and helping to fight against food wastage. Coatings are applied internally on cans to avert the corrosion of metal plates and the migration of metal ions into the food. Polyester coatings were developed to replace epoxy-phenolic coatings as the latest was demonstrated to release Bisphenol A and its derivatives, questioning their safety (Paseiro-Cerrato et al., 2016b; Noureddine El Moussawi et al., 2019; Hwang et al., 2020). The European Framework Regulation (EC) No 1935/2004 (Article 3) states that 'materials and articles should not release their constituents into food under normal or foreseeable conditions which could endanger consumer's health' (EC, 1935/2004). As there are no harmonized regulations for coatings intended to come into contact with food at the European scale, some countries developed their own national regulations, for example, Royal Decree for Varnishes and Coatings, 2016 in Belgium, Real Decreto 847/2011 in Spain, or Commodities Act (2014) in The Netherlands (Commodities Act, 2014; Real Decreto 847/2011; Royal Decree for Varnishes and Coatings, 2016). Nowadays, manufacturers use recommendations from the partial agreement ResAP (2004)1 on coatings intended to come into contact with foodstuffs, which lists all intentionally added substances to be used during the production of coatings. However, the formation of non-intentionally added substances (NIAS) remains inevitable and may occur during all stages of the food can's supply chain: chemical syntheses of coatings, curing, production of cans, filling, sterilization, transport, storage, and recycling (Geueke, 2018).

It has been shown that cyclic and linear oligoesters formed during

* Corresponding author.
E-mail address: isabelle.severin@agrosupdijon.fr (I. Séverin).

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List of abbreviations

2AA	2-Aminoanthracene
9AC	9-Aminoacridine
ACN	Acetonitrile
bw	body weight
BD	1,4-Butanediol
CPA	Cyclophosphamide
DDR	DNA Damage Response
EFSA	European Food Safety Authority
EtOH	Ethanol
FCM	Food Contact Material
GEF	Global Evaluation Factor
GRF	Griseofulvin
iPA	Isophthalic acid
IS	Internal standard
LC-HRMS	Liquid Chromatography-High Resolution Mass Spectrometry
MMS	Methyl methanesulfonate
MMT	Mini Mutagenicity Test
MC	Mitomycin C
m/z	mass-to-charge ratio
NPG	Neopentyl glycol
2NF	Nitrofluorene
NIAS	Non-Intentionally Added Substance
PCgroup	Peak Correlation group
SA	Sodium azide
SML	Specific migration limit
tPA	Terephthalic acid
TCDDM	Tricyclodecanedimethanol
TTC	Threshold of Toxicological Concern
VBL	Vinblastine

coating production (as predictable NIAS) may migrate from coatings to food (Paseiro-Cerrato et al., 2016b; Omer et al., 2019; Lestido-Cardama et al., 2022). Identification and quantification of oligoesters from coating extracts and/or migrates remain difficult due to many possible monomer combinations and isomers, as well as the absence of analytical standards. Moreover, risk assessment associated with oligoesters requires knowledge about their chemical structure and synthesis to assess their toxicity in different bioassays. Liquid chromatography–high-resolution mass spectrometry (LC-HRMS) was previously reported to be a suitable tool for the identification and quantification of oligoesters from packaging extracts (Omer et al., 2018; Pietropaolo et al., 2018; Eckardt et al., 2020).

Regarding unpredictable NIAS able to be released from food contact materials (FCM), ILSI working group recommended to combine analytical methods with a battery of bioassays for safety assessment (ILSI, 2023, 2015). Once identified, oligomers alone (having a molecular weight lower than 1000 Da) can be tested in a battery of *in vitro* bioassays to provide a comprehensive understanding of their toxicity. Nevertheless, it is important to keep in mind that hydrolysis of oligoesters may occur in the gastrointestinal tract leading to the formation of new molecules having different structures that could be more or less hazardous (Eckardt et al., 2019; Ubeda et al., 2020). Analytical chemistry tools are incapable of detecting all NIAS due to their low concentrations and the lack of adequate standards. Testing the genotoxicity of extracts/migrates in *in vitro* bioassays allows for checking the presence of undesirable NIAS which have not been detected or identified by analytical chemistry. Moreover, *in vitro* bioassays advantageously detect cocktail effect of complex mixtures (Severin et al., 2017).

European Food Safety Authority (EFSA) recommended using the bacterial reverse mutation test, *i.e.* the Ames test (OECD 471) and the *in vitro* micronucleus assay (OECD 487) as a requisite step in assessing the mutagenicity of food chemicals. These battery of bioassays can cover all mode of actions of mutagenic chemicals (EFSA Scientific Committee, 2011). However, the standard regulatory Ames test requires a large quantity of substances, which are obviously not available with regards to NIAS because they are present in small quantities in FCM's migrates. Furthermore, a concentration step is mandatory before testing migrates in biotests. Miniaturized methods are more suitable as the amount of test item is reduced (Rainer et al., 2019a). Several miniaturized variants of the Ames test having similar concordance with the traditional method were developed (Diehl et al., 2000; Flamand et al., 2001; Flückiger-Isler and Kamber, 2014; Pant et al., 2016). For example, Flamand et al. (2001) developed miniaturized bacterial reverse mutation test called the Mini Mutagenicity Test (MMT) using 6-well plates (OECD, 2021).

An *in vitro* high-throughput genotoxicity test, namely the DNA Damage Response (DDR) test capable of detecting both DNA-reactive and non-DNA-reactive chemicals using TK6 human lymphoblastoid cells was developed by Litron Laboratories (Bryce et al., 2016). High sensitivity (92.1 %) and specificity (96.2 %) for the DDR test were reported as it measures the induction of several DNA damage response biomarkers associated with clastogenicity (γH2AX), aneuploidy (phospho-histone H3), and genotoxicity (p53) (Bryce et al., 2017). It allows fast reading of the results after 24 h of treatment using flow cytometry.

The objective of this study was to check *in vitro* the presence or absence of genotoxic NIAS issued from polyester coatings in order to provide consumer safety related to the consumption of canned food. The research strategy consisted of preparing migrates of one uncoated and five polyester-coated metal plates using ACN and EtOH 95 %. Then, identification and semi-quantification of identified NIAS were performed using LC-HRMS. Lastly, the genotoxicity and mutagenicity potential of migrates was assessed using the *in vitro* MMT and the DDR tests.

2. Materials and methods

2.1. Chemicals

High-performance liquid chromatography–mass spectrometry grade ACN was purchased from Carlo Erba (Val-de-Reuil, France). EtOH was obtained from Promochem (Picograde®, Wesel, Germany). Nitrofluorene (2NF), sodium azide (SA), mitomycin C (MC), 9-aminoacridine (9AC), 2-aminoanthracene (2AA), methyl methanesulfonate (MMS), griseofulvin (GRF), vinblastine (VBL), cyclophosphamide (CPA), which are positive controls for MMT and DDR tests, were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the MMT test, beta-naphthoflavone and phenobarbital-induced S9 were obtained from Biopredic International (Saint-Grégoire, France). Three linear and cyclic oligoesters composed of neopentyl glycol (NPG) and isophthalic acid (iPA) units (d_4-lin (2NPG+2iPA), d_4-c (2NPG+2iPA), d_8-c (4NPG+4iPA)) were previously synthesized (Cariou et al., 2022). Chemical purities yielded >95 % (NMR estimations) while no impurities could be identified by mass spectrometry except for d_8-c (4NPG+4iPA) containing less than 1 % of d_4-c (2NPG+2iPA).

2.2. Samples

Five polyester-coated tin plate sheets, from the same reference formulation but from different batches, and one non-coated tin plate were prepared by MASSILLY Holding. The reference formulation is commercialized for fatty foodstuffs and is based on two different polyols, namely 1,4-butanediol (BD) and tricyclodecanedimethanol (TCDDM), and two polyacids, namely terephthalic acid (tPA) and maleic

acid from its anhydride precursor. Besides these starting monomers for the polyester resin, the other starting substances of the varnish were not disclosed.

2.3. Migration assays and sample preparation

Two pieces of each sample were cut in a form of a circle (1.02 dm^2) and fixed hermetically inside two different Calipack migration cells (European Standard CEN - EN 1186). Then, 430 mL of ACN or EtOH 95 % were poured into two migration cells and they were incubated at 40 °C for 1 day for ACN samples (non-coated plate: A0, five coated plates: A1, A2, A3, A4, A5) and at 60 °C for 10 days for EtOH 95 % samples (non-coated plate: E0, five coated plates: E1, E2, E3, E4, E5). Mid-polarity solvent ACN was selected as extraction solvent standing for a worst-case scenario, while EtOH 95 % was selected as a practical substitute to the assigned food simulant for foods having a lipophilic character, vegetable oil, according to Commission Regulation (EU) No 10/2011 (EU, 2011). After incubation, the contents of both migration cells containing the same solvent (ACN extracts on the one hand; EtOH 95 % migrates on the other hand) were pooled and concentrated (53.75 times) under a rotary evaporator and then under gentle nitrogen flow at 40 °C.

ACN and EtOH 95 % solvents were treated as described above to provide procedural blanks (AB or EB). All vials were kept at −20 °C before use.

2.4. LC-HRMS data acquisition

For LC-HRMS analysis, 10 μL from 4 mL solution was diluted with 540 μL of ACN or EtOH 95 % (dilution factor of 55). Then, 100 μL were mixed with 10 μL of a standard mixture (4 ng/μL) which contained 3 deuterium-labelled linear and cyclic oligoesters composed of NPG and iPA units used as internal standards (IS). Samples were concentrated until dryness under gentle nitrogen flow and the dried residue was reconstituted in 10 μL of ACN and 10 μL of H_2O. Data acquisition (10 μL) was performed on an Orbitrap Q Exactive instrument fitted with a heated electrospray source (Thermo Fisher Scientific, San José, CA, USA) according to Cariou et al. (2022). Details about the chromatographic separation are provided in the supplementary material. One-dimensional full scan windows were programmed in the negative and positive switching modes over the mass-to-charge ratio (m/z) range 155–1064 at the nominal resolution of 70,000 (full width at half maximum at m/z 200). Targeted-MS^2 experiments were performed as well for structural elucidation, with isolation window set at ±0.4 uma and normalized collision energy set at 10 %, 30 % and 50 %.

2.4.1. Post-acquisition data mining and identification

Full-scan LC-HRMS data were processed *via* an open-source programming *R* environment according to Cariou et al. (2022). Several ion species, such as isotopologues, adducts, or fragments potentially originated from the same compound (peak shape) were paired together for componentisation in the so-called peak correlation group (PCgroup), which were aligned along the sequence. Adduct ion annotations were performed based on mass differences. Xcalibur Qual Browser software (ThermoFisher Scientific) was used to generate an elemental formula for spotted neutral masses of interest. Finally, the NIAS-db software (DOI: 10.15454/HHY2Z2), was used to suggest oligoester combinations. The chromatographic trace and the MS^2 fragmentation patterns were compared to those published by Cariou et al. (2022) for consistency.

2.4.2. Semi-quantification of identified compounds

Since there is no commercially available reference standards for the identified oligoesters, we used deuterium-labelled oligoesters based on NPG and iPA monomers as closest proxies for semi-quantification. The concentrations of identified oligoesters were estimated assuming a relative response factor of 1 compared to the assigned IS. d_4-c (2NPG+2iPA) was the IS for the cyclic oligoester based on 4 monomer units and the average response factor of d_4-l (2NPG+2iPA) and d_8-c (4NPG+4iPA) oligoesters was the IS for the cyclic oligoesters based on 6 monomer units. The summed area of monoisotopic peaks from both dominant $[M + NH_4]^+$ and $[M + Na]^+$ adduct ions was considered. Results were expressed on the basis of the coating surface. Dilution factors during sample preparation were taken into account in final calculations.

2.5. Quantitative structure-activity relationship analysis

Toxtree software, v3.1.0 (Ideaconsult Ltd., Sofia, Bulgaria) was used to determine the Cramer class of identified molecules. The LogP model Meylan/Kowwin of VEGA HUB 1.1.5 software was applied to determine predictive LogP values of identified substances.

2.6. Statistical analysis

The paired sample *t*-test was employed to assess statistical differences (P-value = 0.05) in group means of oligoester concentrations in different solvents (ACN and EtOH 95 %). The statistical tests were conducted using the Origin Pro 2021 software package.

2.7. Mini Mutagenicity Test

2.7.1. Bacteria

Four strains of *Salmonella Typhimurium* (TA98, TA100, TA102, and TA1537) recommended by OECD 471 guideline were used in this study (OECD, 2020). The strains were obtained from Trinova Biochem (Giessen, Germany). After amplification of bacteria from the bacterial disc and phenotype characterization, strains were maintained in frozen stocks (−80 °C, DMSO culture).

2.7.2. Fresh culture preparation

For each experiment, a fresh culture was prepared as follows: one cryotube was thawed and 140 μL of bacterial suspension was introduced in 7 mL of nutritive broth supplemented with 25 μg/mL of ampicillin for TA98, TA100, TA102 strains, and 2 μg/mL tetracycline for TA102 strain. The cryotube was discarded after use. Strains were grown overnight at 37 °C under shaking (120 rpm) until the concentration of bacteria reaches 10^8 bacteria/mL.

2.7.3. Treatment

MMT was performed according to the protocol described by Flamand et al. (2001). The protocol is similar to the direct incorporation method of the Ames test described by OECD 471 guideline, but with minimized reagent's volume. In short, 1.6 mL of top agar was mixed with 80 μL of fresh bacterial culture, 80 μL of test product or positive or negative control, and 400 μL of phosphate buffer (0.2 mM, pH 7.4) for the assay without metabolic activation, or 400 μL of S9 mix (10 %) for the assay with metabolic activation. The tubes were then vortex-mixed and 540 μL of aliquot was transferred onto three wells of 6-well plates containing 5 mL of minimal agar. The plates were inversed after the solidification of the top agar and incubated for 48 h at 37 °C. Revertant colonies were counted manually in three wells and the means and standard deviations were calculated.

A test item was considered positive if the ratio between the number of revertant colonies in the test item and the number of revertant colonies in the negative control treatment was ≥2 for TA100, TA98, and TA102 strains, and ≥3 for TA1537 strain. Positive controls for MMT without S9 mix were 2NF (0.25 μg/well) for TA98, SA (0.4 μg/well) for TA100, MC (0.05 μg/well) for TA102, and 9AC (10 μg/well) for TA1537. 2AA (0.4 μg/well) was the positive control with the S9 mix for all tested strains.

If a negative response was obtained regardless of the strain used by the direct incorporation method, the test was repeated on TA98 and

TA100 strains only using the pre-incubation method taking into account sample limitations. Bacteria were incubated for 20 min with ½ diluted test product (40 μL of the sample was mixed with 40 μL of phosphate buffer) and 400 μL of phosphate buffer or S9 mix before the addition of 1.6 mL of top agar. If a negative response was obtained from the second assay using the pre-incubation method, the test item was concluded as non-mutagenic.

2.7.4. Cytotoxicity

Cytotoxicity was judged when clearing or diminution of the bacterial background lawn was observed under the microscope for spiked and non-spiked assays and the induction factor (the mean of revertant colonies in the sample wells divided to the mean of revertant colonies in solvent control wells) for the non-spiked assay was higher than 0.6. Precipitation due to migrates in the final plates was also estimated.

2.7.5. Spiking experiments with MMT

In order to test the matrix effect of FCM migrates, in parallel to pre-incubation method, experiments were conducted with TA98 and TA100 strains while spiking with the respective positive controls (10 μL, 0.25 μg/well 2NF for TA98 without S9 mix, 0.4 μg/well SA for TA100 without S9 mix, and 0.4 μg/well 2AA for both strains with S9 mix) in the medium.

If no cytotoxicity was observed with migrates and if the spike recovery (number of revertant colonies) for spiked migrates was less than 60 % in comparison with the positive control (2NF for TA98 without S9 mix, 0.4 μg/well SA for TA100 without S9 mix, and 0.4 μg/well 2AA for both strains with S9 mix) and spiked solvent blank (AB for ACN migrates and EB for EtOH 95 % migrates), then a matrix effect was considered (Rainer et al., 2019a).

2.8. DNA damage response test

2.8.1. Cell culture

TK6 human lymphoblastoid cells were purchased from American Type Culture Collection (ATCC, Rockville, USA). Cells were maintained under 37 °C with 95 % relative humidity and 5 % CO_2. The cell culture media (RPMI 10) consisted of RPMI 1640 with 2 g/L of sodium bicarbonate, 10 mL/L of non-essential amino acids, 200 units/mL of penicillin, 50 μg/mL of streptomycin, 0.2 mL of 7.5 N HCl, 10 % *v/v* heat-inactivated horse serum, 0.05 % poloxamer 188, 2 % L-glutamine, 2 % sodium pyruvate, and 0.25 % amphotericin B. The cell concentration was determined every 2 or 3 days, and the cell suspension was maintained at a density of approximately 3 to 7 × 10^5 cells/mL. The doubling time of the TK6 cells was 16–20 h.

2.8.2. Cell treatment

The experiment was conducted using the protocol provided by Litron Laboratories using the MultiFlow DNA Damage Kit – p53, γH2AX, Phospho-Histone H3 Kit (Litron Laboratories, Rochester, NY). Treatments were performed in U-bottom 96-well plates. Two treatment times were carried out: a 4-h short-term treatment (with and without metabolic activation) and a 24-h continuous treatment (without metabolic activation). The metabolic activation system consists of Aroclor-1254-induced rat-liver S9 mix. Each treatment was performed in duplicate (*i.e.*, 2 wells/concentration). MMS (200 μM, clastogen) and GRF (60 μg/mL, aneugen) were used as positive controls for the assay without metabolic activation. CPA (40 μg/mL, clastogen) was used as a positive control for the assay with metabolic activation. The solvents (*i.e.*, ACN and EtOH 95 %) were used as negative controls. Because of technical issues (evaporation), the A1 extract has not been tested in DDR assay.

Briefly, 100 μL of RPMI10 or RMPI10 with 10 % S9 containing 1 % *v/v* test sample, or positive or negative control were added to the corresponding well of U-bottom 96-well plates. Six concentrations of each sample were tested. For the test item, different concentrations were obtained by ½ serial dilutions starting from the highest concentration. Then, 100 μL of cell suspension at 6 × 10^5 cells/mL was added to each well, and plates were incubated at 37 °C for 4 h. For the treatments without S9 mix, after 4 h and 24 h of incubation, cells were mixed and the volume of 50 μL was taken for flow cytometric analysis. For the experiment with S9 mix, after 4 h of incubation, the washing step was introduced to eliminate the S9 mix from the treatment media. For this reason, the content of the wells was transferred into V-bottom 96-well plates followed by centrifugation at 1200 rpm for 6 min, then cells were resuspended in 200 μL fresh RPMI10 media and from this, 50 μL was taken for flow cytometric analysis. The remaining cell suspension was transferred to U-bottom 96-well plates and incubated for an additional 20 h. Lastly, for 24-h continuous treatment without metabolic activation, cells were mixed and a volume of 50 μL was taken for flow cytometric analysis. Cytotoxicity of the samples was estimated using relative population doubling values which should be higher than 50 % after 24 h of treatment.

2.8.3. Spiking experiment with DDR test

To verify the matrix effect which may mask the positive response, in parallel to the non-spiked assay, samples were spiked with respective positive controls. Three concentrations of each sample were tested. MMS (200 μM, clastogen) and GRF (40 μg/mL, aneugen) were used as positive controls for the assay without metabolic activation. CPA (40 μg/mL, clastogen) was used as a positive control for the assay with metabolic activation.

2.8.4. DDR assessment

After 4 h or 24 h of treatment, 50 μL of cell media was transferred to another V-bottom 96-well plate followed by centrifugation. The supernatant was discarded and cells were resuspended in 25 μL RPMI10 media, then 50 μL of "Complete Labelling Solution" (containing nuclei release solution, counting beads, DNA stain, RNase solution, filtered fetal bovine serum, and antibodies) was added and the content of the wells was transferred into another U-bottom 96-well plate. The plate was incubated for 30 min protected from light at ambient temperature before the analysis with DB Fortessa HTS flow cytometry.

2.8.5. Flow cytometry

Analysis was performed on at least 20 μL per well mixed. The parameters of flow cytometry were set up as follows: flow rate of 1 μL/s, mixing volume of 25 μL, mixing speed of 180 μL/s, and wash volume of 400 μL.

2.8.6. Analysis

Analysis of data was performed through the Global Evaluation Factor (GEF) (Bryce et al., 2017). The test item was considered clastogen or aneugen, if at least, two successive concentrations meet or exceed the GEF for at least two out of four clastogen-sensitive (4 h γH2AX, 4 h p53, 24 h γH2AX, and 24 h p53) or aneugen-sensitive (4 h p-H3, 24 h p-H3, 24 h polyploidy, and 24 h p53) biomarkers.

3. Results

3.1. Identification of NIAS

LC-HRMS was used to identify non-volatile compounds migrating from polyester coatings. About 3000 peaks were detected in the 5 coating batches using both solvents, distributed for half in the positive and the negative modes. After componentisation and exclusion of PCgroups arising from the solvent control or uncoated tin plate, only four PCgroups of interest remained, i.e. specific from the coatings, in the positive scan mode only. MS^2 fragmentation experiments showed that these four substances corresponded to cyclic oligoesters involving different combinations of BD, TCDDM, and tPA monomers (4 or 6 units). Three out of the 4 combinations matched (chromatographic traces, MS^2 fragmentation assays) with data previously reported by Cariou et al.

(2022) in coatings: c (2TCDDM+2tPA), c (3TCDDM+3tPA), and c (BD+2TCDDM+3tPA). According to the MS^2 data and the chromatographic trace reported by Eckardt et al. (2020) in press-twist closure, the fourth combination was c (2BD + TCDDM+3tPA). Other combinations from intentionally added monomers such as c (2BD+2tPA), c (3BD+3tPA), or c (BD + TCDDM+2tPA) were not detected. Table 1 shows the chemical formula, retention time (t_R) range, deduced neutral mass, and predicted LogP value of identified oligoesters. Predicted LogP values of all four oligoesters were higher than 11. Representative structures of identified oligoester combinations composed of BD, tPA and TCDDM are presented in Fig. 1. Many structural isomers may arise for each oligoester if taken into account the combination of different isomers of BD, tPA, and the way TCDDM is branched with other isomers which may explain obtained chromatographic humps (Fig. S1).

Semi-quantification of identified oligoesters was done using an internal oligoester's standard mixture. Table 2 shows the mean concentration of identified oligoesters from 5 different coated metal plates (A1-A5 and E1-E5) migrated into two different solvents (ACN and EtOH 95 %). In all samples concentration of c (2TCDDM+2tPA) was around 52.7–85.0 μg/dm^2. For c (BD+2TCDDM+3tPA) and c (3TCDDM+3tPA) the concentrations were 52.6–70.1 μg/dm^2 and 46.2–74.3 μg/dm^2, respectively. c (2BD + TCDDM+3tPA) was present with the lowest concentration in all samples (3.1–5.3 μg/dm^2). The mean concentrations of individual oligoesters in both ACN and EtOH 95 % migrates were compared through a paired sample *t*-test. Statistical significance was observed in the mean concentrations of c (2BD + TCDDM+3tPA) and c (2TCDDM+2tPA) between ACN and EtOH 95 % migrates.

3.2. Genotoxic effects of migrates

3.2.1. MMT

The experiment with MMT was validated as negative and positive control values with and without the S9 mix were inside the laboratorie's historical values (data not shown).

3.2.1.1. Non-spiking experiments. Neither cytotoxicity nor precipitation of migrates was observed in strains in the presence or absence of S9 mix using direct incorporation and pre-incubation methods. Fig. 2 shows that none of the ACN or EtOH 95 % migrates were mutagenic in the four strains of *S. Typhimurium* with the direct incorporation method. Negative data were also observed whatever migrates using TA98 and TA100 strains with the pre-incubation method (Fig. 3).

3.2.1.2. Spiking experiments. For spiking experiments using TA98 strain, the spike recoveries of spiked migrates (except for A5) were equal or higher than 60 % compared with positive control and spiked solvent blank (AB for ACN migrates and EB for EtOH 95 % migrates) with and without S9 mix (Table 3).

For the TA100 strain, the spike recoveries of spiked migrates compared with positive control and spiked solvent blank (AB and EB) without S9 mix was higher than 60 % for all migrates, however, cytotoxicity was observed for all spiked migrates (½ diluted) with S9 mix (Table S1). For this strain, ¼ diluted migrates spiked with positive control were also cytotoxic.

3.2.2. DDR test

3.2.2.1. Non-spiking experiments. The results of the DDR test for ACN and EtOH 95 % migrates are presented in Table 4. The experiment was validated as negative and positive control values with and without the S9 mix were inside the laboratorie's historical values.

There was no cytotoxicity observed when cells were exposed to different migrates in these experiments. None of ACN and EtOH 95 % migrates were clastogenic or aneugenic, all biomarkers data being negative.

3.2.2.2. Spiking experiments. MMS and CPA spiked migrates were not cytotoxic and gave expected positive response with the corresponding positive controls indicating the absence of the matrix effect (Table 4).

Similar results were obtained for all ACN migrates spiked with GRS. However, with EtOH migrates (except for E4), cytotoxicity was observed after 24h of treatment for all GRS-spiked EtOH migrates suggesting that the cytotoxicity was not linked to migrates but rather to the combination of EtOH and GRS for an extended period.

4. Discussion

4.1. Identification of NIAS

In the present study, the genotoxic hazard associated with NIAS of polyester-coated metal plates was estimated. Migration tests were performed based on the recommendations of Commission Regulation (EU) No 10/2011 about contact time, temperature, and food simulants for migration testing (EU, 2011), using EtOH 95 % as food simulant substitute for foods having a lipophilic character. To supplement migration data with food simulant, extraction with mid-polarity solvent ACN as another worst-case scenario was also studied. One cyclic tetramer and three cyclic hexamers composed of different combinations of BD, TCDDM and tPA monomers were identified by LC-HRMS in ACN and EtOH 95 % migrates. A high confidence level 2 according to Schymanski et al. (2014), using acquired MS^2 data and fragmentation database (Cariou et al., 2022). Only a comparison to analytical standards, which have not been synthesized so far, would undoubtedly confirm these assumptions. Hydrolysis of isolated oligoesters would be necessary to determine which monomer isomers are constitutive of these oligomers. In the list of components that coating manufacturer provides to the can manufacturer, only starting substances subjected to specific migration limits (SMLs) as stated in the partial agreement ResAP (2004) are to be disclosed. In our case, tPA, BD and TCDDM are mentioned and we can exclude the intentional presence of their isomers isophthalic acid and 2-methyl-1,3-propanediol (subjected to SMLs) but not ortho-phthalic acid and 1,3-butanediol (not subjected to SMLs). Many structural isomers may arise for each oligoester if taken into account the combination of different isomers of butanediol, phthalic acid, and the way TCDDM and 1,3-butanediol are branched with other isomers.

Numerous studies were published previously assessing the migration from polyester coatings using ACN as an extraction solvent. Several linear and cyclic oligoesters were identified previously in ACN extracts of different vegetable cans coatings (Cariou et al., 2022), polyester-polyurethane-coated metal plates (Bradley et al., 2009; Omer et al., 2018, 2019), polyester-coated tin plate (Lestido-Cardama et al., 2022) and cans (Paseiro-Cerrato et al., 2016a).

Table 1
List of identified NIAS among the most intense PCgroups for which an elemental formula or a structure of cyclic oligoester were suggested.

PC Group	Category	Chemical formula	t_R Range (min)	Deduced neutral mass	NIAS	Predicted LogP value
280	Oligoester	$C_{40}H_{44}O_8$	17.41-17.85	652.3036	c (2TCDDM+2tPA)	11.19
1074	Oligoester	$C_{44}H_{46}O_{12}$	16.43-16.45	766.2986	c (2BD + TCDDM+3tPA)	11.02
281	Oligoester	$C_{52}H_{56}O_{12}$	18.37-18.58	872.3754	c (BD+2TCDDM+3tPA)	12.00
282	Oligoester	$C_{60}H_{66}O_{12}$	20.10–20.81	978.4554	c (3TCDDM+3tPA)	12.00

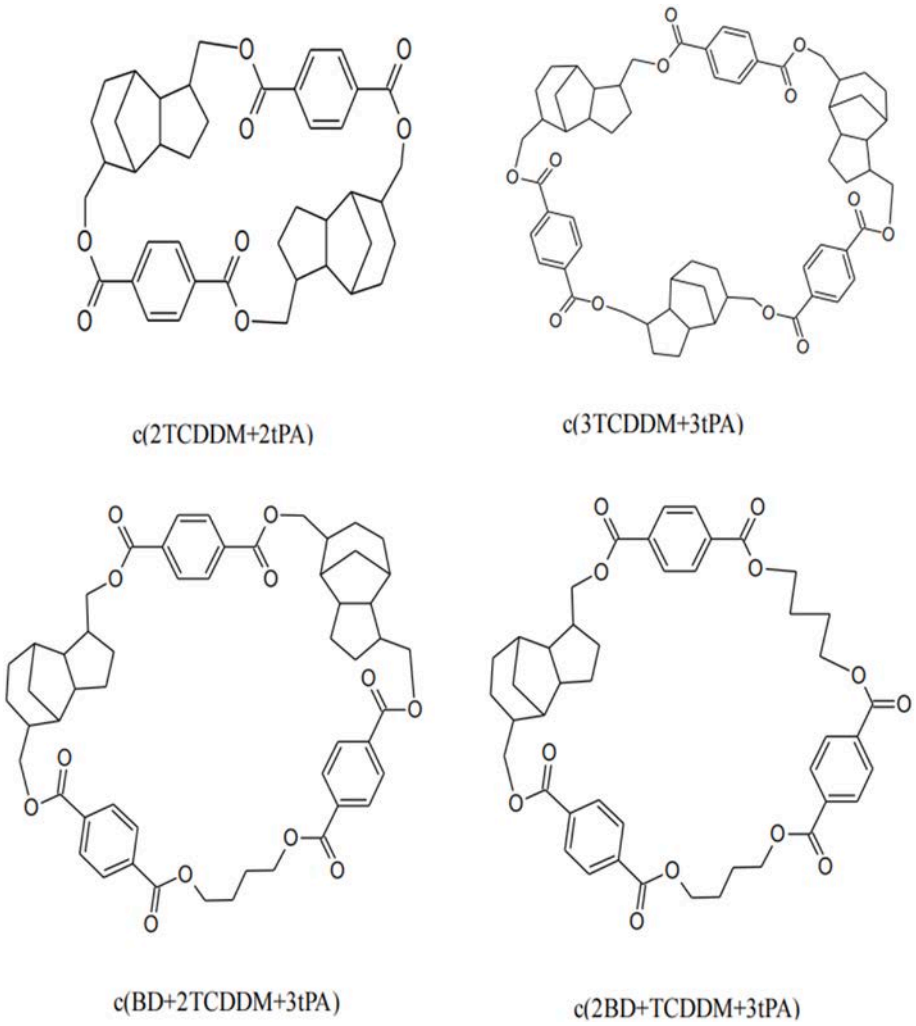


Fig. 1. Representative structures of identified oligoester combinations (many isomers may arise if taken into account different isomers of BD, tPA, and isomerism of TCDDM).

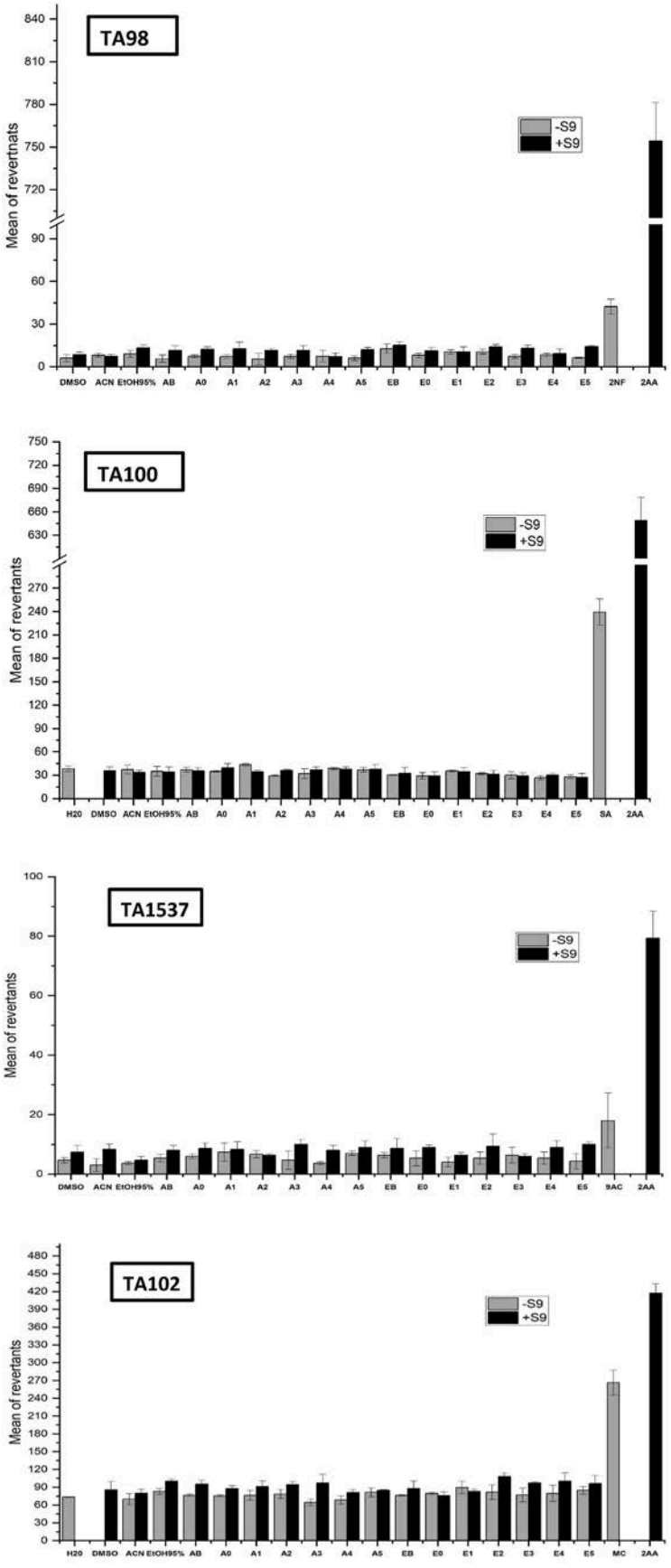
Table 2
Concentration (semi-quantification) of identified oligoesters in polyester coated metal plates migrates (A: extraction to acetonitrile; E: migration to ethanol 95 %).

Sample	Concentration (μg/dm^2)			
	c (BD+2TCDDM+3tPA)	c (3TCDDM+3tPA)	c (2BD + TCDDM+3tPA)	c (2TCDDM+2tPA)
A1	70.1	71.9	6.1	65.0
A2	60.7	62.3	4.6	62.7
A3	60.7	60.8	4.8	62.8
A4	54.3	46.2	4.2	85.0
A5	58.6	46.3	5.3	60.6
Mean of A1-A5	60.9 ± 5.2	57.5 ± 9.9	5.0 ± 0.7	67.2 ± 9.0
E1	55.8	57.8	3.3	52.7
E2	45.7	48.2	3.1	45.4
E3	59.2	65.9	4.2	55.9
E4	65.5	74.3	4.1	72.1
E5	52.6	60.1	3.9	59.5
Mean of E1-E5	55.8 ± 6.6	61.3 ± 8.7	3.7 ± 0.4	57.1 ± 8.8

Previous studies have reported the insignificance of the negative mode in identifying oligoesters from FCM extracts/migrates (Bradley et al., 2009; Schaefer et al., 2004; Omer et al., 2018). In this study, we also recorded the presence of oligomers in the positive mode only and no compounds were identified in the negative mode. However, the results of Paseiro-Cerrato et al. (2016b) showed that it is possible to detect oligoesters in the negative mode.

Only cyclic oligoesters were identified in our study and this was expected too as other groups identified mostly cyclic oligoesters and only a few linear ones in their results (Schaefer et al., 2004; Bradley et al., 2009; Omer et al., 2018, 2019; Cariou et al., 2022; Lestido-Cardama et al., 2022). In contrast, depending on the coating composition the number of linear oligoesters may be higher than the cyclic ones (Paseiro-Cerrato et al., 2016a).

Several studies were conducted using different concentrations of EtOH (10 %, 50 %, 95 %) as food simulants for migration testing of



(caption on next page)

Fig. 2. MMT with ACN and EtOH 95 % migrates tested in four strains of *S. Typhimurium* (TA98, TA100, TA102, TA1537) with and without exogenous metabolic activation system using direct incorporation method (positive controls for assay without S9 mix: 2NF (0.25 μg/well) for TA98, SA (0.4 μg/well) for TA100, MC (0.05 μg/well) for TA102, and 9AC (10 μg/well) for TA1537; positive control for assay with S9 mix: 2AA (0.4 μg/well) for all tested strains; the means of revertants and standard deviations were calculated for three wells).

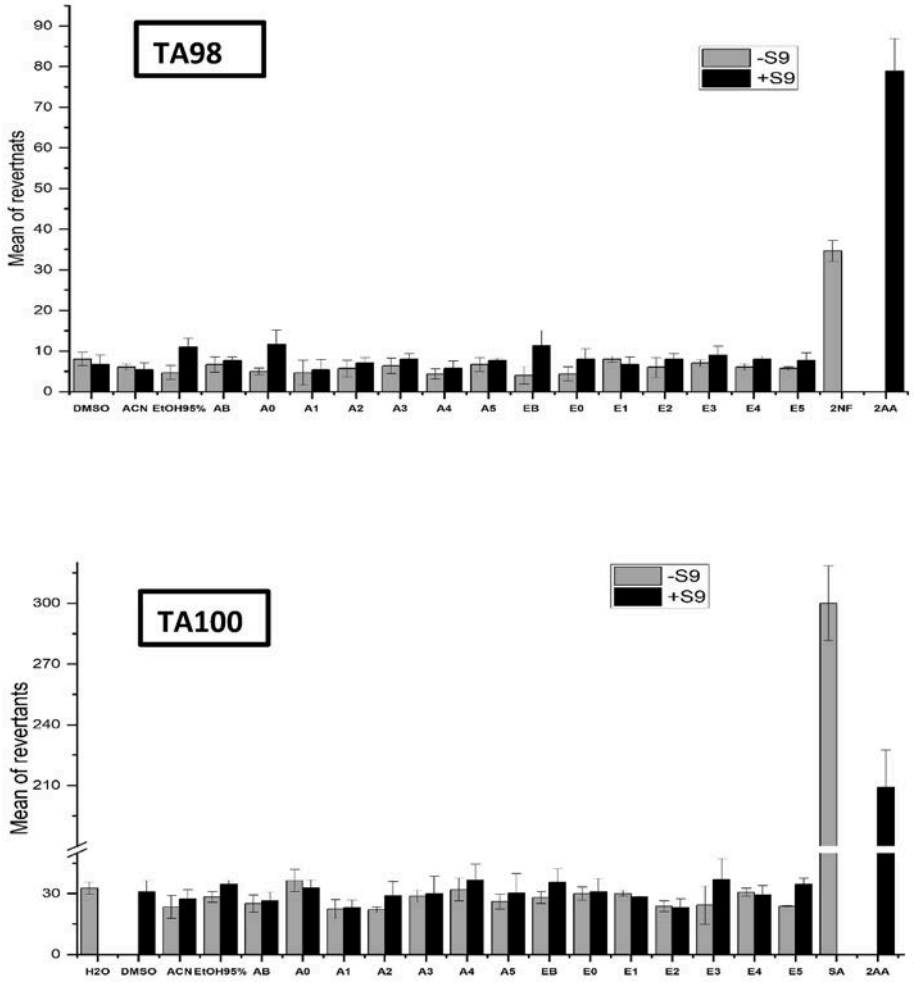


Fig. 3. MMT for ACN and EtOH 95 % migrates tested on TA98 and TA100 strains with and without exogenous metabolic activation system using pre-incubation method (positive controls for assay without S9 mix: 2NF (0.25 μg/well) for TA98, SA (0.4 μg/well) for TA100; positive control for assay with S9 mix: 2AA (0.4 μg/well) for both strains; the means of revertants and standard deviations were calculated for three wells).

polyester coatings (Schaefer et al., 2004; Paseiro-Cerrato et al., 2016b; Driffield et al., 2018). These studies reported that EtOH is an appropriate food simulant for migration testing of polyester coatings. However, data is missing to characterize the suspected ethanolysis of oligoesters in the presence of EtOH, as shown by Brenz et al. (2021). In this study, the four identified oligomers present in ACN migrates were also present in EtOH 95 % migrates at similar concentrations suggesting that no major ethanolysis occurred.

Our results are in accordance with the results existing in the literature, for example Paseiro-Cerrato et al. (2019a) identified two trimers and one tetramer in EtOH 50 % migrates of polyester cans, Pietropaolo et al. (2018) found 5 linear and 20 cyclic oligoesters in two polyester-coated enamel's EtOH 50 % and 95 % extracts, Paseiro-Cerrato et al. (2016b) identified 11 linear and 2 cyclic oligoesters in EtOH 10 % and 50 % migrates of polyester can coatings.

Neither above-mentioned studies detected four oligoesters (c (2TCDDM+2tPA), c (3TCDDM+3tPA), c (BD+2TCDDM+3tPA), and c (2BD + TCDDM+3tPA)) identified in this study. Only Eckardt et al. (2020) was reported the presence of these four oligoesters in polyester–phenolic coated tin plate's ACN and EtOH extracts (extraction was done at 60 °C for 1 h in an ultrasonic bath). There are no toxicological data available in the literature for these oligomers and no migration limits have been established in the legislation meaning that special attention should be paid to assess the safety of these NIAS. The molecular weight of the four identified oligoesters is below 1000 Da, hence, the absorption of these molecules may occur (EFSA, 2008). Also, the predicted LogP values of all oligoesters were higher than 11. Based on the EFSA report log ko/w value below 3 is sufficient evidence for the lack of accumulative potential in the mammalian body, however, a log ko/w of 3 or higher is not a proof that substance will be accumulated in the body, and absence of potential accumulation must be checked (EFSA, 2008; Eckardt et al., 2019). Additionally, Lipinski et al. (2001) developed the 'rule of 5' to estimate the solubility and permeability of substances stating that molecules will have likely poor absorption or permeation when they have more than 5 H-bond donors, 10 H-bond acceptors, a molecular weight greater than 500, and a calculated Log P greater than 5. Lipinski et al. (2001) mentioned that poor absorption or permeability is possible when two parameters are out of the range.

The molecular weights of the four identified oligoesters are higher than 500 Da and predicted LogP values are greater than 5, however,

Table 3
Results of MMT for spiked and non-spiked ACN and EtOH 95 % migrates tested on TA98 strain using pre-incubation method.

Sample	Without S9						With S9					
	Pre-incubation/Non-spiked			Pre-incubation/Spiked			Pre-incubation/Non-spiked			Pre-incubation/Spiked		
	Mean of revertants [a]	IF [b]	IF [c]	Mean of revertants	SR [d] (%)	SR [e] (%)	Mean of revertants	IF [b]	IF [c]	Mean of revertants	SR [d] (%)	SR [e] (%)
DMSO	8.00 ± 1.63						6.67 ± 2.36					
ACN	6.00 ± 0.82			25.67 ± 2.05			5.33 ± 1.70			45.00 ± 2.94		
EtOH 95 %	4.67 ± 1.70			35.67 ± 4.99			11.00 ± 2.16			76.00 ± 2.94		
AB	6.67 ± 1.89	1.11	1.00	34.33 ± 3.09	88.77	**100.00**	7.67 ± 0.94	1.44	1.00	49.33 ± 6.24	90.23	**100.00**
A0	5.00 ± 0.82	0.83	0.75	33.67 ± 4.71	87.07	98.08	11.67 ± 3.40	2.19	1.52	54.33 ± 4.78	99.38	110.14
A1	4.67 ± 3.09	0.78	0.70	28.00 ± 2.16	72.41	81.56	5.33 ± 2.62	1.00	0.69	55.33 ± 5.56	101.21	112.16
A2	5.67 ± 2.05	0.95	0.85	29.33 ± 3.77	75.85	85.44	7.00 ± 1.41	1.31	0.91	50.67 ± 3.86	92.68	102.72
A3	6.33 ± 1.89	1.06	0.95	35.33 ± 6.24	91.36	102.91	8.00 ± 1.41	1.50	1.04	58.67 ± 3.40	107.32	118.93
A4	4.33 ± 1.25	0.72	0.65	34.33 ± 0.47	88.78	100.00	5.67 ± 1.89	1.06	0.74	46.67 ± 6.13	85.37	94.61
A5	6.67 ± 1.70	1.11	1.00	22.67 ± 4.99	58.62	66.04	7.67 ± 0.47	1.44	1.00	62.33 ± 4.19	114.01	126.35
EB	4.00 ± 2.16	0.86	1.00	37.33 ± 2.87	96.53	**100.00**	11.33 ± 3.68	1.03	1.00	83.00 ± 3.56	151.82	**100.00**
E0	4.33 ± 1.70	0.93	1.08	36.33 ± 5.73	93.95	97.32	8.00 ± 2.45	0.73	0.71	80.33 ± 1.25	146.94	96.78
E1	8.00 ± 0.82	1.71	2.00	34.33 ± 2.87	88.78	91.96	6.67 ± 1.89	0.61	0.59	84.00 ± 2.94	153.65	101.20
E2	6.00 ± 2.45	1.28	1.50	38.00 ± 8.00	98.27	101.79	8.00 ± 1.41	0.73	0.71	73.67 ± 7.32	134.75	88.76
E3	7.00 ± 0.82	1.50	1.75	32.33 ± 4.03	83.60	86.61	9.00 ± 2.16	0.82	0.79	87.00 ± 12.75	159.14	104.82
E4	6.00 ± 0.82	1.28	1.50	29.00 ± 4.55	74.99	77.69	8.00 ± 0.82	0.73	0.71	92.00 ± 4.32	168.28	110.84
E5	5.67 ± 0.47	1.21	1.42	31.33 ± 2.87	81.02	83.93	7.67 ± 1.89	0.70	0.68	71.00 ± 2.94	129.87	85.54
2NF	34.67 ± 2.62	4.33		38.67 ± 5.25	**100.00**	150.64						
2AA							79.00 ± 7.87	11.84		54.67 ± 4.50	**100.00**	121.49

[a] The means of revertants and standard deviation calculated for three wells.
[b] IF: induction factor based on solvent control: the mean of revertant colonies in the sample wells was divided to the mean of revertant colonies in solvent control wells (ACN for ACN migrates, EtOH 95 % for EtOH 95 % migrates, or DMSO for 2NF or 2AA).
[c] IF: induction factor based on solvent blank: the mean of revertant colonies in the sample wells was divided to the mean of revertant colonies in solvent blank wells (AB for ACN migrates, EB for EtOH 95 % migrates).
[d] SR: spike recovery based on positive control: the mean of revertant colonies in the sample wells was compared to the mean of revertant colonies in positive control wells (2NF for the assay without S9 mix, and 2AA for the assay with S9 mix).
[e] SR: spike recovery based on spiked solvent blank: the mean of revertant colonies in the sample wells was compared to the mean of revertant colonies in spiked solvent blank (spiked AB for ACN migrates, and spiked EB for EtOH 95 % migrates).

there are no H-bond donors in their structure. For H-bond acceptors (which are expressed in our case as the sum of Os); there are more than 10 Os in three out of the four oligoesters but they all appear as pairs in the ester bond. In this study, one of the parameters (H-bond donors) is out of the range for the four oligoesters and the second parameter (H-bond acceptors) is out of the range only for c (2TCDDM+2tPA). Hence, according to Lipinski's 'rule of 5', c (2TCDDM+2tPA) may show poor absorption with a limited permeability, however, only experimental data could confirm this theory.

Moreover, several studies have shown that cyclic oligoesters may undergo hydrolysis in the gastrointestinal tract resulting in their linear forms with probably different molecular weights, LogP values, H-bond donors and acceptors, and different potency for the accumulation in the body (Eckardt et al., 2019; Ubeda et al., 2020). Further studies are required to investigate the possible gastrointestinal fate of the four identified oligoesters identified in this study.

The threshold of toxicological concern (TTC) concept can be used for food safety assessment when toxicological data are missing and if the NIAS are fully characterized in terms of their chemical structure (Cramer et al., 1976). Before applying the TTC concept, it is mandatory to check first if migrates contain genotoxic substances, and if the mixtures themselves show genotoxic effects as interaction between substances present in the mixture may occur (cocktail effect) (EFSA, 2019). For potential DNA-reactive mutagens and/or carcinogens, TTC value of 0.0025 μg/kg bw per day and for organophosphates and carbamates 0.3 μg/kg body weight (bw) per day was defined. There are also several exclusion categories for which the TTC concept cannot be applied (EFSA, 2019).

In this study, the Cramer decision tree was used to estimate the toxicity of the single identified oligomers. Indeed, using the software Toxtree, the four identified cyclic oligoesters (c (2TCDDM+2tPA), c (3TCDDM+3tPA), c (2BD + TCDDM+3tPA), c (BD+2TCDDM+3tPA)) were classified as Cramer class III substances, indicating that if the calculated or anticipated exposure exceeds the corresponding threshold of 1.5 μg/kg bw per day, potential health concerns might arise.

Quantification of oligoesters migrating from packaging to the food is an important step in the risk assessment of FCMs as it will allow estimating consumers' exposure level to these chemicals. In this study, we showed using semi-quantification that the concentration of three out of four oligoesters in both solvents was in a range of 45.4–85.0 μg/dm^2 (272.4–510 μg/kg food) and for the last one around 3.1–6.1 μg/dm^2 (18.6–36.6 μg/kg food).

It has been reported that the amount of migrated oligoester from polyester-coatings varies largely depending on the extraction solvents, food simulants used, extraction or migration test conditions, coating's formulation, the type of oligoesters, as well as the type of foodstuff intended to be in contact with coatings (Schaefer and Simat, 2004; Driffield et al., 2018; Paseiro-Cerrato et al., 2019a, 2019b; Eckardt et al., 2020; Cariou et al., 2022).

Calculating the realistic exposure of consumers poses a challenge due to variations in canned food consumption across different countries and the diverse range of coatings employed by food packaging manufacturers. Notably data are missing regarding the consumption of canned food exclusively packaged in polyester-coated metal cans.

In this study, we decided to use canned food consumption data from the French population. Roughly 50 kg of canned products are purchased per household per year in France (La conserve, 2018). Taking into account households with only one individual, the exposure amounts to approximately 137g of canned food per person per day. This calculation considers various can coatings, not solely polyester ones. Hence, it is obvious that the consumption of canned food packed only in polyester coated cans will be considerably lower, however, for conservative reasons we will use this value for exposure calculations.

Regarding this study, the exposure by default would be around

Table 4
DDR test results with spiked and non-spiked ACN and EtOH 95 % migrates.

Samples	Clastogen biomarkers				Aneugen biomarkers					
	4 h, 24 h yH2AX		4 h, 24 h p53		4 h, 24 h p-H3		24 h polyploidy		24 h p53	
+/− S9 mix										
	Non-SP[a]	SP (MMS or CP)[b]	Non-SP	SP (MMS or CP)	Non-SP	SP (MMS or CP)	Non-SP	SP (MMS or CP)	Non-SP	SP (MMS or CP)
AB	–	+	–	–	–	–	–	–	–	–
A0	–	+	–	–	–	–	–	–	–	–
A1	–	+	–	–	–					
A2	–	+	–	–	–	–	–	–	–	–
A3	–	+	–	–	–	–	–	–	–	–
A4	–	+	–	–	–	–	–	–	–	–
A5	–	+	–	–	–	–	–	–	–	–
EB	–	+	–	–	–	–	–	–	–	–
E0	–	+	–	–	–	–	–	–	–	–
E1	–	+	–	–	–	–	–	–	–	–
E2	–	+	–	–	–	–	–	–	–	–
E3	–	+	–	–	–	–	–	–	–	–
E4	–	+	–	–	–	–	–	–	–	–
E5	–	+	–	–	–	–	–	–	–	–
MMS/-S9	+	+	–	–	–	–	–	–	–	–
CPA/+S9	+	+	–	–	–	–	–	–	–	–

Samples	Clastogen biomarkers				Aneugen biomarkers					
	4 h, 24 h yH2AX		4 h, 24 h p53		4 h, 24 h p-H3		24 h polyploidy		24 h p53	
- S9 mix										
	Non-SP	SP (GRF)[c]	Non-SP	SP (GRF)	Non-SP	SP (GRF)	Non-SP	SP (GRF)	Non-SP	SP (GRF)
AB	–	–	–	–	–	+	–	+	–	–
A0	–	–	–	–	–	+	–	+	–	–
A1										
A2	–	–	–	–	–	+	–	+	–	–
A3	–	–	–	–	–	+	–	+	–	–
A4	–	–	–	–	–	+	–	+	–	–
A5	–	–	–	–	–	+	–	+	–	–
EB	–	–	–	–	–	Cytotox.	–	Cytotox.	–	Cytotox.
E0	–	–	–	–	–	Cytotox.	–	Cytotox.	–	Cytotox.
E1	–	–	–	–	–	Cytotox.	–	Cytotox.	–	Cytotox.
E2	–	–	–	–	–	Cytotox.	–	Cytotox.	–	Cytotox.
E3	–	–	–	–	–	Cytotox.	–	Cytotox.	–	Cytotox.
E4	–	–	–	–	–	+	–	–	–	+
E5	–	–	–	–	–	Cytotox.	–	Cytotox.	–	Cytotox.
GRF/-S9	–	–	–	–	+	+	+	+	–	–

−: Negative; +: Positive; Cytotox.: Cytotoxicity; Blank space: Untested.
[a] Non-spiked assay.
[b] Spiked assay: clastogen positive control used for spiking in parentheses (MMS for the assay without S9 mix or CP for the assay with S9 mix).
[c] Spiked assay: aneugen positive control used for spiking in parentheses (GRF for the assay without S9 mix).

0.77–0.84 μg/kg bw per day for c (BD+2TCDDM+3tPA), c (3TCDDM+3tPA), c (2TCDDM+2tPA) and 0.05 μg/kg bw per day for c (2BD + TCDDM+3tPA) (calculated from the mean concentration of oligoester in EtOH 95 % migrates).

The assessed exposure to four cyclic oligoesters remains below the TTC reference value (1.5 μg/kg bw/day), underscoring the absence of safety concern. Additionally, it is noteworthy to mention that the utilization of ACN and EtOH 95 % for migration testing tends to overstate the degree of chemical migration from polyester coatings into food.

4.2. Mixtures effects

Few data are available in the literature regarding the genotoxicity of the polyester oligoesters. To test whether oligoesters can act as mutagenic chemicals, they need to be synthesized (which is a high-cost and time-consuming process) before testing them as a single substance in the battery of regulatory genotoxicity bioassays.

In the study conducted by Ubeda et al. (2020), the endocrine activity of two cyclic oligoesters migrating from multilayer packaging was assessed through *in vitro* assays. The findings revealed a mild androgen receptor antagonism activity, with no observed aryl hydrocarbon receptor activity or binding to the thyroid hormone transport protein. It is worth noting that, although these oligoesters share a cyclic structure with our compounds, they are derived from different monomers. Consequently, "read-across" cannot be applied due to substantial structural differences. It is important to highlight that this study did not assess the genotoxicity of the oligoesters under investigation.

Testing the genotoxicity of FCMs migrates in *in vitro* bioassays is important as it gives supplementary information about the presence of other genotoxic NIAS which have not been detected by the analytical chemistry and allows the detection of the cocktail effect of complex mixtures. Rainer et al. (2018) showed that the Ames test is capable of detecting only a small percentage of genotoxic substances (at levels of 0.01 mg kg^{-1}), hence, it should be combined with other *in vitro* genotoxicity bioassays and analytical methods.

In this study, the genotoxicity of the ACN or EtOH 95 % migrates, which contain the four oligoesters, was tested using two bioassays (MTT and DDR) in complement to analytical analysis as a whole approach.

All migrates were negative in the MMT in all experimental conditions. The biological threshold and the sensitivity/specificity of used bioassays play a crucial role in hazard assessment of mixtures regarding DNA direct-acting chemicals, emphasizing the importance of the concentration step (Severin et al., 2017). Furthermore, it is possible that a positive effect is hidden due to the matrix effect (e.g., due to quenching). To check this drawback, spiking experiments with the MMT assay were performed in parallel on TA98 and TA100 strains using the pre-incubation method. The spike recovery for all spiked migrates was higher than 60 % (compared to spiked solvent blank: AB or EB) without

any cytotoxicity in the TA98 strain indicating the absence of any matrix effect. Similar results were obtained for all spiked migrates in the TA100 strain but only without the S9 mix. Indeed, cytotoxicity was observed with spiking assays with S9 mix even when migrates were further diluted. Cytotoxicity could be due to a combination of various substances/parameters (with low cytotoxic potential when tested alone) with a strong cytotoxicity when combined together (synergistic effect). In our case, it could be linked to the combination of S9 mix with the positive control (2AA dissolved in DMSO), and the presence of ACN or EtOH 95 %. Cytotoxic effect of S9 is a well-known challenge when using cell lines (Hinson et al., 1994; Oliveira et al., 2021).

Regarding the absence of mutagenicity, our data are in accordance with the literature. In one study polyester-coated metal strip's EtOH 95 % migrates were examined for their mutagenicity using the Ames II-Test with *S. Typhimurium strains* (TA98 and TA100) and no mutagenicity was observed. However, in this study, migrates were tested without metabolic activation system (Mittag et al., 2006).

The mutagenicity of several can coatings extracts was also examined but the type of coatings was not specified. For example, the genotoxicity of 30 different packaging materials belonging to three main material categories: coatings, plastics, and composite materials was examined by Rainer et al. (2019a). A migration test with EtOH 95 % as food simulant was conducted for 10 days at 60 °C. Migrates were concentrated approximately 300-fold and genotoxicity was assessed on Ames MPF assay using *S. Typhimurium* strains TA98 and TA100 in the presence and absence of Aroclor-induced rat liver homogenate (S9-fraction). None of the coating extracts showed a mutagenic response in this assay and one out of 7 tested coating migrates showed a reduction of the activity of the spiked positive control on both strains used with and without S9 mix indicating matrix-induced inhibitory effect (Rainer et al., 2019b). In another study, the genotoxic effect of six chemically different tin cans was investigated on newly developed reversed phases-high-performance thin-layer chromatography-UV/Vis/FLD–SOS–Umu-C assay using *S. Typhimurium* TA1535 modified strain (contains pSK1002 plasmid). Migration was performed with EtOH 95 % at 60 °C for 10 days for one tin can. The other 5 cans were extracted with *n*-hexane/acetone 1:1 (*v/v*) at 25 °C for 16 h. No genotoxic effect was observed in can coating migrates or in the different coating extracts (Meyer, 2020). Lastly, Séverin et al. (2016) also observed a negative response for mutagenicity when testing sol-gel coating migrates (EtOH 10 % for one coating and isooctane for another) on Ames test (OECD 471) using four strains of *S. Typhimurium* (TA98, TA100, TA135 and TA1537) and one strain of *Escherichia coli* (WP2uvrA pKM101). They observed a negative response in micronucleus assay (OECD 487) on the HepG2 cell line. Interestingly, initially the genotoxicity of one sol-gel coating's precursors when tested alone on the Ames test was positive in the TA100 and TA1535 strains suggesting that after the coating formulation and application to polycarbonate, genotoxic potential of some coating's precursors may disappear (Lionti et al., 2014).

The MMT is only sensitive to gene mutations but unable to detect other mutagens with different modes of action, such as chemicals causing structural and numerical chromosome aberrations. Therefore, there is a need to use a battery of bioassays as recommended by EFSA to cover all modes of action of genotoxicity and mutagenicity (EFSA Scientific Committee et al., 2019).

In this study, samples were also tested in the DDR test to cover other modes of action of mutagenicity, such as clastogenicity and aneugenicity. It allows to determine the overall genotoxicity of the tested item *via* the activation of the p53 oncogene (Bryce et al., 2017). To our knowledge, this is the first time that the DDR test was used to test the mutagenicity of the FCM's migrates.

The DDR test, similar to the MMT, gave a negative response for all ACN and EtOH 95 % migrates revealing that the risk related to *in vitro* genotoxicity of polyester-coated cans migrates appears to be low. No matrix effect was associated with migrates in the DDR test, as all spiked migrates showed positive responses similar to their corresponding positive controls. Only GRF-spiked EtOH migrates showed cytotoxicity after 24 h of treatment which was probably due to the combination of solvent (EtOH) with GRF for an extended period.

It is important to highlight that in this study samples were concentrated 53.75 times following migration tests. Subsequently, they were subjected to analysis using LC-HRMS and tested in the MMT and DDR tests. Dilution of samples is inevitable when testing samples on different bioassays. Samples were diluted 27 (direct-incorporation method) or 54 times (pre-incubation method) depending the method used in MMT test and 100 times in DDR test. Therefore, the concentration of four oligoesters (as well as other non-detected/non identified NIAS) in the final treatment media will be lower than in the initial concentrated mixture. In comparison with mixture analysed by LC-HRMS (53.75 times concentrated), in bioassays samples are concentrated only 2 (direct-incorporation method) or 1 (pre-incubation method) times in MMT test and diluted 1.86 times in DDR test.

5. Conclusion

The safety of polyester–coated metal plates was investigated with regard to the migration of chemicals from the coating into food. Four cyclic oligomers (NIAS) identified in FCM migrates were classified in our study as Cramer class III substances with an estimated exposure (calculated for French population only) below the TTC value of 1.5 μg/kg bw per day highlighting no safety concern.

ACN and EtOH 95 % migrates were negative *in vitro* in the MMT and the DDR test, meaning that under conditions tested there was no genotoxic effect associated to migrates. In addition, no matrix effect was related to migrates.

Further investigations on the other toxicity endpoints (e.g., endocrine-disrupting activity) of ACN and EtOH 95 % migrates are recommended to give a final conclusion about the safety of polyester coatings. Gas chromatography–mass spectrometry analyses are required to identify other predictable or unpredictable NIAS able to migrate from polyester coating. Lastly, investigation on the gastrointestinal fate of identified oligoesters is necessary to better assess the hazard linked to polyester coatings.

CRediT authorship contribution statement

Ruzanna Hayrapetyan: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Ronan Cariou:** Formal analysis, Resources, Writing – original draft, Writing – review & editing. **Anne Platel:** Data curation, Formal analysis. **Julie Santos:** Data curation. **Ludovic Huot:** Data curation. **Véronique Monneraye:** Funding acquisition, Supervision, Validation. **Marie-Christine Chagnon:** Conceptualization, Formal analysis, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **Isabelle Séverin:** Conceptualization, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2024.114484.

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Discussion

Oligoesters are known NIAS from polyester coatings. Several linear and cyclic oligoesters were identified previously in different vegetable cans coatings (Cariou *et al.*, 2022), polyester-polyurethane-coated metal plates (Omer *et al*., 2018, 2019; Bradley *et al*., 2009), polyester-coated tin plate (Lestido-Cardama et al., 2022a) and cans (Paseiro-Cerrato *et al*., 2016a).

Four cyclic oligoesters identified in this study in ACN extracts and EtOH 95% migrates have molecular weight below 1000 Da indicating that absorption of these molecules may occur (EFSA, 2008). Additionally, all identified oligoesters have predicted log $K_{o/w}$ values exceeding 11. According to the EFSA, a log $K_{o/w}$ below 3 is considered sufficient to rule out bioaccumulation in the human body. However, values at or above 3 do not confirm bioaccumulation potential, and further assessment is required to determine whether such accumulation may occur (EFSA, 2008). Applying Lipinski's 'rule of 5' it was suggested that c (2TCDDM+2tPA) may show poor absorption with a limited permeability, however, only experimental data could confirm this theory (Lipinski *et al*., 2001).

No toxicological data are currently available in the literature for the identified oligomers, and no SMLs have been established by legislation. This highlights the need for careful safety assessment of these NIAS. All four oligoesters were classified as Cramer Class III, indicating a high level of concern regarding their potential toxicity. The exposure by default (calculated for French population only) would be around 0.77–0.84 µg/kg bw per day for c (BD+2TCDDM+3tPA), c (3TCDDM+3tPA), c (2TCDDM+2tPA) and 0.05 µg/kg bw per day for c (2BD +TCDDM+3tPA) (calculated from the mean concentration of oligoester in EtOH 95 % migrates) which is below the TTC value of 1.5 µ g/kg b.w./day, suggesting a no safety concern.

ACN and EtOH 95% migrates tested negative for genotoxicity in both the MMT and the DDR assay, indicating no genotoxic effects under the conditions applied. Additionally, no matrix effects were observed. However, further studies are recommended to evaluate other toxicological endpoints, such as endocrine-disrupting potential, to draw definitive conclusions on the safety of polyester coatings. Complementary GC-MS analyses are needed to identify additional known or unknown NIAS (refer to Chapter 3) that may migrate from the coating. Moreover, investigating the

gastrointestinal fate (refer to Chapter 5) of the identified oligoesters is essential for a more comprehensive hazard assessment.

Chapter 3- Article 2

Hazard assessment of fenozan, a released non-intentionally added substance from polyester-based can coating

Summary

Introduction

In Chapter 2, we identified non-volatile NIAS, specifically cyclic oligoesters, originating from polyester coatings. However, oligoesters are not the only substances of concern when it comes to migration from can coatings. Several studies have shown that volatile and semi-volatile chemicals may also migrate from these coatings into food (Vázquez-Loureiro *et al.*, 2021; Lestido Cardama *et al*., 2019; Domeño *et al*., 2017). For instance, Vázquez-Loureiro *et al*. (2021) reported the migration of various volatile and non-volatile compounds—including aldehydes, alcohols, ethers, alkenes, and phthalic compounds—from polymeric coatings used in metal food and beverage cans. Notably, they also identified 7,9-di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione, a degradation product of the antioxidant Irganox 1010, as a NIAS.

Building on our previous findings, we have conducted complementary GC-HRMS analyses to identify additional known and unknown NIAS that may migrate from the coating. This study focuses on the identification of volatile and semi-volatile NIAS from polyester-based coatings to evaluate their potential hazards and ensure consumer safety regarding exposure from canned food.

Materials and Methods

Five polyester-coated tinplate samples (the same as those used in Chapter 2) were tested, along with one uncoated control. For sample preparation, two solvents were employed: ACN as a worst-case extraction medium, and EtOH 95% as a food simulant. Extractions and migration tests were performed following the same procedures described in Chapter 2. Samples then were concentrated and analyzed using GC-HRMS. The presence of an identified NIAS (fenozan) was confirmed using an analytical standard.

The Danish (Q)SAR Database was used to predict the thyroid hormonal activity of the identified compounds.

To assess the toxicity of the identified compound, a series of *in vitro* bioassays were conducted, including the Ames test, micronucleus assay, and iodide uptake assay.

The Ames test was carried out in accordance with OECD Guideline 471, using three *Salmonella typhimurium* strains (TA98, TA100, and TA1535) and one *Escherichia coli* strain (WP2), both with and without metabolic activation (S9 mix). Both the direct incorporation and pre-incubation methods were applied.

The micronucleus assay was performed on human HepG2 cells, with and without S9 metabolic activation. Cytotoxicity and genotoxicity were assessed using flow cytometry.

The iodide uptake assay was conducted using rat thyroid FRTL-5 cells to evaluate the impact of fenozan on iodide uptake, as an indicator of potential anti-thyroid activity. Cytotoxicity was measured using the MTT reduction assay.

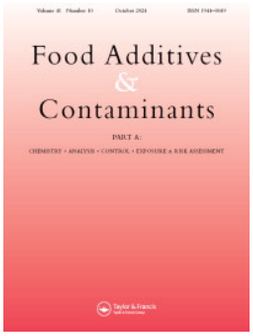
Results

GC-HRMS analysis identified the presence of 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionic acid (commonly known as fenozan; CAS RN: 20170-32-5), a degradation (hydrolysis) product of an antioxidant, at an estimated concentration of ~0.2 µg/dm² based on semi-quantitative analysis. Fenozan was detected in both ACN extracts and EtOH 95% migrates. Its identity was confirmed using an analytical reference standard, achieving Level 1 confidence on the Schymanski identification scale (Schymanski *et al.*, 2014).

In the Ames test, fenozan yielded negative results across three *Salmonella typhimurium* strains (TA98, TA100, and TA1535) and one *Escherichia coli* strain (WP2), with and without metabolic activation (S9 mix), using both the direct incorporation and pre-incubation methods. Cytotoxic effects were observed at high concentrations.

The *in vitro* micronucleus assay performed on HepG2 cells also produced a negative result following a 4-hour treatment with S9 and a 48-hour treatment without S9. Cytotoxicity was noted at the highest tested concentration.

In the iodide uptake assay, fenozan exhibited a weak yet statistically significant inhibitory effect, beginning at 800 µM, with 47% inhibition observed at 1000 µM and an IC_{50} value of 1152 µM. No significant cytotoxicity was observed in this assay.



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Hazard assessment of fenozan, a released non-intentionally added substance from polyester-based can coating

Ruzanna Hayrapetyan[a], Isabelle Séverin[a], Olga Matviichuk[b,c], Lorine Da Costa[a], Cristina Juan[d], Ana Juan-Garcia[d], Hélène Moche[e], Anne Platel[e], Ronan Cariou[b] and Marie-Christine Chagnon[a]

[a]CTM UMR1231, Nutrition Physiology and Toxicology Team (NUTox), Dijon, France; [b]Oniris, INRAE, LABERCA, Nantes, France; [c]Metal Packaging Research Laboratory (LEREM), Montataire, France; [d]Universitat de València, València, Spain; [e]ULR 4483, IMPECS – IMPact de l'Environnement Chimique sur la Santé Humaine, Univ. Lille, CHU Lille, Institut Pasteur de Lille, Lille, France

ABSTRACT

Since the safety of new-generation polyester-based internal coatings regarding the migration of non-intentionally added substances (NIAS) is poorly documented, studies are needed to identify NIAS originating from these food-contact materials (FCM). The aim of this study was to identify volatile and semi-volatile NIAS from polyester-based coatings in order to assess their hazard and ensure consumers' safety with regard to exposure from canned food. Extraction and migration tests were carried out on a single polyester-coated tin plate (5 batches) using two solvents: acetonitrile and ethanol 95%, then FCM's extracts and migrates were analysed by GC-MS. An antioxidant degradation (hydrolysis) product, 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionic acid or fenozan (CAS RN: 20170-32-5), was identified and confirmed by reference standard in all migrates. To assess fenozan's toxicity, several *in vitro* bioassays, such as the Ames test (to assess point mutation), the micronucleus assay (to detect chromosomal aberrations), and the iodide uptake assay (to study one mode of action for thyroid disruption) were conducted. Fenozan was negative in the Ames test on three strains of *S. typhimurium* (TA98, TA100, and TA1535) and on one strain of *E.coli* (WP2), with and without metabolic activation system (S9 mix) using direct incorporation and pre-incubation methods. The *in vitro* micronucleus assay conducted on HepG2 cells also exhibited a negative response following a 4-hour treatment with the S9 mix, and a 48-hour treatment without the S9 mix. A weak inhibitory effect was obtained when testing fenozan in the iodide uptake assay using rat thyroid FRTL-5 cells. Significant inhibition started from 800 µM of fenozan, with a maximal inhibition of almost 47% at 1000 µM. The findings indicate that fenozan exhibits an anti-thyroid activity *in vitro*.

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Introduction

Various chemical substances are used worldwide in the manufacture of food packaging, including can coatings. It is a challenging task for packaging manufacturers to design food-contact internal can coatings with the expected physicochemical properties and minimized levels of chemical migration. Consumers can be exposed to chemicals migrating from food packaging to food, in particular NIAS with no or few toxicological data. In the EU coatings along with other FCMs are subject to the framework Regulation EC 1935/2004 (European Community 1935). To date, no harmonized regulations exist that specifically regulate coatings intended to come into contact with foodstuffs. Therefore, manufacturers rely on industry guidelines (CEPE code of practice) which are based on the Council Resolution ResAP (2004)1 (n.d.). This document provides a list of starting substances or intentionally added substances (IAS), such as monomers or additives (e.g. antioxidants, stabilizers, and plasticizers) that can be used to manufacture coatings. Furthermore, the migration of NIAS which are defined as contaminants, degradation products, and impurities of starting substances remains a high concern for the safety of food packaging

CONTACT Isabelle Séverin isabelle.severin@agrosupdijon.fr CTM UMR1231, Nutrition Physiology and Toxicology Team (NUTox), Dijon F-21000, France
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because of the gap in knowledge about their existence, origin, formation, identification, quantification methods (due to the absence of standards), as well as associated toxicities.

Migration of several cyclic and linear oligomers (as predictable NIAS) from can coatings has already been reported (Louise Bradley et al. 2009; Omer et al. 2019, 2018; Cariou et al. 2022; Lestido-Cardama et al. 2022; Hayrapetyan et al. 2024). However, oligoesters are not the only concern regarding the migration from can coatings. It has been also shown that several volatile and semi-volatile chemicals may also migrate from can coatings to food (Domeño et al. 2017; Lestido Cardama et al. 2019; Vázquez-Loureiro et al. 2021). For example, Vázquez-Loureiro et al. (2021) detected the migration of different volatile and non-volatile compounds including aldehydes, alcohols, ethers, alkenes, or phthalic compounds from polymeric coatings used in metal food and beverage cans. Among these, a degradation product of the antioxidant Irganox 1010 namely 7,9-di-tert-butyl-1-oxaspiro [4.5]deca-6,9-diene-2,8-dione was also identified as a NIAS.

Polymers may oxidize during synthesis, processing, transport, or final usage, producing free radicals that can interact with oxygen to create hydroperoxides and eventually lose their physicochemical features. In order to delay oxidative processes and preserve the qualities of coatings while increasing their shelf life, antioxidants are added during coating manufacturing. Numerous synthetic phenolic antioxidants (SPAs) are used to produce can coatings. They share a relatively similar structure composed of a phenolic ring substituted with hindered alkyl groups in the ortho position. SPAs can react with free radicals by donating an atom of hydrogen, resulting in a stable product. Antioxidant free radicals are sufficiently stable on their own and do not appear to initiate or spread a chain reaction, which stops the autoxidation chain reaction. 2,6-di-tert-butyl-4-methylphenol (BHT), a typical SPA congener, has lately been recommended for replacement by high molecular SPAs because of their reduced migratory potential (Liu and Mabury 2020a). Several studies have reported the migration of degradation products of SPAs from different plastic materials can coatings (Vázquez-Loureiro et al. 2021), plastic films (Bertoldo and Ciardelli 2004; Vera et al. 2018; Ji et al. 2024), kitchenware (Hu et al. 2021; Song et al. 2022; Wrona et al. 2023), plastic pipes (Brocca et al. 2002; Vera et al. 2018; Diera et al. 2023). They are ubiquitous in indoor and outdoor environments and have various exposure pathways, such as food intake, dust ingestion, or inhalation (Liu and Mabury 2020a).

The toxicity of degradation products of antioxidants is not well evaluated and further investigation is required in order to assess the hazard and risk related to these antioxidants. It was hypothesized that there is a concern for the thyroid effect due to the exposure of BHT (structural analogue of several degradation products of antioxidants) as several rat studies showed changes to thyroid physiology following exposure to this chemical (ANSES 2016). Thyroid hormones play an important role in the regulation of metabolism, growth, and development. Iodide uptake from blood into thyroid follicular cells is the first step in the biosynthesis of thyroid hormones thyroxine (T4) and triiodothyronine (T3) in the thyroid gland. This transport is mediated by the sodium iodide symporter (NIS) which plays a key role in thyroid diseases such as thyroid cancer, autoimmune diseases, and congenital hypothyroidism (Dohán et al. 2003; Riesco-Eizaguirre and Santisteban 2006; Darrouzet et al. 2014). Therefore, assessing the impact of substances on iodide uptake is an essential step for the evaluation of their anti-thyroid activity.

Testing genotoxicity of degradation products of antioxidants is crucial step in the safety evaluation of these chemicals. The European Food Safety Authority (EFSA) recommends using a combination of two regulatory bioassays, namely the bacterial Ames test and the *in vitro* micronucleus assay, as the initial approach to assess the mutagenicity of chemicals. These two tests are capable of addressing all potential modes of actions associated with mutagenic chemicals (EFSA Scientific Committee 2011).

The objective of this study was to identify volatile and semi-volatile NIAS migrating from polyester-coated metal plates to evaluate the hazard associated with this type of coating. Five batches of one single polyester-coated metal plate's acetonitrile (ACN) extracts and ethanol (EtOH)

95% migrates were analysed by GC – high-resolution mass spectrometry (HRMS). The genotoxicity of fenozan, a hydrolysis product of several antioxidants identified as NIAS, was assessed using relevant *in vitro* bioassays. As alerts for sodium/iodide symporter (NIS) activity (Leadscope model) were obtained using the Danish (Q)SAR Database, an iodide uptake assay was conducted to confirm potential anti-thyroid activity of the substance.

Materials and methods

Samples

Five tin plates were coated with different batches of a single polyester-based formulation. In addition, one uncoated tin plate was prepared as a control. Coated tin plates were used to produce cans. The reference formulation was composed of two polyols, 1,4-butanediol and tricyclodecanedimethanol, as well as two polyacids, terephthalic acid and maleic acid from its anhydride precursor. Other starting substances of the varnish were not disclosed.

Extraction/migration assays and sample preparation

Two pieces of each batch of polyesters-coated plates were cut into a disk shape (1.02 dm^2) and fixed hermetically inside two different Calipack migration cells (European Standard CEN – EN 1186-1 2002). Then, 430 mL of ACN (Carlo Erba, Val-de-Reuil, France) or EtOH 95% (Promochem, Picograde®, Wesel, Germany) were poured into two migration cells and were incubated at 40 °C for 1 d for ACN samples or at 60 °C for 10 d for EtOH 95% samples. Mid-polarity solvent ACN was selected as the extraction solvent standing for a worst-case scenario, while EtOH 95% was selected as a practical and recognised substitute to the assigned food simulant for foods having a lipophilic character, vegetable oil, according to Commission Regulation (EU) No 10/2011 (European Union 2011). After incubation, the contents of both migration cells containing the same solvent (ACN extracts on the one hand; EtOH 95% migrates on the other hand) were pooled and concentrated (53.75 times) under a rotary evaporator and then under gentle nitrogen flow at +40 °C.

For the control, extractions and migration tests were performed on one non-coated tin plate using ACN (A0) and EtOH 95% (E0). Finally, ACN and EtOH 95% solvents were treated as described above to provide procedural blanks (AB or EB).

GC-HRMS analysis

For the GC-HRMS analysis, 50 µL each of the five coated ACN extracts and EtOH 95% migrants were mixed to obtain two pooled samples, one in ACN (from now on A1-5) and one in EtOH 95% (from now on E1-5). Then, 10 ng of the D_4-labelled cyclic tetraester composed of neopentyl glycol and isophthalic acid monomers were added as an external standard. Volumes of 250 µL were taken from the remaining control sample (A0, AB, E0, EB) to which 10 ng of deuterated standards were also added. All migrates were then concentrated under a gentle nitrogen stream and reconstituted in 10 µL of toluene (Sigma-Aldrich).

Data acquisition

Extracts were analysed by GC-HRMS using a TRACE 1310 GC system fitted with a TriPlus RSH auto sampler and coupled to an Orbitrap Q Exactive mass spectrometer (Thermo, Fischer Scientific). Two microliters were injected in the splitless mode (1 min). The inlet temperature was set at +300 °C. The stationary phase was a DB-5MS column (30 m × 0.25 mm, 0.25 µm). The mobile phase was helium (1 mL/min). The temperature gradient started at +100 °C (2 min) and rose to +310 °C (+10 °C/min, 10 min). The transfer line temperature was set at +325 °C. Ionisation was performed in the electron impact mode at 70 eV. The source temperature was set at +300 °C. The acquisition was operated in one-dimensional full scan mode over the mass/charge (*m/z)* range of 120–800 and the resolving power was set at 120,000 FWHM @200.

Post-acquisition data processing

Xcalibur software, in particular its Qual Browser utility, was used to visually check the

chromatograms and spectra from the raw data. The pre-processing of the raw data set obtained for the sequence performed on the GC-Q-Orbitrap was then handled using *RStudio* (version 2022.07.1 + 554), an open-source integrated development environment for the *R* programming language. Firstly, the proprietary raw format files were converted to the open *mzXML* format using *msConvert* (v3.0.22265), an open-source command-line utility provided by ProteoWizard for conversion between various MS data formats, so that they can be used for further processing. The data processing was carried out using a script written in-house. The idea of MS data pre-processing is to transform a large amount of raw spectral data into a much smaller and statistically manageable set of features. The operations behind this script were to identify the chromatographic peaks that differed from the background noise (peak picking), to pair signals that could arise from the same compound in a mass spectrum (coeluted ions, so-called componentisation step), to integrate them, and to align (pair) them along the sequence. Data processing was performed using the *xcms* and *metaMS* packages. The allowed retention time (RT) shift between the same molecule in different samples was set at 0.05 min. The minimum number of ions in a mass spectrum to consider it as a molecule was set to 5. The fraction and the absolute number of samples, in which a pseudospectrum is present before it is considered an *unknown,* were set at 0.5 and 1, respectively. The output table consisted of *unknowns* characterised by the following information the sample containing these *unknowns*, RT and the intensity (area). It was prepared and exported to an Excel® sheet for manual data processing and analysis. Finally, the resulting pseudospectra were compared with the NIST standard reference database (version 2.3, build May 2014).

MS^2 fragmentation experiment

To confirm the obtained correspondence with the NIST database, an MS^2 fragmentation was carried out. This analysis was carried out using the HCD cell at 10%, 30%, and 50% of the normalized collision energy (NCE) after filtering the precursor molecular ion $[M]^{+\bullet}$ in the quadrupole at ±0.4 mDa (low resolution). The resulting ions were analysed in the Orbitrap set at 120,000 FWHM@200.

Quantitative structure-activity relationship analysis

Danish (Q)SAR Database (n.d.) (http://qsar.food.dtu.dk/) was used to predict the thyroid hormonal activity of identified molecules. CAS RN (20170-32-5) of fenozan was used to search information in the Danish (Q)SAR Database.

In vitro bioassays

The bacterial reverse mutation test or Ames test (OECD 471)

Reagents for the Ames test were prepared with chemicals from Sigma-Aldrich (St. Louis, MO, USA). Seven concentrations (5, 3, 2.5, 1.6, 0.5, 0.16, and 0.05 mg/plate) of fenozan (Synthonix, North Carolina, US) were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and tested with Ames test according to OECD 471 guideline on three strains of *Salmonella typhimurium* (*S. typhimurium* TA98, TA100, and TA1535) and one strain of *Escherichia coli* (*E. coli* WP2) with and without S9 mix (beta-naphthoflavone and phenobarbital-induced S9 purchased from Biopredic International) using direct incorporation and pre-incubation methods (OECD, 2020). The strains (Trinova Biochem) were maintained in frozen stocks (−80 °C, DMSO culture) and were grown overnight in the fresh growth medium (7 mL of nutritive broth supplemented with 25 µg/mL of ampicillin for *S. typhimurium* TA98, TA100, and *E. coli* WP2 strain).

Positive controls were used to validate the experiment. The positive controls without the S9 mix were nitrofluorene (2NF) (1.0 µg/plate; Sigma-Aldrich) for TA98, sodium azide (SA) (2.0 µg/plate; Sigma-Aldrich) for TA100 and TA1535, and 4-nitroquinoline 1-oxide (4NQO) (1.0 µg/plate; Sigma-Aldrich) for *E.coli* WP2. The positive control with the S9 mix was 2-aminoanthracene (2AA) (2.5 µg/plate for TA98, TA100, and TA1535 and 25.0 µg/plate for *E. coli WP2;* Sigma-Aldrich) for all strains. DMSO was used as a negative solvent control in this experiment.

For the direct incorporation method, 2 mL of previously melted top agar was mixed with 500 µL of sterile phosphate buffer (0.1 mM, pH 7.4, for the protocol without the S9 mix) or S9 mix (for the protocol with the S9, 10%). Then, 100 µL of fenozan solution or DMSO (negative control) or mutagen (positive control), as well as 100 µL of bacteria (~10^8 viable cells) were added. The obtained mixture was vortexed and poured on the surface of a minimal glucose agar plate. For the pre-incubation method, 100 µL of bacteria were incubated for 20 min with 50 µL of fenozan solution or DMSO or positive control, and 500 µL of phosphate buffer (0.1 mM, pH 7.4) or S9 mix. After incubation, 2 mL of top agar was added to each tube and the content was poured onto the surface of the Petri dish containing the minimal glucose agar. After solidification of the top agar, Petri dishes were incubated at 37 °C for 48 h.

The number of revertant colonies was then counted and compared with the number of spontaneous revertant colonies developed on the solvent control plates. Three replicates were conducted for each sample. The average number of revertant colonies and the standard deviation (SD) of three replicates for samples, positive and negative controls were calculated. A result was considered positive if the number of revertant colonies was two-fold higher than the number of revertant colonies in the solvent control plates for TA98, TA100, and *E.coli* WP2 strains, and three-fold for TA1535. Otherwise, a negative result was defined.

Micronucleus assay

Cell culture and maintenance- Human hepatoma cells (HepG2) obtained from European Collection of Authenticated Cell Cultures (85011430) were cultured in MEM medium (21090-022, Gibco) supplemented with 2 mM L-glutamine (25030-024, Gibco), 1% non-essential aminoacids (11140-068, Gibco) and 10% Fetal Bovine Serum (S132507, Dutscher) in a humidified atmosphere of 5% CO_2 and at 37 °C. All cells used in the experiments were maintained and expanded in 75 cm^2 cell culture plastic flasks. Cells were divided every 4/5 days at 3×10^6 cells/flask by trypsination until 70%–80% confluency. Only cells from passages 2–12 were used in the experiments.

Treatment- HepG2 cells were seeded in a 24-well plate at the density of 200,000 cells/well. After 24 h, the cells were treated 48 h (1.5-times HepG2 doubling time (30 h)) in the absence of metabolic activation, or 4 h with S9 metabolic activation followed by a 44-hour recovery period. The S9 mix contained the following components glucose-6-phosphate (180 mg/mL), NADP (25 mg/mL), KCl (150 mM) and S9 fraction mixed in the ratio 1:1:1:2. The final concentration of the liver homogenate in the medium supplemented with 0.5% Fetal Bovine Serum was 2%. Cells were treated with three different concentrations of fenozan (0.25; 0.1; 0.01 mg/mL) or solvent control (1% DMSO) or positive control mitomycin C (0.05 µg/mL; CAS RN 50-07-7; Sigma-Aldrich) and vinblastin (0.0025 µg/mL; CAS RN 143-67-9; Sigma-Aldrich) without metabolic activation, and cyclophosphamide (CPA) (15 µg/mL; CAS RN 6055-19-2; Sigma-Aldrich) with metabolic activation. Concentrations of dosing solutions and the time of exposure were based on OECD 487 guideline (OECD, 2023). Acceptable cytotoxicity was 55%. Cytotoxicity was determined thanks to 'Counting beads' (C16508, Invitrogen). By scoring these 'counting beads' on the flow cytometer, the number of EMA(-)SYTOX(+) 'healthy' nuclei per well can be assessed. The relative survival was calculated from nuclei-to-counting beads ratios (Instruction manual of *In Vitro* MicroFlow kit® (Litron Laboratories, Rochester, NY)). Cytotoxicity was observed at 0.5 mg/mL fenozan solution, the highest concentration was determined by dividing the cytotoxic dose by two. HepG2 treatments were repeated independently at least two times with and without metabolic activation.

Staining and lysis- Staining and lysis were performed as described in the instruction manual of *In Vitro* MicroFlow kit® (Litron Laboratories, Rochester, NY) and in Guo et al. (2020), Barbezan et al. (2019), Juan-García et al. (2018) and Juan-García et al. 2019), Bryce et al. (2010). Briefly, after 72 h in culture, the plate was placed on ice for 20 min. After medium removing, cells were stained with ethidium bromide monoazide or EMA dye (E1374, Thermo-Fisher Scientific) at a concentration of 8.5 µg/mL diluted in PBS with 2% foetal bovine serum. Plates were exposed to visible light for 30 min on ice for photoactivation

of EMA dye to stain the DNA of non-viable cells. EMA dye was removed and cells were rinsed with 1 mL of PBS with 2% foetal bovine serum. The first lysing solution containing sodium chloride (0.584 mg/mL), sodium citrate dihydrate (1 mg/mL), IGEPAL (0.3 µL/mL), RNAse (0.5 mg/mL) and SYTOX Green (Thermo-Fisher Scientific) fluorescent dye (0.12 µM) (S34860, Thermo-Fisher Scientific) was added to each well. The plate was mixed gently and incubated for 1 h at +37 °C in the dark. Next, cells received the second lysis solution with sucrose (85.6 mg/mL), citric acid anhydrous (1.5 g/mL), SYTOX (0.12 µM) and 1 drop/10 mL of lysis solution of fluorescent beads (C16508, Life Technologies). Finally, the plate was incubated during 30 min at room temperature in the dark and cells were collected for flow cytometric analysis.

Flow cytometry – Micronucleus were scored using a LSRFortessa® flow cytometer (BD Biosciences) and FACSDiva software. Based on the acquisition of at least 20,000 EMA(-) SYTOX(+) « healthy » nuclei per well, the percentage of micronucleus and viability (relative survival) were calculated from nuclei-to-counting beads ratios. A positive outcome was determined if at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control, the increase is dose-related in at least one experimental condition when evaluated with an appropriate trend test and any of the results are outside the distribution of the historical negative control data.

Iodide uptake assay

Iodide uptake assay was performed based on an iodide-catalyzed Sandell-Kolthoff reaction between arsenic (III) and cerium (IV), following the method described by Waltz et al. (2010) with a few modifications. Rat thyroid FRTL-5 cells were purchased from CLS Cell Lines Services GmbH (Eppelheim, Germany) and cultured in Coon's F-12 medium (Sigma-Aldrich, St-Quentin-Fallavier, France) supplemented with 5% FBS (Gibco, Illkirch, France), 100 U/mL penicillin, 100 µg/mL streptomycin, 10 µg/mL insulin (Sigma-Aldrich, St-Quentin-Fallavier, France), 10 nM hydrocortisone (Sigma-Aldrich, St-Quentin-Fallavier, France), 10 ng/mL Gly-His-Lys acetate (Sigma-Aldrich, St-Quentin-Fallavier, France), 1 mU/mL bovine thyroid stimulating hormone (TSH, Sigma-Aldrich, St-Quentin-Fallavier, France) and 5 µg/mL transferrine (Sigma-Aldrich, St-Quentin-Fallavier, France) at 37 °C and 5% CO_2. Cells were subcultured every 3–4 d. Upon reaching *ca.* 80% confluency, 200 µL of a suspension at 250,000 cells/mL were seeded in columns 3–10 of duplicate 96-well flat-bottomed plates for assessment of iodide uptake and cell viability.

Three days after plating, culture medium in the plates was replaced by 80 µL of Coon's F-12 medium at room temperature. 10 µL of 10X fenozan solutions (7 or 8 concentrations) in Coon's F-12 medium were transferred in triplicate wells. DMSO and sodium perchlorate (7 concentrations) in Coon's F-12 medium were also added as solvent control and reference substance, respectively. Then 10 µL of NaI solution at 100 µM were immediately added. The plates were incubated protected from light at room temperature for 1 h. The iodide uptake assay plate was then washed 3 times with 200 µL of cold Hank's Balanced Salt Solution (HBSS, Gibco, Illkirch, France) supplemented with 10 mM HEPES (Sigma-Aldrich, St-Quentin-Fallavier, France) without entirely emptying the wells. After that, the supernatant was removed and 100 µL of water were added to columns 3–10. 100 µL of NaI standards (0, 100, 200, 300, 400, 500, 600, and 700 nM) were distributed in quadruplicate wells in columns 1–2 and 11–12. 100 µL of 24 mM sodium arsenite (III) solution and 100 µL of 10.5 mM ammonium cerium (IV) sulfate solution were added in all columns and the plate was incubated for 21 min at 25 °C. Optical density at 405 nm (OD_{405}) was then immediately measured using a Spectrostar Nano (BMG LabTech, Champigny s/Marne, France). A calibration curve was prepared by plotting the logarithmic conversion of the means of OD_{405} ($n = 4$) *vs.* iodide standard concentrations. The iodide concentrations in treated wells were determined after linear regression of the calibration curve.

Cell viability was estimated from cell metabolism as measured using the 3-(4,5-dim ethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay. After a 1-h incubation at room temperature, the supernatant

was removed from the wells of the cytotoxicity assay plate and 100 µL of MTT at 0.5 mg/mL in DMEM without phenol red were added. After a 2-h incubation at 37 °C, supernatant was removed and 100 µL of acidified isopropanol was added. The plate was shaken until formazan crystals were dissolved and optical density at 570 and 640 nm was measured using a Spectrostar Nano (BMG LabTech, Champigny s/Marne, France).

Iodide uptake and cell viability data were normalized to solvent control values and expressed as relative iodide concentration (%) and relative viability (%). A significant threshold of 30% inhibition was chosen for iodide uptake assay and only concentrations inducing less than 20% decrease in cell viability were included from the analysis. IC_{50} values were derived from concentration-response curves fitted with a four-parameter logistic curve using the statistical software GraphPad Prism 9 (GraphPad Software, LLC, San Diego, CA, USA) with the top and bottom values constrained to 100 and 0, respectively.

Absence of interference with the Sandell-Kolthoff reaction was checked by subjecting fenozan stock solution or DMSO to the Sandell-Kolthoff reaction in presence of a fixed NaI concentration. Determined iodide concentration in presence of fenozan was compared to the one for DMSO. No significant interference was observed (results are not shown).

Results

Identification of NIAS

Data acquisition by GC-HRMS and its preprocessing using in-house written script resulted in a list of aligned peak groups (named *unknowns*). Analysis of the signals obtained in the ACN extracts and EtOH 95% migrates, after excluding the signals present in all controls and blanks, gave 27 signals for EtOH 95% migrates and 37 for the ACN extracts, from which 13 were specific of EtOH migrates and 5 of ACN extracts. As a result of this manual filtering, 23 *unknowns* were selected (Table S1). Then, extracted ion chromatograms (EICs) and mass spectra of the 23 *unknowns* were compared with the NIST database. It is unlikely that one of the 23 *unknowns* was an oligoester having a phthalic acid as a monomer unit, as its specific signal for phthalic anhydride ion at *m/z* 149.0233 was absent. The match scores with the best NIST candidates ranged between 10% and 67% for 20 out of the 23 *unknowns* of interest, which was considered too low for consideration.

Only the three NIST matches with a probability of more than 70% were taken into account, namely *unknowns* 93, 84, and 71. *Unknown* 93, whose intensity was the lowest of the three, matched 4,4′-dihydroxy-2,2′,6,6′-tetrachlorobiphenyl (86%), but visual examination of the mass spectrum led to the rejection of this hypothesis due to the absence of both chlorine isotopes within the molecular ion peak. *Unknown* 84 matching cholesta-4,6-dien-3-ol, (3á) (89%), which is a by-product of vitamin D3, also failed to find logical confirmation on inspection of the mass spectrum. Lastly, *unknown* 71, the most intense signal of interest, matched 3,5-di-tert-butyl-4-hydroxyphenyl propionic acid (86%) or fenozan (CAS RN 20170-32-5, Figure 1). In order to confirm this correspondence, an analytical standard of fenozan was purchased and compared to the *unknown* 71 in the MS^1 and MS^2 experiments. A comparison of the RT and fragmentation spectra at three different collision energies proved that the observed signal and that of the standard were identical (Figure 2). Thus, the confidence level of the identification improved to the highest level on the Schymanski scale (Schymanski et al. 2014).

A rough semi-quantification of fenozan by comparing with the standard response led to ~0.2 µg/dm^2 in both ACN and EtOH 95% migrates.

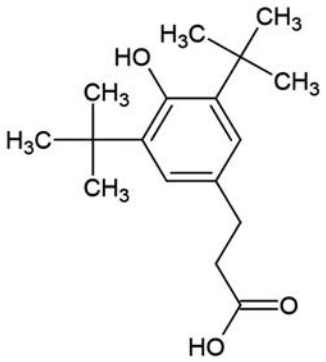

Figure 1. The structure of 3,5-di-tert-butyl-4-hydroxyphenyl propionic acid or fenozan (CAS RN: 20170-32-5) identified in ACN extracts and EtOH 95% migrates.

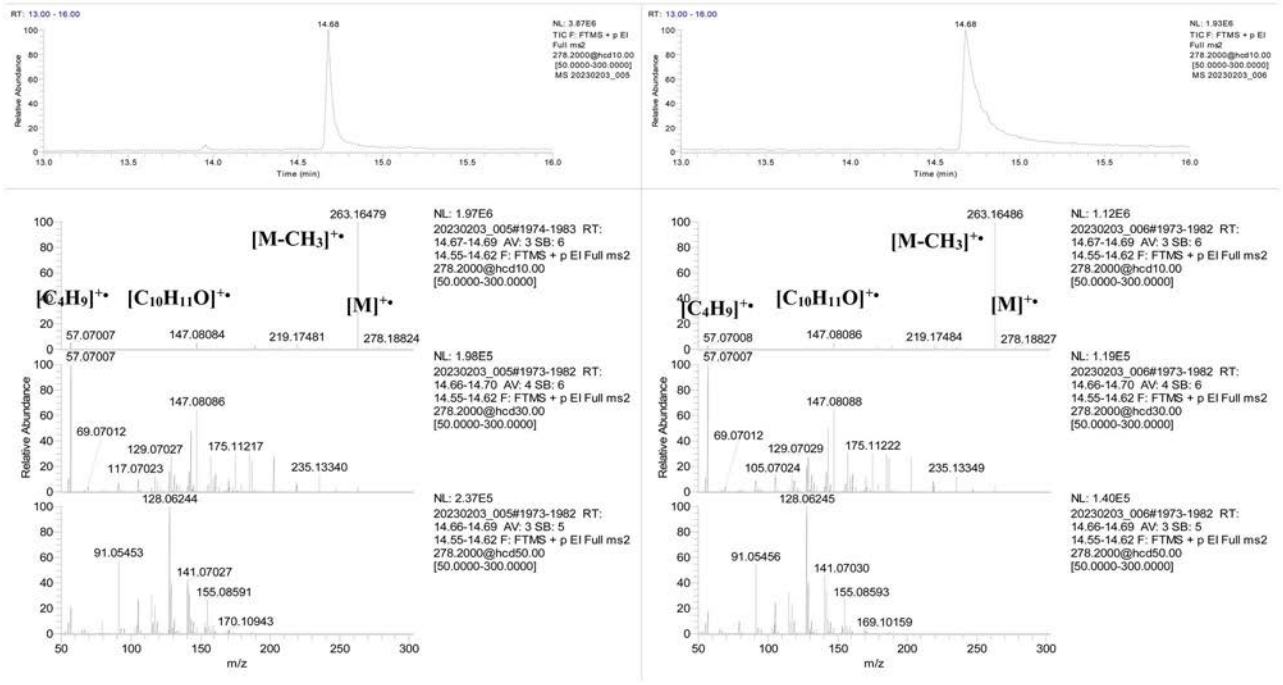

Figure 2. Confirmation at level 1 (by Schymanski scale) of the identity of *unknown* 71 as fenozan. Left: A1–A5 ACN extracts; Right: fenozan standard (1 ng/µL); Top: MS^2 total ion chromatogram obtained at 10% NCE with molecular ion as precursor (m/z 278.2 ± 0.4 mDa); Bottom: MS^2 mass spectra obtained at 10% (top), 30% (middle), and 50% (bottom) NCE.

In vitro bioassays

Bacterial reverse mutation test or Ames test

The results of the Ames test were validated because the values of the negative and positive controls, with and without S9 mix, were inside the laboratory's historical values (data not shown). Cytotoxicity was observed at concentrations of 2.5, 3, and 5 mg/plate with and without S9 mix in TA98 strain using direct incorporation and pre-incubation methods. In addition, a cytotoxic effect was detected for the same concentrations without the S9 mix condition in TA100 strain only for pre-incubation method. No cytotoxicity was detected in any of the other strains, whatever the conditions or methods used.

The results of the Ames test using three strains of *S. typhimurium* (TA98, TA100, TA1535) and one strain of *E. coli* (WP2), with and without S9 mix, were negative in both direct incubation and pre-incubation methods (Tables 1 and 2).

Micronucleus assay

The results of the *in vitro* micronucleus assay were validated because the values of negative and positive controls, with and without the S9 mix, were inside the laboratory's historical values (data not shown). No cytotoxicity was observed in the viability assay when testing three different concentrations of fenozan (Figure 3). Cytotoxicity was observed at higher concentrations, with viability lower than 55% (data not shown). *In vitro* micronucleus assay showed negative results for both the 4-hour treatment with S9 mix and the 48-hour treatment without S9 mix (Figure *Iodide uptake assay*

Iodide concentration in solvent controls and IC_{50} values for sodium perchlorate were within the laboratory's historical values (data not shown). No decrease in cell viability was noted whatever the concentration of fenozan. Fenozan induced statistically significant iodide uptake inhibition from the concentration of 800 µM, reaching 47% at the highest concentration of 1000 µM (Figure 4), with an IC_{50} of 1152 µM. Noteworthy, statistically significant effects were only noted at concentrations where a cloudiness was observed.

Discussion

Migration of fenozan from different FCMs

In this study, the migration of volatile and semi-volatile chemicals from can's polyester

Table 1. Results of the Ames test of fenozan using direct incorporation method.

	S. typhimurium TA98		*S. typhimurium* TA100		*S. typhimurium* TA 1535		*E. coli* WP2	
	without S9	with S9	without S9	with S9	without S9	with S9	without S9	with S9
Sample	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
H_2O			105.3 ± 12.7		17.0 ± 2.9			
DMSO	25.0 ± 2.5	25.3 ± 7.4	164.0 ± 12.0	158.0 ± 9.6	16.7 ± 1.3	18.7 ± 4.2	87.0 ± 10.2	113.7 ± 15.3
Fenozan 0.05 mg/plate	21.7 ± 5.6	21.7 ± 5.3	154.7 ± 9.5	140.3 ± 16.5	21.0 ± 1.6	12.0 ± 1.6	85.3 ± 8.3	92.0 ± 10.4
Fenozan 0.16 mg/plate	20.3 ± 2.6	19.7 ± 7.7	160.0 ± 1.6	122.3 ± 16.1	39.7 ± 9.2	13.0 ± 1.6	89.7 ± 10.3	117.7 ± 5.0
Fenozan 0.5 mg/plate	15.3 ± 2.1	24.0 ± 5.1	143.0 ± 11.5	151.3 ± 2.1	18.0 ± 3.0	12.3 ± 2.4	85.0 ± 4.6	119.3 ± 13.9
Fenozan 1.6 mg/plate	14.7 ± 10.4	24.7 ± 4.3	157.0 ± 13.6	133.0 ± 9.2	16.7 ± 1.9	11.7 ± 0.5	79.0 ± 6.5	112.3 ± 5.7
Fenozan 2.5 mg/plate	16.7 ± 7.4[a]	23.3 ± 2.4[a]	148.0 ± 6.4	135.3 ± 2.4	26.0 ± 4.6	10.7 ± 0.5	80.0 ± 1.6	109.7 ± 15.9
Fenozan 3 mg/plate	10.7 ± 4.1[a]	30.7 ± 2.9[a]	154.7 ± 11.6	143.7 ± 11.2	17.0 ± 0.0	12.3 ± 2.4	84.7 ± 9.7	124.0 ± 8.3
Fenozan 5 mg/plate	17.0 ± 3.6[a]	20.3 ± 4.5[a]	151.3 ± 2.6	142.7 ± 9.7	17.7 ± 6.6	11.0 ± 0.8	83.0 ± 7.2	122.7 ± 4.8
SA	–	–	712.0 ± 159.0	–	290.0 ± 58.9	–	–	–
2NF	256.0 ± 46.6	–	–	–	–	–	–	–
4NQO	–	–	–	–	–	–	1858.0 ± 188.5	–
2AA	–	852.7 ± 18.6	–	793.3 ± 130.6	–	135.7 ± 11.3	–	1962.0 ± 241.9

[a]Indicates that cytotoxicity was observed, positive controls for assay without S9 mix: 2NF for TA98, SA for TA100 and TA1535, and 4NQO for *E. coli* WP2; positive control for assay with S9 mix: 2AA for all tested strains; the means of revertants and standard deviations (SD) were calculated for three replicates ($n = 3$).

Table 2. Results of the Ames test of fenozan using pre-incubation methods.

	S. typhimurium TA98		*S. typhimurium* TA100		*S. typhimurium* TA1535		*E. coli* WP2	
	without S9	with S9	without S9	with S9	without S9	with S9	without S9	with S9
Sample	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
H_2O			130.0 ± 4.1		11.0 ± 3.2			
DMSO	12.7 ± 2.5	12.7 ± 2.5	117.0 ± 8.5	135.0 ± 17.7	8.3 ± 1.8	11.3 ± 1.7	151.0 ± 9.4	231.3 ± 13.1
Fenozan 0.05 mg/plate	16.0 ± 2.8	22.7 ± 4.7	127.0 ± 22.6	129.3 ± 14.8	9.3 ± 2.9	11.0 ± 3.7	147.0 ± 15.5	214.0 ± 11.8
Fenozan 0.16 mg/plate	8.0 ± 2.2	16.3 ± 4.5	125.0 ± 11.1	119.3 ± 12.8	8.7 ± 2.3	5.7 ± 2.5	109.3 ± 4.5	204.7 ± 10.4
Fenozan 0.5 mg/plate	16.7 ± 1.3	21.0 ± 6.4	109.7 ± 9.6	128.7 ± 10.3	6.3 ± 2.2	6.0 ± 1.4	153.3 ± 4.8	189.7 ± 15.5
Fenozan 1.6 mg/plate	14.3 ± 3.3	24.3 ± 5.9	115.3 ± 7.4	123.3 ± 6.2	11.3 ± 1.8	7.7 ± 4.6	118.7 ± 25.8	189.7 ± 14.9
Fenozan 2.5 mg/plate	11.7 ± 4.1[a]	18.7 ± 5.3[a]	113.3 ± 5.3[a]	124.0 ± 9.0	8.0 ± 1.9	9.0 ± 0,8	166.0 ± 5.4	196.0 ± 11.4
Fenozan 3 mg/plate	11.0 ± 2.2[a]	22.0 ± 2.8[a]	120.5 ± 4.5[a]	99.7 ± 16.2	8.0 ± 1.4	7.0 ± 2.5	171.3 ± 6.3	190.0 ± 15.3
Fenozan 5 mg/plate	12.3 ± 4.5[a]	18.7 ± 5.6[a]	102.7 ± 12.0[a]	120.3 ± 12.2	7.0 ± 3.1	9.7 ± 1.7	140.0 ± 15.3	134.7 ± 24.8
SA	–	–	356.0 ± 3.3	–	372.0 ± 18.7	–	–	–
2NF	205.3 ± 29.9	–	–	–	–	–	–	–
4NQO	–	–	–	–	–	–	2621.3 ± 245.8	–
2AA	–	829.7 ± 76.4	–	1210.7 ± 263.9	–	105.7 ± 10.7	–	1693.3 ± 72.1

[a]Indicates that cytotoxicity was observed, positive controls for assay without S9 mix: 2NF for TA98, SA for TA100 and TA1535, and 4NQO for *E. coli* WP2; positive control for assay with S9 mix: 2AA for all tested strains; the means of revertants and standard deviations (SD) were calculated for three replicates ($n = 3$).

coatings was investigated by conducting migration tests with 95% EtOH as food simulant and extractions with mid-polarity solvent ACN. 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionic acid also known as fenozan was identified (~0.2 µg/dm^2 after a rough semi-quantification by comparing with the standard response) by GC-HRMS and its presence was confirmed by the reference standard.

Fenozan was reported as a degradation (hydrolysis) product of different sterically hindered phenolic antioxidants, such as Irganox 1010, and Irganox 1024 (Haider and Karlsson 2002; Bertoldo and Ciardelli 2004; Liu and Mabury 2021; Ji et al. 2024). In a study conducted by Bertoldo and Ciardelli (2004), water extraction and degradation of sterically hindered phenolic antioxidant Irganox 1010 from films made of three different materials (isotactic polypropylene (PP) homopolymer and two heterophasic PP/ethylene-propylene monomers copolymers, under isothermal conditions at 40 °C, 50 °C, and 70 °C) was investigated. HPLC was used to track the antioxidant concentration over time both in the film and in the water (dissolved antioxidant). They observed that the amount of Irganox 1010 in water increased initially at 50 °C and 70 °C, reached a maximum value, then declined over the course of a few days to become undetectable. In contrast, the hydrolysis product, fenozan, was found in the water solutions, and its concentration increased over time as the water-polymer contact period increased.

Vera et al. (2018) reported migration of fenozan from twenty-six PP films from various European manufacturers used for food packaging. PP films (6 dm^2/1 kg simulant) were subjected to migration tests using EtOH 10% and 95%, 3% acetic acid,

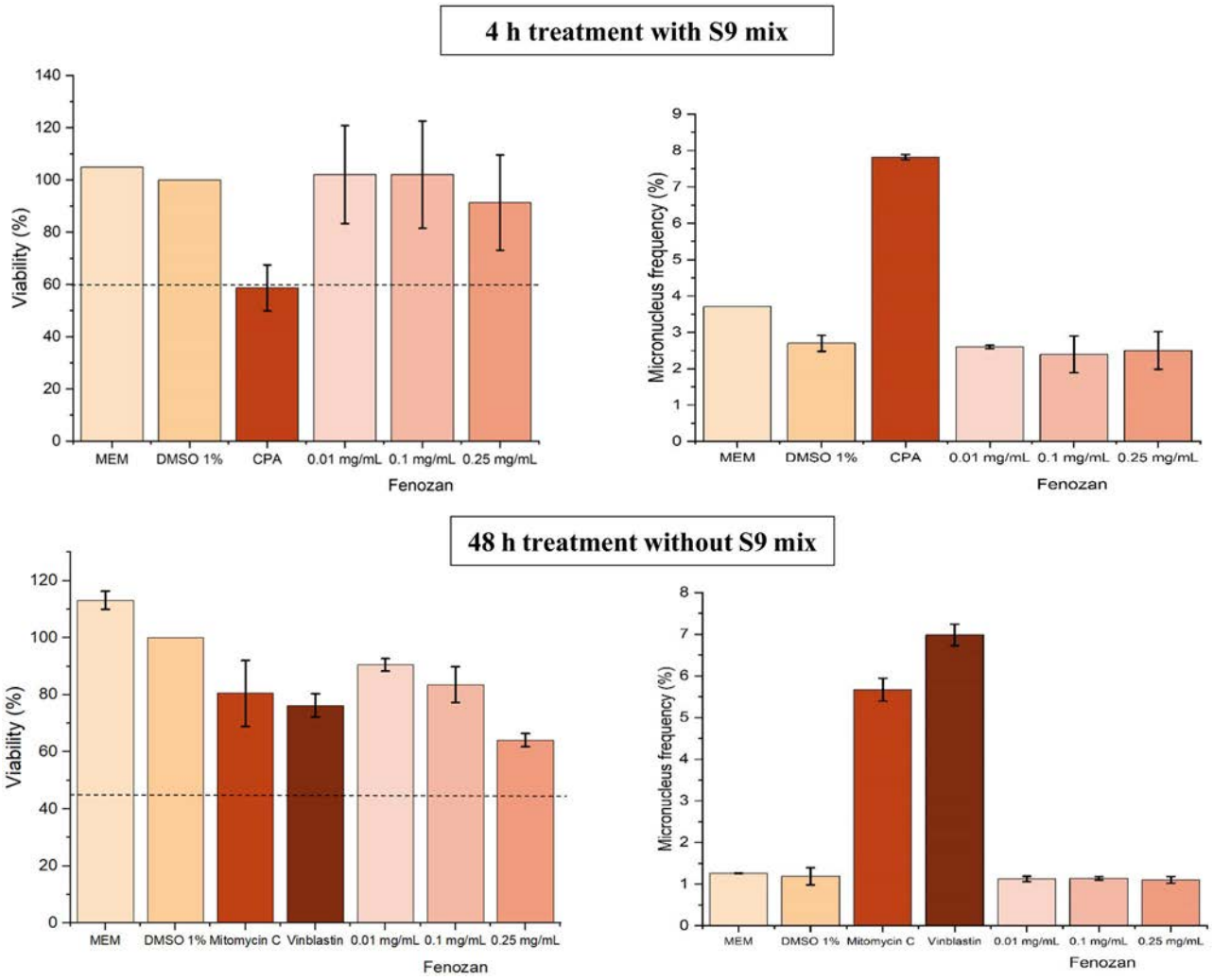

Figure 3. Results of *in vitro* micronucleus assay in HepG2 cells for a 4-h treatment with S9 mix and a 48-h treatment without S9 mix (positive controls for assay without S9 mix: mitomycin C, vinblastine, positive control for assay with S9 mix: CPA, the data points represent the mean ± SD from at least two independent experiments ($n = 2$).

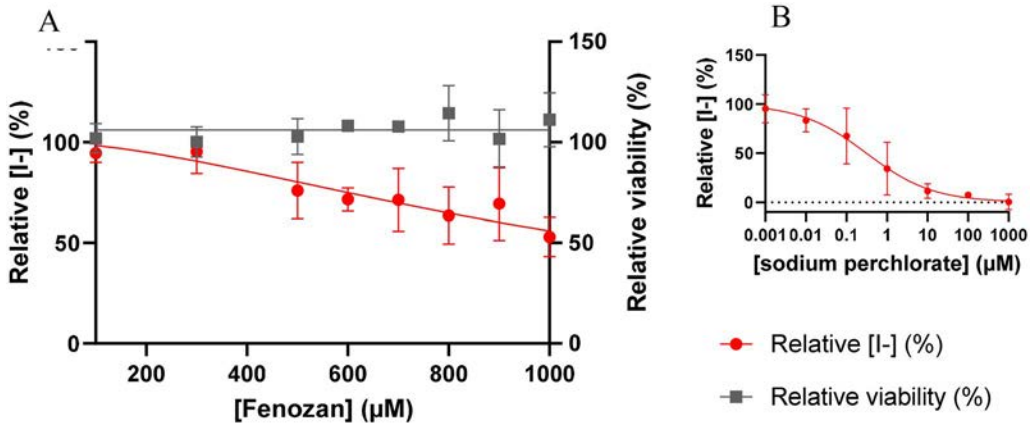

Figure 4. Results of iodide (I^-) uptake assay in FRTL-5 cells exposed for 1 h to fenozan (A) and perchlorate (B) used as positive control. Each data point represents the mean ± SD of 2–4 independent experiments.

and Tenax®, depending on the type of food intended to come into contact with the films, during 10 d at 60 °C. Ultra-high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) was used to analyse samples after migration experiments.

The identification of seventy-six compounds revealed that sixteeen of them were IAS, including numerous antioxidants like Irganox 1010, 1076, and 3104, Irgafos 168, and Chimassorb 81. These chemicals were likely employed to make PP films. Among identified chemicals, fifty-seven were NIAS of which ten were degradation products of antioxidants having similar structures constituted by a group 3,5-di-*tert*-butyl-4-hydroxyphenyl. Fenozan was also identified as a degradation product of antioxidants in three PP films and its presence was validated using the reference standard (Vera et al. 2018).

Moreover, the loss of antioxidants Irganox 1010 and Irgafos 168 was investigated from medium-density polyethylene (MDPE) films exposed to a variety of environmental conditions (during a period of 4 years at 25 °C) compost for the study of biotic degradation, aqueous media at pH 5 and 7 to observe chemical hydrolysis, and open-air and direct sunlight for the study of photo-oxidation. Polymeric films were then extracted with chloroform under ultrasonic treatment under 60 °C for 45 min, samples were then filtered and analysed by HPLC to detect extracted antioxidants and GC-MS to identify low molecular weight degradation products. Fenozan was found in all exposure conditions but was more abundant in water with a pH 5, then in compost and water with a pH 7, but least abundant in sunlight and air. It is important to note that fenozan was not found in unaged films (Haider and Karlsson 2002).

The migration of fenozan from kitchenware was also reported by different groups. For example, Hu et al. (2021) carried out migration tests on 5 polyamide (PA) kitchen utensils using different food simulants (10% and 95% (v/v) EtOH, 3% (w/v) acetic acid) at 70 °C for 2 h to assess the risk of non-volatile substances able to migrate into food. Thirty-six compounds were identified by UPLC-QTOF-MS in migrants of different food simulants including fenozan having similar concentration (55.4–56.1 μg/dm^2) in three food simulants. The amount of fenozan migrating from PA kitchenware was significantly higher than the amount of fenozan migrating in our study (~0.2 μg/dm^2).

In another study conducted by Song et al. (2022), fenozan, 3,5-di-tert-butyl-4-hydroxybenzaldehyde, and 3,5-di-tert-butyl-4-hydroxyacetophenone were among 95 identified compounds by ion-mobility HRMS from the EtOH 95% migrants of PA spatula, which were proposed as degradation products of antioxidants Irganox 1024 and Irganox 1098 because the presence of these antioxidants was also confirmed with reference standards, leading to the conclusion that they were a part of the PA spatula formulation.

Additionally, fenozan was found in EtOH 10% migrants of 'natural' bamboo dishes sold on the market by a Spanish supplier for use in food applications, but the concentration was below its limit of detection (Wrona et al. 2023). Fenozan migrate not only from food packaging or kitchenware but also from plastic pipes used for drinking water distribution systems (Brocca et al. 2002; Löschner et al. 2011; Diera et al. 2023).

Except intake of food or drinking water, there are other sources of human exposure to fenozan. It has been shown that fenozan was detected in 1 out of 80 indoor dust samples from Toronto, Canada with a concentration of 68.9 μg/kg, indicating that it is an environmental pollutant and exposure to fenozan is also possible by breathing or swelling the indoor dust (Liu and Mabury, 2020b).

Safety evaluation of fenozan

Since bioavailability refers to a substance's capacity to be orally absorbed, it is crucial to know the bioavailability of substances when performing risk assessment. Compounds with low toxicity but high bioavailability may represent a health concern at some exposure levels. Understanding fenozan's bioavailability is crucial since it is a released NIAS to which humans are exposed. The log $k_{o/w}$ value of fenozan is equal to 4.7 (National Center for Biotechnology Information 2024). According to EFSA, the log $k_{o/w}$ value below 3 indicates no accumulative potential of the substance in the mammalian body. However, a value of 3 or above does not necessarily mean accumulation will occur; further investigation is necessary to confirm the absence of potential accumulation (Silano et al. 2008).

Direct intake of fenozan is one possible exposure pathway as it is ubiquitously prevalent in the environment due to the degradation of

antioxidants used for different applications. However, the major exposure pathway to fenozan remains *via* the consumption of food or water (Liu and Mabury 2020b).

Prokopov, Shukil', and Berlyand (2006) studied the *in vivo* bioavailability of fenozan in rabbits by oral administration in the form of tablets and suppositories in a single dose of 100 mg/kg (fenozan was in the form of 0.1-g tablets also containing starch and calcium stearate as auxiliary components and 0.2-g rectal suppositories). GC-MS was used to track the level of fenozan in the blood over time. Fenozan was absorbed rather quickly and was relatively slowly excreted from the body, having absolute bioavailability *in vivo* of 46.8% and 51.4% for tablets and rectal suppositories, respectively.

In another study, it was demonstrated that after peroral administration (introduced *per os* at a dose of 100 mg/kg in the form of a solution with Tween-80), fenozan was rapidly absorbed by rabbit and the blood maximal concentration reached its peak within 12 min after delivery. Additionally, fenozan was slowly eliminated from the rabbit's organism during 11.3 and 11.8 h (the mean RT), with 57.8% bioavailability (Prokopov, Shukil', Berlyand, and Veselovskaya 2006).

Considering literature data on fenozan's bioavailability, we can conclude that this molecule may be rapidly absorbed by the human body and slowly eliminated from it, underscoring the significance of the safety assessment of this antioxidant's degradation product. The metabolic pathway of fenozan in the human body is important to investigate in order to assess the toxicities of its metabolites.

In the study of Liu and Mabury (2021), the exposure level of seven 3-(3,5-di-tert-butyl-4-hyd roxyphenyl) propionate antioxidants (AO 1010, AO 1024, AO 1035, AO 1076, AO 1098, AO 1135, AO 697) was measured in human urine samples. No traces of the aforementioned antioxidants, either before or after β-glucuronidase hydrolysis, were found in 50 samples of human urine. In the rat metabolism studies with two representative antioxidants (AO 1010 and AO 1024), none of the two representative congeners were detected in the rat blood and urine. However, high concentrations of both antioxidants were detected in feces indicating that gastrointestinal track is their primary excretion pathway. Fenozan, a breakdown (hydrolysis) product of these antioxidants, was also found in rat's blood (starting from 0.5 h) and urine after dosing. According to this study, the main metabolite of AO 1010 and AO 1024 in rat blood was fenozan. As further evidence of widespread human exposure to the seven aforementioned antioxidants, fenozan was found in 88% of human urine samples before hydrolysis (free fenozan, geometric mean 0.69 ng/mL) and 98% of the samples after hydrolysis (conjugated fenozan, geometric mean 10.2 ng/mL).

To our best knowledge, the toxicity of fenozan is not completely evaluated till now with data being proprietary and not accessible to the public. On the European Chemicals Agency website, it is mentioned that fenozan is toxic to aquatic life with long-lasting effects and is harmful if swallowed. It is also a pre-registered substance with REACH EC Number 243-556-1 and listed in Annex III which is the criteria for 1–10 tonnes registered substances (European Chemicals Agency-ECHA).

In this study, several *in vitro* bioassays were performed to evaluate the toxicity of fenozan. First, to assess genotoxicity, the combined use of two regulatory bioassays, the bacterial Ames test and *in vitro* micronucleus assay, are recommended by EFSA for first step mutagenicity testing of chemicals as these two bioassays can cover all mode of actions of mutagenic chemicals (EFSA Scientific Committee 2011). None of the tested fenozan concentrations were positive in the Ames tests under our experimental conditions. Similarly, fenozan did not induce any positive response in the *in vitro* micronucleus assay.

Interestingly, the anti-carcinogenic effect of fenozan was demonstrated by different authors. Bespalov et al. (2016) studied the anti-carcinogenic effect of fenozan on albino LIO rats and SHR mice of local breeding with a high incidence of spontaneous tumours (more than a third of the population at the end of their life). A therapeutic dose of 5 mg/kg of fenozan in sunflower oil was administrated three times per week for 18 months. By lowering the total incidence and multiplicity of tumors in rats and mice, fenozan was demonstrated anti-carcinogenic effects in this study.

In another study, the effect of fenozan was investigated on the development of spontaneous leukemia in AKR mice having a high frequency (65%–95%) of spontaneous leukemia at the age of 6–11 months. Fenozan when administered at two doses (10^{-4} mol/kg (therapeutic) and 10^{-14} mol/kg (ultra-low)), exhibited antitumor activity at both doses. However, the therapeutic dose proved more efficient in increasing the life span of the short-lived subpopulation, while the ultra-low dose increased the life span of the long-living subpopulation (Erokhin et al. 2007).

The French Agency for Food, Environmental and Occupational Health and Safety hypothesized the role of BHT (an analogue of fenozan) in endocrine disruption. It was reported that its steric bulk is expected to prevent direct activation of estrogenic and androgenic receptors. However, *in vitro* data indicate that BHT affects these receptors, raising questions about its impact on these pathways. However, after exposure to BHT, a thyroid effect was observed, as several rat studies showed changes to thyroid physiology (ANSES 2016). De Abrew et al. (2022) used the next-generation risk assessment approach to examine the endocrine-disrupting effects of BHT and its analogues, including fenozan, at five concentrations on four different cell lines A549, HCC1428, HepG2, and MCF7. Cells were treated with test chemical for 6 h, and then RNA was extracted from cells and subsequently analysed using the L1000 gene array. A toxicogenomic analysis was carried out to identify potential modes of action linked to endocrine disruption among BHT and similar compounds on the basis of their genomic profiles. The aim was to compare the unique genomic signature of BHT with a complete external database containing signatures of various substances, including numerous well-known endocrine disruptors. The findings did not associate either BHT or its analogues, such as fenozan, with substances exhibiting estrogenic, androgenic, thyroid, or steroidogenic activities.

The Danish (Q)SAR Database was used in our study to predict (anti-)thyroid activity of fenozan. In particular, fenozan showed positive prediction for sodium/iodide symporter (NIS) inhibition (Leadscope model). To confirm this prediction, the *in vitro* iodide uptake assay was performed in FRTL-5 cells in this study.

For the first time, fenozan was shown to induce an inhibitory effect in the iodide uptake assay. Compared to perchlorate, a strong iodide inhibitor, the IC50 of fenozan was about 3840-fold higher, indicating its weak iodide inhibition activity. However, these results suggest that fenozan may have anti-thyroid activity, emphasizing the need for further investigation of its potential in thyroid hormone balance disruption.

Conclusion and perspectives

The non-targeted analysis of ACN extracts and EtOH 95% migrates of polyester coated metal plates by GC-HRMS revealed the presence of an antioxidant degradation product fenozan. Fenozan was not mutagenic in the Ames test using four strains, with and without the S9 mix using direct incorporation and pre-incubation methods. Similarly, fenozan showed no genotoxic activity in the *in vitro* micronucleus test, either following a 4-hour treatment with the S9 mix or a 48-hour treatment without the S9 mix.

A weak inhibitory effect was observed when testing fenozan in the *in vitro* iodide uptake assay suggesting that it could affect thyroid hormone synthesis. *In vivo* Larval Amphibian Growth and Development Assay (LAGDA) could be performed to better understand the potential consequences of NIS inhibition by fenozan (OECD 2015).

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Disclosure statement

No conflicts of interest were reported by authors.

ORCID

Isabelle Séverin http://orcid.org/0000-0002-8103-3896

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Discussion

In this study, the degradation product of common sterically hindered phenolic antioxidants, namely fenozan, was identified. Fenozan has previously been reported as a hydrolysis product of several antioxidants, including Irganox 1010 and Irganox 1024 (Ji *et al.*, 2024; Liu and Mabury, 2021; Bertoldo and Ciardelli, 2004; Haider and Karlsson, 2002).

To the best of our knowledge, the toxicological profile of fenozan has not been fully evaluated to date, with existing data being proprietary and not publicly accessible. According to the literature, the Log $k_{o/w}$ value of fenozan is approximately 4.7. The EFSA considers substances with a Log $k_{o/w}$ below 3 to have no bioaccumulation potential in the mammalian body. However, a Log $k_{o/w}$ of 3 or higher does not inherently indicate accumulation; further studies are required to confirm the compound's bioaccumulative potential (EFSA, 2008). Reported oral bioavailability of fenozan ranges from 46.8% to 57.8%, suggesting it may be rapidly absorbed and slowly eliminated from the body (Prokopov *et al*., 2006a, Prokopov *et al*., 2006b).

Following EFSA's guidance on evaluating the genotoxicity of chemical substances (EFSA, 2019a), *in vitro* bioassays including the Ames test and micronucleus assay (using flow cytometric analysis) were performed. Both assays yielded negative results, indicating that fenozan does not exhibit genotoxic properties under the conditions tested. Interestingly, some literature suggests that fenozan may even exhibit anti-carcinogenic properties (Bespalov *et al*., 2016, Erokhin *et al*., 2007).

Additionally, an iodide uptake assay was conducted to verify *in silico* alert for sodium/iodide symporter (NIS) inhibition, as indicated by the Danish (Q)SAR Database. Fenozan exhibited weak inhibitory activity in the iodide uptake assay, with an IC_{50} value approximately 3,840 times higher than that of perchlorate—a well-known and potent inhibitor of iodide uptake—when tested in rat thyroid FRTL-5 cells. Although the inhibitory effect was weak, it may be involved in thyroid-disrupting activity.

ANSES has reported that BHT—a structural analogue of fenozan—can cause alterations in thyroid physiology in rats (ANSES, 2016a). To better elucidate the possible consequences of NIS inhibition by fenozan and its relevance to thyroid toxicity, *in vivo* Larval Amphibian Growth and Development Assay (LAGDA) testing under OECD TG 241 is recommended (OECD, 2015).

Given its potential biological activity, widespread occurrence, and the absence of harmonized regulatory standards for can coatings in the EU, a comprehensive toxicological assessment of fenozan is essential for consumer safety.

Chapter 4

Potential carcinogenicity testing of polyester coatings extracts and migrates

Summary

Introduction

In silico analysis revealed tumor-promoting alerts for four identified cyclic oligoesters found in polyester ACN extracts and EtOH 95% migrates (see Chapters 1 and 2). Since these substances are not commercially available, their potential carcinogenicity was assessed using the CTA with Bhas 42 cells as a screening method. This approach also aimed to evaluate any cocktail effects and potential carcinogenic activity of unidentified compounds present in the mixtures.

Materials and methods

Two modes of carcinogenic action—initiation and promotion—were investigated using the CTA. Prior to conducting the transformation assay, cell growth assays were performed to assess whether the FCM extracts and migrates exhibited any cytotoxicity and to determine suitable dilution ranges for further testing. Subsequently, the FCM extracts and migrates were tested at various dilution levels in the CTA to assess potential dose-response effects.

The sample preparation and extraction/migration protocols used in this study followed the procedures outlined in Chapter 2, with one key modification: samples were concentrated 107.5-fold rather than 53.75-fold using a gentle nitrogen stream. An additional preparation step was required for ACN extracts, as ACN is not compatible with cell culture. These extracts (3 mL) were concentrated nearly to dryness (approximately 100 µL) under nitrogen and then reconstituted in EtOH 95% to a final volume of 3 mL, resulting in a final ACN concentration of 3.33%.

Results

The results of the cell growth assays demonstrated that none of the undiluted FCM extracts or migrates exhibited cytotoxic effects. Experimental validity was confirmed, as the values for both negative and positive controls fell within the laboratory's historical control range. Furthermore,

all FCM extracts and migrates tested negative in both the initiation and promotion assays across all tested concentrations (undiluted, ½ dilution, and ¼ dilution) in both experimental replicates.

Discussion and conclusion

Before implementing the CTA in the PackTox lab, the bioassay setup and validation using positive and negative controls were necessary. The significantly higher number of transformed foci in positive controls confirmed proper assay performance. Negative results in this study suggest that polyester extracts and migrates lack tumor-initiating or -promoting activity under the test conditions. However, the dilution of samples—initially concentrated 107.5 times and then diluted 200-fold—resulted in a final 1.86-fold dilution, which may impact the results.

While CTA is a useful tool for identifying carcinogenic potential, it cannot fully replace long-term *in vivo* tests. Limitations include subjective scoring, inter-operator variability, and poor reproducibility across labs, often due to differing cell culture conditions (Hayrapetyan *et al*., 2023). Notably, this study appears to be the first to apply CTA to FCM extracts and migrates. A related study by Ohmori and Kawamura (2009) showed that 2 paper contaminants lacked initiating potential but displayed possible promoting activity.

Further research is needed to evaluate CTA's reliability for FCM testing, especially considering the high dilution during the assay. Proper sample concentration and minimal chemical loss during preparation are essential for accurate results.

In conclusion, no carcinogenic potential was observed for the tested oligoester-containing extracts and migrates. However, results remain preliminary, and assay standardization for FCMs is still required.

ARTICLE

1. Introduction

Due to *in silico* alerts for tumor-promoting activity obtained for the four identified cyclic oligoesters (refer to Chapter 1 and 2) present in polyester extracts and migrates, these mixtures were tested on CTA using Bhas 42 cells to detect their potential carcinogenicity (refer to paragraph 'Bhas 42 CTA' of bibliographic report) (screening approach) as these substances were not commercially available. It also provides an additional information about cocktail effect and carcinogenic potential of any non-identified chemicals present in the mixture. With CTA, two modes of action (initiation and promotion) of carcinogenicity were studied. The cell growth assays were performed prior to the transformation ones to determine if cytotoxicity was associated with FCMs extracts and migrates and to define dose range (dilution factors) of samples to test further in the transformation assay. Then, FCMs extracts and migrates with different dilution factors were tested on CTA to observe any dose-response effect.

2. Material and methods

2.1. Migration assays and sample preparation

Samples and extraction/migration protocols used in this study are the once described in the Chapter 2.

The only difference was that they were concentrated 107.5 times instead of 53.75 times under gentle nitrogen flow. Additional step was added for ACN extracts as this solvent is not compatible for cell culture. ACN extracts (3mL) were concentrated near to dryness (100 µL) under a gentle nitrogen flow and diluted with EtOH 95% to reach a volume of 3mL (in the final sample, ACN was 3.33%)**.** ACN and EtOH 95 % solvents were treated as described above to provide procedural blanks (AB or EB). All vials were kept at − 20 °C before use.

2.2. Cell culture

Harlan Laboratories (Rossdorf, Germany) delivered Bhas 42 cells free from bacteria, fungi and mycoplasma as a gift. From frozen master stock, cells were expanded in Eagle's minimum essential medium (Gibco^{TM}, Paisley, UK) supplemented with 10% fetal bovine serum (FBS)

(Dominique Dutcher, South American origin), 1% penicillin G sodium (10 000 units /mL) /streptomycin sulfate (10 000 µg/mL) (P/S) (Gibco™, New York, USA), and 1% L-glutamine (Gibco™, New York, USA) (M10F) and cultured in a humidified 5% CO_2 atmosphere at 37 °C until 70% confluence was achieved and then cells were trypsinized and subcultured until the confluence was reached to 70 %. To prepare a working stock, cells were trypsinized again before being suspended at a density of 5 x 10^5 cells /mL in freshly prepared M10F containing 5% DMSO (Sigma Aldrich, St. Louis, MO, USA). Large batches of 0.5 mL aliquots were cryopreserved and stored in liquid nitrogen at -80 °C. For each experiment, a thawed cryotube from the working stock was used for the cell growth and transformation assay.

M10F was used for cell expansion, cell storage, and the first culture after thawing. Dulbecco's modified Eagle's medium/Ham's F12 (Gibco™, New York, USA) supplemented with 5% FBS and 1% P/S (DF5F) was used for the cell growth and transformation assays as well as routine passages, and subculturing of cells.

Two different batches of FBS (Dominique Dutcher and Hycultec, both having South American origin) were prescreened in order to selected the one with the ability to support a low frequency of spontaneous transformed foci formation in the negative control and to induce a high frequency of transformed foci formation in the positive control.

For each cytotoxicity and CTA (initiation and promotion assays), one cryotube of the frozen Bhas 42 cells from working stock was rapidly thawed and suspended in 6mL of M10F media and transferred in 25cm^2 of cell culture flask. After 24h of incubation, media was replaced by 6 mL DF5F and cells were incubated for 48-72h depending on the cell growth, up to about 70% confluence.

Then, cells were trypsinized, suspended in DF5F media and seeded in 75cm^2 cell culture flask with a density of 10^4 cells/mL and incubated until cells reach ≤70% confluence. After incubation, cells were trypsinized again and suspended in DF5F, this cell suspension was used for the cell growth and initiation/ promotion assays.

The positive control for initiation assay was 3-methylcholanthrene (MCA) (Sigma-Aldrich, France) (1µg/mL final concentration). TPA (Sigma-Aldrich, France) (0.05µg/mL final concentration) was the positive control for promotion assay. Positive controls were dissolved in DMSO, the final

concentration in the media was 0.5%. EtOH is solvent control for all EtOH migrates, and EtOH with 3.33% ACN is solvent control for all ACN migrates.

2.3. Transformation assay

Six wells were prepared for each treatment group. Each initiation and promotion assay were performed including blank control (DF5F), 0.5% vehicle control (DMSO for MCA and TPA, EtOH for EtOH migrates, and EtOH with 3.33% ACN for ACN extracts), positive control (1 µg/mL MCA for initiation assay and 0.05µg/mL TPA for promotion assay), and 0.5% FCM migrates (non-diluted, ½ and ¼ diluted). Experiments were performed using the protocol described by OECD guidance (OECD, 2017a). Each experiment was performed twice.

2.3.1. Initiation assay in a 6-well format

For the initiation assay, cells were suspended in DF5F at a density of 2.10^3 cells/mL and seeded onto each well of 6-well microplates in 2 mL volume (4000 cells/well, day 0).

After 24 h from seeding (day 1), the medium was replaced with fresh media containing nothing (DF5F: blank control), or 0.5 % (in the final volume) vehicle control (DMSO for MCA, EtOH with 3.33% ACN for ACN extracts, and EtOH for EtOH migrates), or positive control (MCA dissolved in DMSO with a final concentration of 1µg/mL), or FCM migrate. After 72h of the exposure period, treatment medium was removed and the cells were rinsed with 2mL of Dulbecco's phosphate buffered saline (DPBS) (Gibco™, New York, USA) and the medium was replaced by a fresh media without the test chemical/ negative or positive control (day 4). Cells were subsequently maintained in DF5F medium until day 21, replacing the media on days 7, 10, and 14. At day 21, medium was removed and cells were rinsed with 2mL of DPBS and fixed with 2mL of methanol (Sigma Aldrich, St. Louis, MO, USA), cooled dawn at +4 °C for 10 min, cells were then stained with 5% Giemsa's (Sigma Aldrich, St. Louis, MO, USA) solution for 30 minutes, then rinsed with 2mL of distilled water and dried under the open air.

2.3.2. Promotion assay in a 6-well format

For the promotion assay, cells were suspended in DF5F at a density of 7000 cells/mL and seeded onto each well of 6-well microplates in 2 mL volume (14 000 cells/well, day 0). Cells were maintained for 4 days without a medium change. On days 4, 7, and 10, the culture medium was

replaced with fresh medium containing nothing (DF5F: blank control), or 0.5 % vehicle control (DMSO for TPA, EtOH with 3.33% ACN for ACN extracts, and EtOH for EtOH migrates), or positive control (TPA dissolved in DMSO with a final concentration of 0.05µg/mL), or 0.5% FCM migrate. The exposure period was continued for 10 days until day 14. On day 14 the medium was removed, and cells were rinsed with 2mL of DPBS and subsequently maintained in DF5F medium without the test item/negative or positive control till day 21. On day 21, cells were fixed and stained as described above.

2.3.3. Cell growth assay

The cell growth assays were performed prior to the transformation assays to determine if cytotoxicity is associated with non-diluted FCM extracts and migrates and if yes, to define doses (dilution factors) of extracts and migrates to test in the transformation assay.

In addition to a prior cell growth assay, a concurrent growth assay was also performed in parallel to every initiation and promotion assay to estimate the effect of each treatment on cell growth and survival.

Three wells were prepared for each test concentration. The cells were seeded and treated with a sample in the same way as in the initiation and promotion assay. On day 7 cells were fixed with EtOH 96% (*Merck KGaA, Darmstadt, Germany)* cooled dawn at +4 °C for 10 minutes, then EtOH was removed, and cells were stained with 0.1% cristal violet (CV) (Sigma Aldrich, St. Louis, MO, USA) solution for 15 minutes under darkness. Then, cells were rinsed with distilled water and air-dried for a few minutes before extracting CV incorporated in the cells using 2 mL of extraction solution (composed of 0.02 M HCl and 50% EtOH) for 10 min. From this, 200 µL was transferred into three wells of 96-well microplates and the optical density of the extract was measured at 540-570 nm. The mean optical density and standard deviation (SD) for three wells were calculated for each treatment group and the survival rate of cells treated with a chemical were expressed as a percentage of the control cells.

Figure 3-8 shows the timeline for the initiation and promotion test component of the transformation assay for 6-well format by OECD guidance (OECD, 2017a).

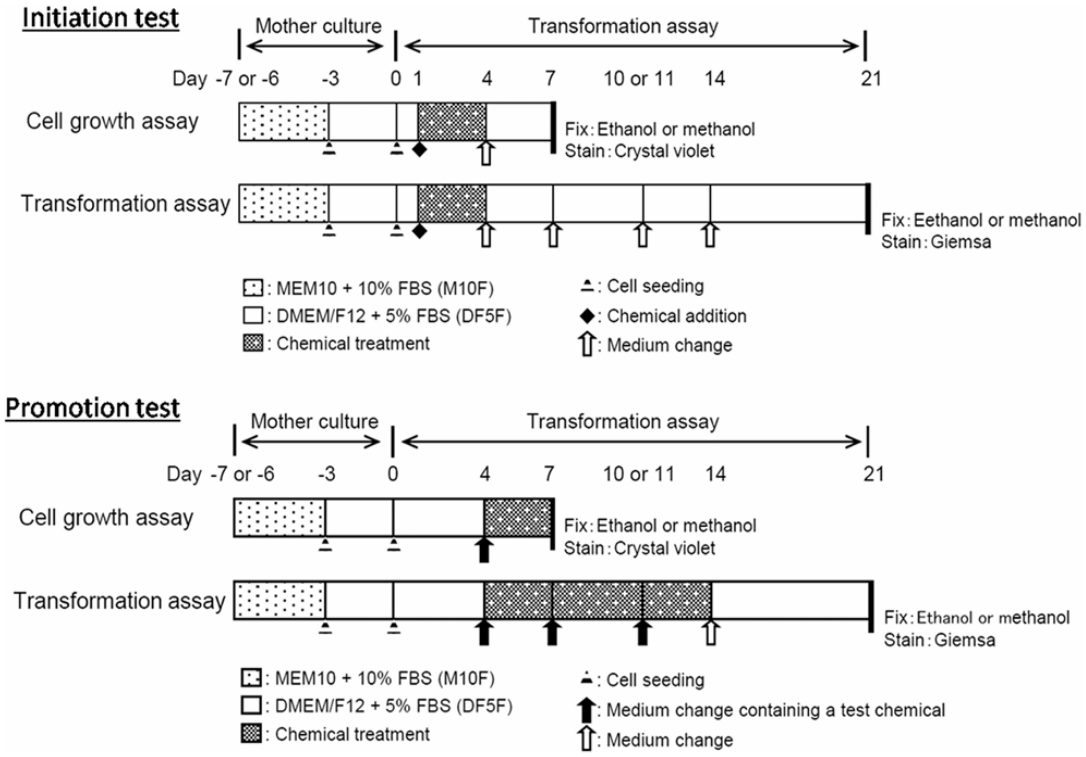

Figure 3 - 8. Time line for the initiation and promotion test component of the transformation assay (6-well format) (OECD, 2017a)

2.4. Determination of transformation frequency and statistical analysis

Transformed foci were counted in each well of 6-well plate and the mean and SD of transformed foci in 6 wells were calculated for all treatment groups. The following morphological characteristics were used to distinguish transformed foci: (a) more than 100 cells; (b) spindle-shaped cells exhibiting an appearance different from contact-inhibited monolayer cells; (c) deep basophilic staining; and (d) random orientation of cells, especially noticeable at the edge of foci (criss- cross misalignment of individual cells), (e) dense multi-layering of cells (piling up), and (f) invasive growth into the surrounding confluent monolayer of contact-inhibited cells. Examples of transformed and non-transformed foci are shown in Figure 3-9.

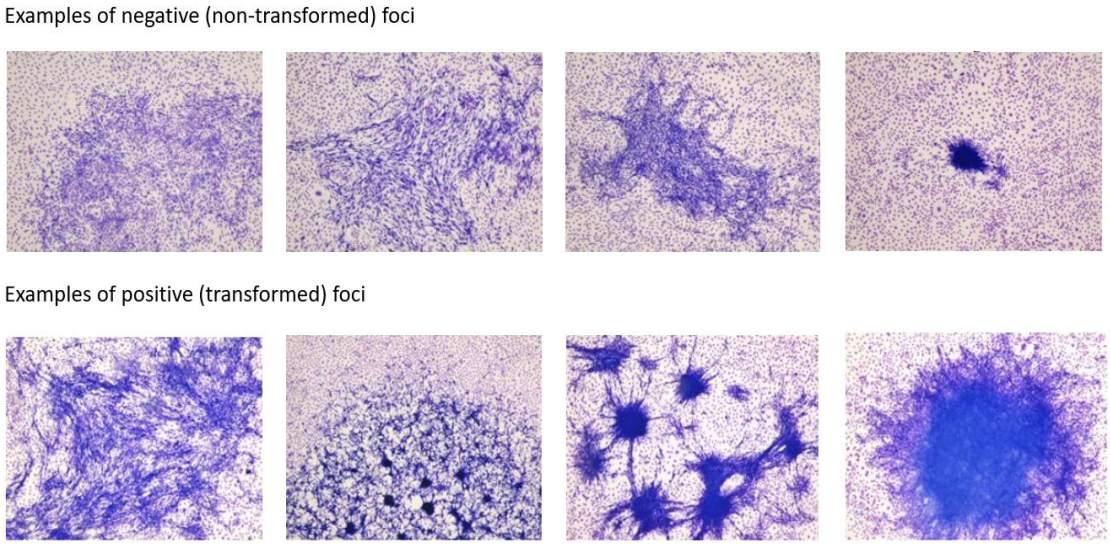

Figure 3 - 9. Examples of transformed and non-transformed foci in Bhas 42 CTA (OECD, 2017a)

To determine migrate-induced transformation frequency, multiple comparison one-sided Dunnett's test ($p<0.05$, upper-sided) was used which compares all treatment groups with corresponding solvent control. Depending on the outcomes of the F-test for homoscedasticity (homogeneity of variance), the one-sided Student's t-test or Aspin-Welch test ($p<0.05$, upper-sided) was used to assess the statistical significance of the positive control's value from solvent control value. Graphpad software 10.3.0 was used to do statistical analysis.

3. Results

In this study, extracts and migrates from polyester coatings were tested on CTA as a screening method to verify *in silico* predictions of tumor-promoting activity of four identified oligoesters present in the mixture.

Table 3-7 presents historical data for positive and negative controls. The mean number of transformed foci of the blank control (DF5F) and solvent control (DMSO) range from 3.11 to 5.11. The mean transformed foci for the positive controls (MCA and TPA) fall between 12.78 and 13.33, which is significantly higher than the negative controls.

Table 3 - 7. Historical data of negative and positive controls used in cell transformation assay

	DF5F	DMSO (0.5%)	Positive control
Initiation			
Mean of transformed foci	3.11	3.44	13.33[a]
SD	1.79	0.83	1.80
Number of wells	9	9	12
Promotion			
Mean of transformed foci	5.11	4.93	12.78[b]
SD	1.59	1.84	2.78
Number of wells	9	15	18

[a] positive control for the initiation assay is MCA (1µg/mL)

[b] positive control for the promotion assay is TPA (0.05µg/mL)

The cell growth assay results indicated that none of the undiluted extracts and migrates exhibited cytotoxicity, with cell growth percentages comparable to the blank control in both the initiation and promotion assays (results not shown).

To evaluate dose-response effects, undiluted, ½ diluted, and ¼ diluted FCMs ACN extracts and EtOH 95% migrates were then tested in the CTA.

The results of CTA for non-diluted migrates are shown in Table 3-8 (the results of diluted samples are not shown as all of them were negative). Experiments were validated as negative and positive controls values were inside the laboratories historical control values. Negative results were obtained for all FCMs extracts and migrates during initiation and promotion assay for all tested doses (non-diluted, ½, and ¼ diluted) in both repetitions (Table 3-8).

Table 3 - 8. The results of CTA for non-diluted ACN extracts and EtOH 95% migrates of polyester coatings (the mean and SD were calculated for 6 wells (n=6) of 2 independent experiments)

Sample	Initiation			Promotion		
	Foci/ well [a]	CG (%) [b]	Judgment	Foci/ well	CG (%)	Judgment
DF5F	2.83 ± 1.34	100.00		6.17 ± 0.69	100.00	
EtOH (0.5%) [c]	3.17 ± 0.90	98.38		5.50 ± 1.38	99.43	
EtOH (0.5%) with 3.33% ACN [c]	3.17 ± 1.07	99.87		6.67 ± 0.94	93.93	
DMSO (0.5%) [c]	3.50 ± 0.96			6.83 ± 0.90		
MCA (0.5% DMSO) [d]	**12.83 ± 1.77**		+			
TPA (0.5% DMSO) [d]				**16.17 ± 1.07**		+
AB	4.67 ± 0.47	135.11	-	5.67 ± 0.75	103.53	-
A0	4.17 ± 1.57	144.99	-	5.50 ± 1.26	108.3	-
A1	3.83 ± 1.21	133.57	-	5.50 ± 0.50	103.34	-
A2	5.33 ± 1.49	105.57	-	5.83 ± 0.90	99.25	-
A3	4.67 ± 1.8	100.23	-	6.00 ± 1.41	99.28	-
A4	4.50 ± 1.50	104.4	-	5.50 ± 0.96	99.40	-
A5	4.00 ± 1.00	92.11	-	6.83 ± 0.69	97.64	-
EB	2.67 ± 0.94	91.77	-	5.83 ± 1.34	96.54	-
E0	3.00 ± 1.53	97.56	-	5.17 ± 0.90	93.34	-
E1	4.83 ± 1.07	98.38	-	5.50 ± 1.12	104.92	-
E2	4.50 ± 0.5	92.76	-	5.83 ± 1.77	99.51	-
E3	3.00 ± 1.15	98.27	-	6.00 ± 0.58	94.05	-
E4	3.17 ± 0.37	93.89	-	5.17 ± 0.37	105.31	-
E5	3.80 ± 0.75	96.64	-	6.17 ± 1.21	87.41	-

Not tested

[a] the mean of transformed foci/well ± SD
[b] % of cell growth compared to blank control
[c] solvent control: final solvent concentration in the working culture in parentheses
[d] positive control: final solvent concentration in the working culture in parentheses

4. Discussion

As the CTA had not been implemented in the PackTox laboratory before, it is essential to first set up the bioassay and generate historical data using both positive and negative reference substances to demonstrate its performance. MCA was used as the positive control for the initiation assay, while TPA served as the positive control for the promotion assay. The mean number of transformed foci in the positive controls was significantly higher than in the corresponding solvent controls, confirming the proper performance of the bioassay in the PackTox laboratory.

Negative results obtained in this study indicate that polyester extracts and migrates do not present tumor-promoting or -initating activity under tested experimental conditions. Dilution of the samples during bioassay, as well as biological threshold and the sensitivity/specificity

of the bioassay is another important point that should be considered when negative results are observed. Significant dilution of FCMs extracts and migrates during CTA may result in a negative response, making proper sample concentration a critical factor for the bioassay's outcome. For example, in this study samples were initially concentrated by a factor of 107.5 and then diluted 200-fold during the test, resulting in a final dilution factor of 1.86. Considering the 1.86-fold dilution of samples, the highest tested doses of different oligoesters present in the samples will be as follows: 28.33–45.70 µg/dm² for c(2TCDDM+2tPA), 24.57–37.69 µg/dm² for c(BD+2TCDDM+3tPA), 24.84–39.95µg/dm² for c(3TCDDM+3tPA), and 1.67–3.28 µg/dm² for c(2BD+TCDDM+3tPA).

The CTA was used in this study as it is a powerful bioassay used to test the carcinogenic potential of both tumor-initiating and tumor-promoting chemicals. However, this bioassay cannot replace the 2-year *in vivo* carcinogenicity test, as it cannot fully replicate the complex process of carcinogenesis observed in living organisms. Additionally, CTA assays have several drawbacks. The most significant drawback is the visual detection of transformed foci, which introduces subjective scoring and leads to variations between different operators (refer to Annex 5) (Hayrapetyan *et al*., 2023). Another issue is the reproducibility between laboratories (Hayrapetyan *et al*., 2023).

These variations between experiments can be attributed to differences in cell maintenance conditions, particularly the serum batch and the duration of the cell culture period (Hayrapetyan *et al*., 2023).

To our best knowledge, this is the first time that carcinogenic potential of FCM's extracts and migrates were tested in CTA assay. However, in the study conducted by Ohmori and Kawamura (2009) initiation and promotion potential of two contaminants (abietic and dehydroabietic acids) from virgin paper products and recycled paper products used for food contact applications were investigated using CTA. The results showed that these molecules did not possess initiation potential, however, they may have tumor-promoting potential as they induced transformed foci dose dependently in the concentration range 20–60 nmol ml^{-1}.

It is crucial to understand the bioassay's ability to detect both genotoxic and non-genotoxic carcinogens at levels found in FCMs extracts and migrates. Since no studies have investigated the use of this bioassay to test the potential carcinogenicity of FCMs extracts and migrates, further investigation is needed to determine the reliability of the CTA for testing FCMs extracts

and migrates. Another important factor to consider is the high dilution (200 times) that occurs during testing, indicating the importance of pre-concentration step during sample preparation. During sample pre-concentration, preventing chemical dilution is crucial to ensure accurate representation of FCMs migration and human exposure. Minimizing chemical loss and avoiding sample loss during extraction is essential.

5. Conclusion

In this study coating's extracts and migrates containing the four cyclic oligoesters were tested on CTA to verify *in silico* predictions for potential tumor-promoting activity of these molecules as a first screening approach. Both initiation and promotion assays yielded negative results, suggesting no carcinogenic potential of these mixtures in tested experimental conditions. However, the approach used in this study was preliminary as this bioassay is not yet standardized for the use of FCMs extracts and migrates and the biological threshold needs to be determined.

Chapter 5

Identification of non-volatile non-intentionally added substances by LC/ESI-HRMS from the polyester-based lacquer's ACN extracts and investigation of their gastrointestinal fate

Summary

Introduction

In previous chapters, four cyclic oligoesters (c(2TCDDM+2PA), c(2BD+TCDDM+3PA), c(BD+2TCDDM+3PA), and c(3TCDDM+3PA)) were identified from polyester coating ACN extracts and EtOH 95% migrates using LC-HRMS (see Chapter 2). It is essential to investigate the gastrointestinal fate of these identified NIAS, as they may metabolize in the human body via digestive enzymes which may potentially affect their toxicity. Research shows that cyclic oligomers can hydrolyze into linear counterparts or shorter oligomers during simulated intestinal digestion (Eckardt *et al*., 2019). Furthermore, cyclic oligoesters are classified as Cramer class III substances, indicating higher toxicity potential, while their linear forms are classified as Cramer class I, suggesting lower toxicity (Lestido-Cardama *et al.*, 2022b; Eckardt *et al*., 2020, 2019). This study aims to identify non-volatile NIAS from polyester coatings and investigate their gastrointestinal fate.

Materials and methods

To investigate the gastrointestinal fate of the previously identified oligoesters, an additional ACN extraction (by grating) was performed on the polyester coating (see Chapter 2) to obtain more concentrated extracts for detecting other parent compounds not previously identified. Each coated metal plate was grated (4 cm^2) and placed in a 15 mL vial with 4 mL of ACN. After 24 hours of incubation at 40 °C, the vials were centrifuged, and the supernatants were pooled into a new 20 mL vial and concentrated under a gentle nitrogen stream to 4 mL. This extract is referred to as CA_{mix} in the manuscript. CA_{mix} was subjected to simulated intestinal digestion following a modified protocol from Eckardt *et al.* (2019), and parent compounds and expected transformation products were monitored using LC-HRMS. A portion of CA_{mix} was concentrated 50 times to enable the detection of chemicals in the extract at very low concentrations, referred to as parent compounds.

Results

Using a non-targeted LC/ESI-HRMS approach in both positive and negative ionization modes, 18 substances (parent compounds) were identified in the ACN extract (concentrated 50 times to detect trace amounts of substances) with varying levels of confidence. For oligoesters, 9 cyclic and 5 linear combinations were hypothesized.

Esterase activity in the intestinal fluid simulant was assessed to evaluate the pancreatin enzyme's enzymatic activity. The results showed that the pancreatin enzyme in the simulant was suitable for conducting the *in vitro* intestinal digestion assay of the polyester coatings' ACN extract.

Following *in vitro* intestinal digestion of the non-concentrated ACN extract, previously identified parent compounds and potential degradation products were monitored by LC/ESI-HRMS. Monitoring revealed that oligoesters containing only TCDDM and PA appeared resistant to pancreatin hydrolysis. However, oligoesters with BD-PA and TCDDM-AA ester bonds were readily hydrolyzed by pancreatin.

In total, 8 degradation products (all linear oligoesters) were identified with varying confidence levels. Suspected linear oligoesters increased in the presence of pancreatin, suggesting further degradation of some. Smaller degradation products might have been formed, but the chromatographic and analytical methods used were unable to detect them. Among the monomers, only suspected TCDDM (parent compound) was monitored, showing no increase, indicating that TCDDM is likely not the final degradation product.

Discussion

This preliminary study shows that oligoesters degrade into linear oligoesters, raising concerns about the degradation products that may be absorbed through the intestinal barrier. Previous research has demonstrated that cyclic oligoesters hydrolyze into their linear forms, which tend to be less toxic. For example, cyclic oligoesters were classified as Cramer class III (high toxicity), while their linear forms were classed as Cramer class I (lower toxicity) (Lestido-Cardama *et al.,* 2022b; Eckardt *et al.*, 2020, 2019).

These findings align with literature. Eckardt *et al.* (2019) observed that cyclic oligomers quickly hydrolyze into their linear forms under intestinal conditions, with more than 75% of the initial amount degrading within the first hour. Further hydrolysis to shorter linear oligomers

occurred, though complete monomer degradation was not observed. Similarly, Ubeda *et al*. (2020) found that two cyclic oligoesters from polyurethane showed decreased concentrations and increased hydrolyzed products during digestion.

Although the preliminary results for the gastrointestinal fate of these oligoesters are promising, the study's limitation of only one replicate (n=1) prevents statistical analysis of enzyme effects. Future research with more replicates is needed to confirm the findings. Toxicological analysis is necessary for the identified parent compounds and their degradation products to assess their absorption potential and toxicity. Using *in silico* QSAR tools for toxicity prediction can help prioritize molecules for further testing in bioassays, aiding risk assessment and quantification.

ARTICLE

1. Introduction

The gastrointestinal tract which includes the oral cavity, pharynx, oesophagus, stomach, small intestine, large intestine, and anal canal, is responsible for the ingestion and digestion of food, absorption of nutrients, secretion of water and enzymes, and excretion of waste products (Ogobuiro *et al*., 2024). Digestive enzymes, for example, amylase, pepsin, and pancreatin, are crucial for chemical metabolism within the human body and can influence the toxicity of substances. These enzymes facilitate the breakdown and transformation of various compounds, potentially resulting in the formation of by-products with distinct toxicological properties.

Studies have demonstrated that cyclic polyester oligomers, which are predictable NIAS, can be hydrolyzed into their linear forms, and for some, further into shorter-chained linear oligomers when exposed to simulated intestinal digestion. However, degradation down to the monomer level was not observed (Eckardt *et al*., 2019). In addition, cyclic oligoesters identified in various studies were classified as Cramer class III substances with the probability of higher toxicity level and their linear forms as Cramer class I substances with the probability of lower toxicity level (Lestido-Cardama *et al*., 2022b; Eckardt *et al*., 2020, 2019). These results indicate that when chemicals are exposed to gastrointestinal enzymes, their toxicity as well as the absorption may change. This underscores the necessity of investigating gastrointestinal fate of identified NIAS. It is also considered by EFSA as relevant information for defining the physical and chemical properties of migrating substances from FCMs (EFSA, 2016).

EFSA described a method to measure the extent of hydrolysis of plastics monomers and additives *in vitro* using standard digestive fluid simulants, in order to assess whether the constituents break down into innocuous substances (EFSA, 2008). The guideline details the composition of various digestive fluid simulants, including saliva, gastric juice, and intestinal fluid. For esters, testing should begin with the intestinal fluid simulant. If complete hydrolysis is observed, further testing with other simulants is unnecessary (EFSA, 2008). Most non-volatile NIAS from polyester coatings are oligoesters which are formed during the esterification reaction between PO and Pa. To demonstrate the hydrolysis of these chemicals, an *in vitro* digestion assay with gastrointestinal fluid is necessary.

It is important to mention that protocols that simulate human's entire digestion process *in vitro* also exist (e.g., INFOGEST protocol) and are preferred methods as they represent more accurately the digestion process and the metabolism of chemicals in human body (Brodkorb *et al*., 2019; Minekus *et al*., 2014).

The objective of this study is to identify non-volatile NIAS arising from the polyester coatings and investigate their gastrointestinal fate.

Four cyclic oligoesters (c(2TCDDM+2PA), c(2BD+TCDDM+3PA), c(BD+2TCDDM+3PA) and c(3TCDDM+3PA)) were identified from polyester coatings ACN extracts and EtOH 95% migrates by LC-HRMS (refer to Chapter 2). In order to investigate gastrointestinal fate of previously identified oligoesters another ACN extraction (by grating) was conducted on previously described polyester coating (refer to Chapter 2) to obtain more concentrated extracts for detection of other parent compounds not detected previously. Then, ACN extract was subjected to simulated intestinal digestion according to a modified protocol described by Eckardt *et al.* (2019), and parent and expected transformation products were monitored using LC-HRMS.

2. Materials and methods

2.1. Samples

Five metal plates coated with distinct batches of a single reference polyester-based lacquer formulation and one control uncoated plate used in this study were described in Chapter 2.

2.2. Sample preparation

The coating of each coated metal plate was grated (4 cm^2) and placed in a vial of 15 mL with 4 mL of ACN. After incubation for 24 h at 40 °C, the vials were centrifuged, the supernatants pooled into a new vial (20 mL of total volume) and concentrated under a gentle stream of nitrogen until 4 mL. The obtained extract will be so-called CA_{mix} in the present manuscript. CA_{mix} was directly used (without any concentration step) for *in vitro* digestion assay. However, a portion of CA_{mix} was concentrated (50 times) to enable the detection of chemicals in the extract at very low concentrations. These chemicals were called parent compounds.

2.3. Identification of parent compounds from concentrated CA_{mix}

2.3.1. LC/ESI(+/-)-Orbitrap data acquisitions

For one-dimensional data acquisition (MS^1), a CA_{mix} extract aliquote (1 mL) was mixed with 10µL of dStd mix (mix of three deuterium-labelled oligoesters: D4-lin(2NPG+2iPA), D4-

c(2NPG+2iPA) and D8-c(4NPG+4iPA)) at 1 ng/µL and concentrated until dryness under a gentle nitrogen flow, then dry residue was reconstituted in 20 µL of ACN (concentration factor 50). From this, 5 µL were injected on a LC Q Exactive (Orbitrap) instrument fitted with an ESI source operated in the positive mode and then in the negative mode. The parameters were those described in Cariou *et al*. (2022) pending slight modifications: (i) there was no sodium acetate added into the mobile phase, (ii) the polarity was positive or negative, (iii) there was no lock mass since experience showed that the calibration was stable enough. Worth of note, the absence of sodium acetate influences the adduct ion ratios compared to those described in Cariou *et al*. (2022), by not favouring the $[M + Na]^+$ adduct.

Solvent blank (ACN) and deuterium-labelled standards (d-Std) were also injected along with CA_{mix} samples.

2.3.2. Post-acquisition data processing in the positive mode

Two approaches were applied to pinpoint signals of interest: non-targeted analysis and suspect screening. The output lists of signals of interest were then merged and further MS^2 fragmentation experiments were performed in order to increase the identification confidence level.

2.3.2.1. Non-targeted analysis

First, a non-targeted approach was applied. The pre-processing of the MS^1 raw data file was handled using Workflow4Metabolomics (W4M), an open-source, collaborative online computational metabolomics platform installed in the web-based platform Galaxy. The W4M platform offers fully developed LC/MS and GC/MS pipelines using the Galaxy framework for data analysis, including preprocessing, normalisation, quality control, statistical analysis and annotation. These workflows are modular and extensible and consist of the same existing components used for the pre-processing of the GC/EI-HRMS data (e.g., *xcms* and *CAMERA* packages), as well as a whole set of additional tools. For reference, the *xcms* package was used for the integration of signals and the *CAMERA* package was used for the componentisation step (creation, so-called PCgroup from coeluted ions: isotopic contributions, adducts and fragments).

The W4M LC/MS workflow was used to pre-process the resulting dataset prior to the CAMERA annotation step. To do this, the proprietary raw format files were converted to the open format *mzXML* (using *msConvert* – an open-source command line utility for converting

between different MS formats) as described in the previous report. In terms of the parameters used at each step of LC/MS workflow – the default parameters were applied except in the chromatographic peak detection step, the "centWave" extraction method was used for peak detection.

CA_{mix} was processed along with ACN and dStd-Mix injections, allowing to manually remove signals arising from the mobile phase from the output data table. The output data table was sorted in the decreasing order of intensity, assuming that the concentrations would follow a similar trend.

Selected PCgroups were then manually examined and most features were annotated as either an adduct or a ^{13}C isotopic contribution. Adducts were mainly $[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$, with $[M + NH_4]^+$ as a major adduct type. The annotation of the adducts allowed determining the monoisotopic neutral mass of the considered PCgroup. Manual control and annotation of the data table was achieved up to a reasonable compromise between allocated time and signal intensities.

2.3.2.2. Suspect screening

Second, a suspect screening approach was applied, in order to test for the presence of oligoester combinations that may be missed in bioinformatics pre-processing. Indeed, the automated scripts do not always take into account all peaks, especially those that are non-Gaussian chromatographic peaks. In addition, low-sensitivity signals are usually lost. Most oligoester combinations involve various isomers arising from isomeric monomers or TCDDM asymmetry, leading to non-Gaussian peaks. In that case, peak-picked features might not be properly componentized and aligned.

Several combinations of oligoesters were tested using LABERCA's in-house script that integrated selected *m/z* signals based on the *pracma* package. For each suspect, $[M + H]^+$, $[M + NH_4]^+$ and $[M + Na]^+$ adducts were tested. This list comprised cyclic oligoesters, including those already identified by Cariou *et al*. (2022), as well as linear ones (potential degradation products of cyclic oligoesters) (Table 3-9). Acid-terminated combinations might not ionize appropriately in the positive mode.

Table 3 - 9. List of targeted ion m/z within the suspect screening approach

Suspect combination	Targeted ion *m/z*		
	$[M + H]^+$	$[M + NH_4]^+$	$[M + Na]^+$
lin(BD+TCDDM+2PA)	565.24321	582.26976	587.22516
lin(BD+2TCDDM+3PA)	891.39502	908.42157	913.37697
lin(3TCDDM+3PA)	997.47327	1014.49982	1019.45522
lin(2BD+TCDDM+3PA)	785.31677	802.34332	807.29872
lin(BD+TCDDM+PA)	417.22717	434.25372	439.20911
lin(BD+2PA)	387.10745	404.1340	409.08939
lin(BD+PA)	239.09140	256.11795	261.07335
lin(TCDDM+2PA)	493.18570	510.21225	515.16764
lin(TCDDM+PA)	345.16965	362.19620	367.15160
lin(2TCDDM+2PA)	671.32146	688.34801	693.30341
lin(2TCDDM+PA)	523.30542	540.33197	545.28736
lin(2TCDDM+3PA)	819.33751	836.36406	841.31945
lin(3TCDDM+2PA)	849.45723	866.48378	871.43917
lin(2BD+TCDDM+2PA)	637.30073	654.32728	659.28267
lin(2BD+2PA)	459.16496	476.19151	481.14691
lin(2BD+PA)	311.14892	328.17547	333.13086
lin(2BD+3PA)	607.18101	624.20756	629.16295
lin(3BD+3PA)	679.23852	696.26507	701.22047
lin(3BD+2PA)	531.22248	548.24903	553.20442
lin(4BD+3PA)	751.29604	768.32259	773.27798
c(2BD+2PA)	441.15440	458.18095	463.13634
c(BD+TCDDM+2PA)	547.23265	564.2592	569.21459
lin(2TCDDM+MA+AA)	601.33711	618.36366	623.31906
lin(TCDDM+MA+AA)	423.20135	440.2279	445.18329
lin(TCDDM+AA)	325.20095	342.22750	347.18290
lin(TCDDM+MA)	295.15400	312.18055	317.13595
c(TCDDM+AA)	307.19039	324.21694	329.17233
c(TCDDM+MA)	277.14344	294.16999	299.12538
lin(2TCDDM+2AA)	631.38406	648.41061	653.36601
lin(3TCDDM+3AA)	937.56717	954.59372	959.54912
lin(3TCDDM+2AA)	809.51983	826.54638	831.50177
lin(2TCDDM+3AA)	759.43141	776.45796	781.41335
lin(2TCDDM+2MA)	571.29016	588.31671	593.27211

2.3.3. Post-acquisition data processing in the negative mode

Only a non-targeted approach was applied to the ESI(-) data, using a similar approach as for ESI(+).

CA_{mix} was processed along with an injection of ACN containing three ISs, allowing to manually remove signals arising from the mobile phase and the standards from the output data table. Like in positive mode, the output data table was sorted in the decreasing order of intensity, assuming that the concentrations would follow a similar trend. Selected PCgroups were then manually reviewed as described for the ESI(+) part.

First, adducts were annotated. They were mainly identified as $[M - H]^-$ and $[M + CH_3COOH - H]^-$. In some cases, other adducts such as $[M + Cl]^-$ or $[M + NO_3]^-$ were

observed. For some substances, an adduct ion at [M – H + 173.96] was observed as well. A literature search allowed to annotate it as $[M + H_6Si_2O_7 – H]^-$ (Su *et al.*, 2020). Despite the diversity of ions found, a significant number of adducts could not be annotated (36%), which were considered $[M – H]^-$ for formula search.

2.3.4. MS^2 fragmentation experiments

Thirteen MS^2 fragmentation experiments were performed in order to increase the confidence level of the pinpointed NIAS in the positive and negative modes. Loaded extract was adjusted in order to diminish ion suppression and to preserve the analytical column. A CA_{mix} extract aliquote (1 mL) was thus reconstituted in 100 µL of ACN/H_2O (1:1, *v/v*) (concentration factor 10). Two to seven microliters (depending on the intensity of the response during MS^1) were injected for MS^2 experiments. The parameters were those described in Cariou *et al*. (2022), including normalized collision energy of 10%, 30% and 50%. The selected precursors corresponded to $[M - H]^-$, $[M + H]^+$ or $[M + NH_4]^+$ adducts. Resulting mass spectra were manually reviewed (chromatographic shape) and annotated (ion formula, fragment annotation).

2.4. *In vitro* digestion of CA_{mix} and identification of transformation products

2.4.1. Intestinal-fluid simulant

Intestinal-fluid simulant was prepared according to the note for the guidance provided by the EFSA (EFSA, 2008). Potassium dihydrogen orthophosphate (KH_2PO_4) (Sigma-Aldrich, France) (1.36 g) was added in 50 mL of water, then 38 mL of 0.2 M sodium hydroxide (NaOH) (Sigma-Aldrich, France) and 80 mL of water was added in the volumetric flask and the solution was shaken briefly. Next, 2 g of pancreatin extract (Sigma-Aldrich, France) was added into a 30 mL of water in a separate vial and the content stirred until a stiff, homogenous paste was obtained. The pancreatin paste was transferred to the volumetric flask by rinsing the beaker and funnel with water. Lastly, 0.1 g of sodium taurocholate (Sigma-Aldrich, France) was added in the flask and flask was gently shaken. The volume of the solution was adjusted to 200 mL and shake thoroughly to mix. The pH of the solution was adjusted to 7.5 ± 0.1 with 0.2 M NaOH.

Blank intestinal fluid was prepared exactly the same way as intestinal fluid without the addition of pancreatin. Intestinal and blank intestinal fluids were placed at -20 °C and thawed at room temperature before the experiment.

2.4.2. In vitro digestion assay

To perform the *in vitro* digestion assay, three variable parameters were tested as outlined below:

1. ACN or CA_{mix};
2. Intestinal fluid ("IF") or blank intestinal fluid ("noIF");
3. Incubation time (0, 2, or 4 h).

ACN was used as a solvent control alongside CA_{mix} in the assay because CA_{mix} was prepared *via* ACN extraction of a polyester coating. Both CA_{mix} and the solvent control (ACN) were tested with and without (blank intestinal fluid) intestinal fluid simulant to monitor digestion caused by the pancreatin enzyme while avoiding non-enzymatic hydrolysis of the identified parent compounds. Additionally, different incubation periods (0, 2, and 4 h) were selected to track the digestion kinetics of the parent molecules and the production kinetics of degradation products.

In addition, ACN, standards (Std), and CA_{mix} were injected. Two assays (without or with pancreatin; "IF" or "noIF") were added to assess the potential matrix effect in which the CA_{mix} was added after the addition of ACN for stopping the reaction (for 4h of incubation condition). The 2 latter assays were used for normalisation of the data (Table 3-10, condition 13 and 14). Table 3-10 shows all tested conditions used during *in vitro* digestion assay.

Table 3 - 10. Tested conditions for *in vitro* digestion assay

Solvent blank	ACN
Standard	Std
ACN extract used for digestion assay	CA_{mix}
Condition 1	ACN+noIF, 0h
Condition 2	ACN+IF, 0h
Condition 3	ACN+noIF, 2h
Condition 4	ACN+IF, 2h
Condition 5	ACN+noIF, 4h
Condition 6	ACN+IF, 4h
Condition 7	CA_{mix}+ noIF, 0h
Condition 8	CA_{mix}+ IF, 0h
Condition 9	CA_{mix}+ noIF, 2h
Condition 10	CA_{mix}+ IF, 2h

Condition 11	CA_{mix}+ noIF, 4h
Condition 12	CA_{mix}+ IF, 4h
Condition 13 (to test matrix effect)	noIF, 4h, CA_{mix}
Condition 14 (to test matrix effect)	IF, 4h, CA_{mix}

In vitro digestion was performed with the modified protocol described by Eckardt *et al.* (2019). CA_{mix} or ACN (100 µL) were added in 6 mL tubes and were concentrated until dryness under nitrogen flow, then the dry residue was reconstituted with 10 µL of ACN followed by the addition of 1 mL of intestinal or blank intestinal fluid. Tubes were incubated for 0 h, 2 h, and 4 h at 37 °C with agitation under darkness. To stop the reaction 1 mL of ACN was added in each tube. Then, 10 min ultrasonic treatment was applied to precipitate the enzyme. To clarify samples from enzyme residue a small portion of Na_2SO_4 was added until phase separation was visible. Tubes were then centrifuged at 2500 g for 5 min and 250 µL of supernatant was transferred in new vials.

Subsequently, 10 ng of IS (dStd mix) was added to each tube (mixture at 1 ng/µL each in 10 µL ACN). The extracts were concentrated to dryness and the dry residue was reconstituted in 20 µL of ACN. Finally, the vials were centrifuged again at 2500 g for 5 min and the supernatants were transferred to new vials. The samples were injected (5 µL) into the LC/ESI-HRMS instrument. *In vitro* digestion protocol used in this study is shown in Figure 3-10.

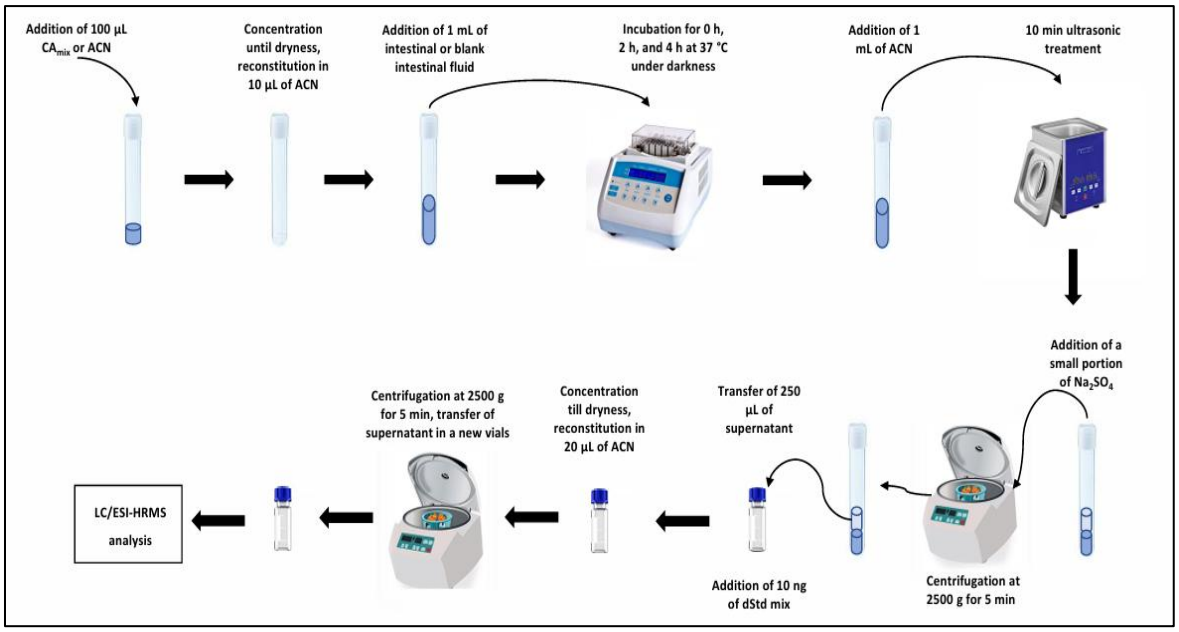

Figure 3 - 10. Illustration of *in vitro* digestion protocol (personal interpretation)

2.4.3. LC/ESI-Orbitrap data acquisitions

LC Q Exactive (Orbitrap) instrument parameters were those described in paragraph 2.3.1.

2.4.4. Post-acquisition data processing

The files in proprietary raw format have been converted to the open format *mzXML* (using *msConvert* - an open-source command-line utility for converting between various MS data formats provided by ProteoWizard 3.0.22265) so that they can be used for further processing. Then, an in-house script was applied for integrating the peaks corresponding to the targeted compounds. The script was handled in the *RStudio* (version 2022.07.1+554), the open-source integrated development environment for the *R* programming language and involved the *pracma* package.

The set of 18 substances previously identified in 50 times concentrated CA_{mix} (14 in the positive mode, 4 in the negative mode) were monitored. They were considered as parent compounds, although linear combinations could be degradation products from cyclic and/or longer linear combinations. For each substance, 1 to 3 adduct ions were selected according to Annex 3 and integrated according to their *m/z* (±2 ppm) and tR range.

In addition, all possible linear oligoester combinations that could stand as degradation products from the set of parent oligoesters were determined and manually screened for signals in the positive and negative modes. In case a signal was observed, the in-house script was applied at the observed tR range. Fragmentation of these chemicals was also performed to increase identification confidence.

2.4.5. Determination of esterase activity

Esterase activity of intestinal fluid was determined by measuring the extinction of 4-nitrophenyl butyrate (p-NPB) incubated with intestinal or blank intestinal fluid according to Eckardt *et al*. (2019). Pancreas converts p-NPB to p-nitrophenol (p-NP) in basic environment (Figure 3-11). The production of p-NP was followed by measuring the absorbance at 400 nm during 10 min. External calibration of p-NP was used to calculate the concentration of produced p-NP.

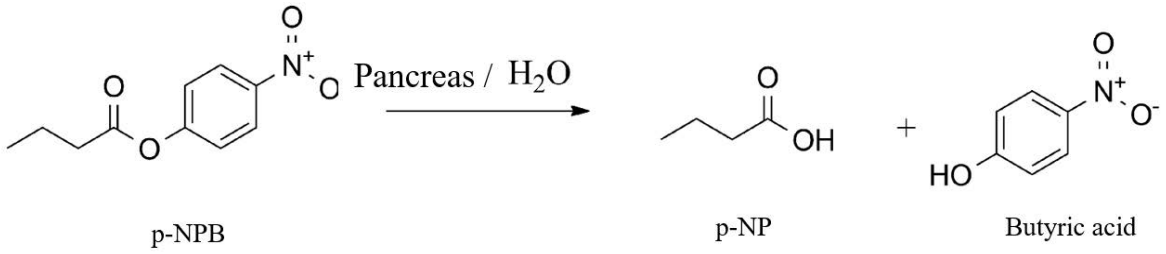

Figure 3 - 11. Hydrolysis of p-nitrophenyl butyrate (p-NPB) to p-nitrophenol (p-NP) (personal interpretation)

Reference solutions of p-NPB (Sigma-Aldrich, France) and p-NP (Sigma-Aldrich, France) which is a hydrolysis product of p-NPB were freshly prepared before the experiment at 717 µmol/L. p-NPB solution was prepared by dissolving 15 mg p-NPB in 1 mL DMSO (Sigma-Aldrich, France) and reaching the volume of 100 mL with the blank intestinal fluid. To prepare p-NP reference solution, 9.97 mg of p-NP were dissolved in 1 mL of DMSO and the volume reached 100 mL with blank intestinal fluid. Several concentrations (10-645 µmol/L) of p-NPB were prepared by two-fold serial dilution. External calibration of p-NP at the concentration of 10 to 250 µmol/L by two-fold dilution was also prepared.

For the assay, 180 µL of p-NPB (final concentrations ranging 10-645 µmol/L) or p-NP (10 to 250 µmol/L) were incubated with 20 µL of the intestinal or blank intestinal fluid in transparent 96-well plates for 10 min.

Michaelis Menten enzyme kinetics was recorded by measuring the amount of produced p-NP (using the external calibration of p-NP). Pancreatin (Sigma-Aldrich, France) converts p-NPB into p-NP, which is measurable by spectrophotometry at 400 nm. The absorbance was measured at 0 second, as well as every 60 seconds for a duration of 10 min at 25.5 ± 0.5 °C using FLUO star OPTIMA.

The specific esterase activity of the intestinal fluid expressed in units (U) per weight pancreatin was calculated from the Michaelis Menten kinetics by dividing the maximum reaction rate (V_{max}) by the amount of pancreatin used in the incubation assay.

3. Results

3.1. Identification of parent compounds from concentrated CA_{mix}

3.1.1. Positive mode

Figure 3-12 shows the total ion chromatogram obtained for the concentrated CA_{mix} in the positive mode. The chromatogram looks overloaded at this step.

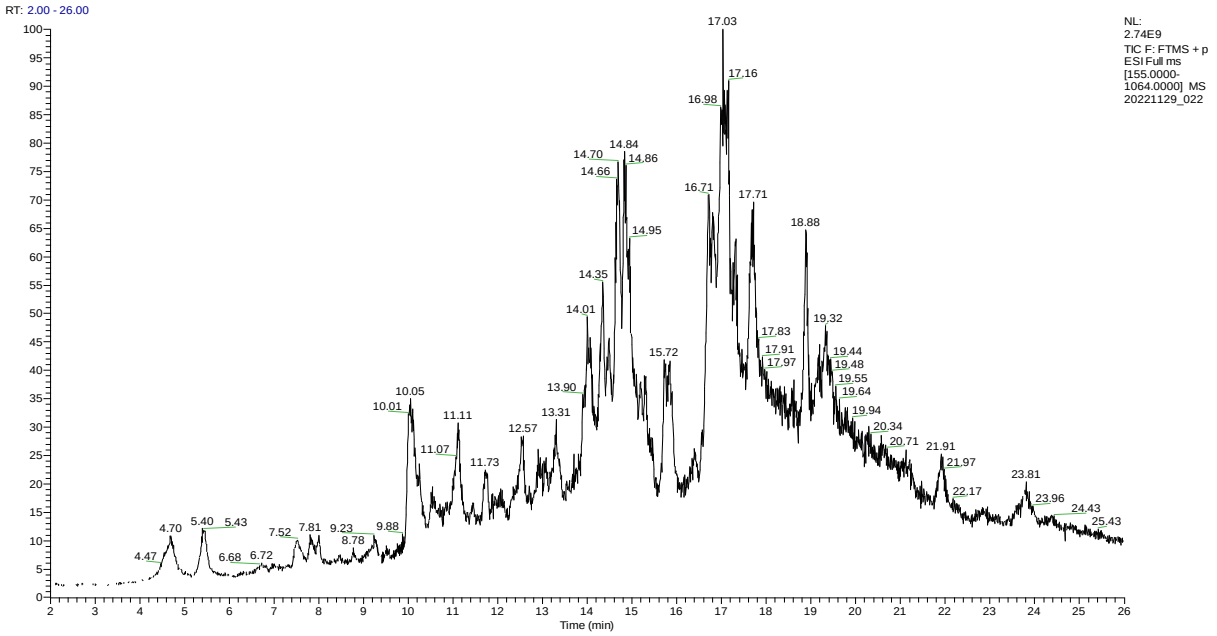

Figure 3 - 12. Total ion chromatogram obtained for the CA_{mix} in the positive mode

3.1.1.1. Non-targeted analysis

Manual control and annotation of the data table was achieved up to 53.4% of the total cumulative area. In total, 35 most intense PCgroups (corresponding to 165 ions) were reviewed in details (Table 3-11).

Table 3 - 11. Summary of the completion of the manual review of the signals obtained by LC-ESI(+)-HRMS in the concentrated CA_{mix} extract

Number of signals	3315
Cumulative intensity	1.29E+12
Contribution of 35 annotated ion groups	53.4%
…of which Signals present in the blank	51.6%
…of which coating-specific signals (8 PCgroups)	1.8%

A total of 8 PC groups were annotated as compounds of interest. The calculated monoisotopic neutral masses were matched against the theoretical database of oligoester combinations "NIAS-db 1.0" (Cariou *et al.*, 2022). Table 3-12 shows the calculated neutral mass, hypothesised chemical formula, and structures.

Of these 8 PCgroups, 4 matched the compounds previously identified: c(2TCDDM+2PA), c(2BD+TCDDM+3PA), c(BD+2TCDDM+3PA), and c(3TCDDM+3PA). This confirmed their prevalence in the sample extracts.

For 2 other PCgroups, the theoretical combination could correspond to cyclic tetra- and hexamers of TCDDM and AA, which remained consistent according to the knowledge of the resin formulation.

Table 3 - 12. List of PC groups of interest pinpointed with the non-targeted approach in the positive mode

PCgroup	Neutral mass	Chemical formula	Hypothesised Structure
1	652.3036	$C_{40}H_{44}O_8$	c(2TCDDM+2PA)
17	766.2989	$C_{44}H_{46}O_{12}$	c(2BD+TCDDM+3PA)
4	872.3751	$C_{52}H_{56}O_{12}$	c(BD+2TCDDM+3PA)
5	978.4554	$C_{60}H_{66}O_{12}$	c(3TCDDM+3PA)
3	612.3661	$C_{36}H_{52}O_8$	c(2TCDDM+2AA)
10	918.5497	$C_{54}H_{78}O_{12}$	c(3TCDDM+3AA)
549	582.3193	$C_{34}H_{46}O_8$	*c(BD+TCDDM+SeA+PA)
8	-	-	unknown

*Eventually refuted

Another combination could also involve three expected monomers (BD, TCDDM and PA), along with an unexpected diacid (SeA). Such a combination is unusual and as it has not been reported before in the literature. This hypothesis has been refuted on the basis of MS^2 data (see below).

For the last PC group, the neutral mass could not be determined due to the lack of associated adducts. Thus, no oligoester combination could be hypothesised.

3.1.1.2. Suspect screening

The suspect screening resulted in 6 additional combinations hypothetically present in CA_{mix}, among them 5 linear combinations (Table 3-13).

Table 3 - 13. List of compounds of interest pinpointed with the suspect screening approach in the positive mode

Combination	Neutral mass	Chemical formula
c(BD+TCDDM+2PA)	546.2254	$C_{32}H_{34}O_8$
lin(3TCDDM+2PA)	848.4500	$C_{52}H_{64}O_{10}$
lin(TCDDM+AA)	324.1936	$C_{18}H_{28}O_5$
lin(2TCDDM+2AA)	630.3767	$C_{36}H_{54}O_9$
lin(3TCDDM+2AA)	808.5125	$C_{48}H_{72}O_{10}$
lin(2TCDDM+MA+AA)	600.3298	$C_{34}H_{48}O_9$

3.1.1.3. Fragmentation results and identification level

In total, 14 compounds of interest were pinpointed in the positive dataset, among them 13 with hypothesised oligoester structures.

Levelling up the confidence level in those identifications according to the so-called Schymanski scale (Schymanski *et al.*, 2014) required MS^2 analysis. In total, 10 out of the 14 compounds of interest were subjected to MS^2 experiments.

Detailed information on the MS^1 and MS^2 assay results is provided in the Annex 3. A summary of the confidence levels is provided in Table 3-14.

Table 3 - 14. Summary of the oligoesters identified in the polyester-based coating by ESI(+) with associated confidence levels and their cumulative surface area in the studied extract

Confidence level	Compound	Adducts intensities			Cumulative area
		$[M + H]^+$	$[M + NH_4]^+$	$[M + Na]^+$	
1	*c(2TCDDM+2PA)	5.30E+09	4.94E+10	2.20E+09	5.69E+10
2a	c(BD+TCDDM+2PA)	8.81E+07	4.98E+08	2.86E+08	8.72E+08
	*c(2BD+TCDDM+3PA)	2.09E+07	1.57E+09	3.80E+08	1.97E+09
	*c(BD+2TCDDM+3PA)	1.29E+08	1.32E+10	7.33E+07	1.34E+10
	*c(3TCDDM+3PA)	1.34E+08	5.85E+09	9.08E+06	5.99E+09
	c(2TCDDM+2AA)	2.09E+08	1.50E+10	2.04E+10	3.56E+10
	c(3TCDDM+3AA)	2.24E+06	2.25E+09	1.78E+09	4.03E+09
	lin(TCDDM+AA)	6.30E+07	1.02E+08	8.32E+07	2.48E+08
	lin(2TCDDM+2AA)	5.36E+07	2.01E+08	6.55E+07	3.20E+08
	lin(3TCDDM+2AA)	9.64E+07	6.31E+08	1.07E+09	1.80E+09
	lin(2TCDDM+MA+AA)	5.31E+07	3.93E+07	2.23E+07	1.15E+08
2b	c(2TCDDM+MA+AA)	1.72E+08	2.47E+09	9.73E+08	3.62E+09
4	lin(3TCDDM+2PA)	5.27E+07	7.22E+07	3.37E+07	1.59E+08
5	Unknown 8				2.60E+08

*: originally identified in the Chapter 2

The combination c(2TCDDM+2PA) was confirmed at confidence level 1 according to the analytical standard c(2TCDDM+2iPA) provided by LABERCA (synthesized in the frame of the ANR project (ANR-21-CE21-0005_OLIGO). Although the MS^2 spectra matched, the PA isomer was maintained as undefined.

Ten oligoester combinations were identified at confidence level 2a. It means that the MS^2 data matched, that no other structural hypotheses could be done according to the available information and that they have been previously reported in the literature (Cariou *et al*., 2022) and/or are consistent with the know formulation monomers. In the latter case, 4 combinations were reported for the first time: c(2BD+TCDDM+3PA), c(2TCDDM+2AA), c(3TCDDM+3AA) and c(2TCDDM+MA+AA).

In the case of c(2TCDDM+MA+AA), the corresponding PC group 549 was initially hypothesized as c(BD+TCDDM+SeA+PA). Although TCDDM fragments were observed, none of the fragments obtained after MS^2 analysis corresponded to SeA. In addition, fragments likely corresponding to AA were observed. Thus, considering the observed fragments, only one possible combination was hypothesized as being c(2TCDDM+MA+AA). Maleic acid (MA) is a known starting substance from its anhydride precursor but it was disregarded so far since no oligoester involving this monomer has ever been reported. Obtained MS^2 data was consistent with this hypothesis, fragments being annotated as derived from TCDDM, AA, and MA monomers.

No fragmentation data was obtained for the linear combination lin(3TCDDM+2PA) matched during the suspect screening. Indeed, the signal intensity of selected precursors ($[M + NH_4]^+$ and $[M + H]^+$) remained too low, so it was not possible to improve the confidence at a level better than 4.

Last, the fragmentation of the unknown chemical (PC group 8) showed that it was not an oligoester. Identified fragments could correspond to stearic, palmitic, and myristic acid fragments, but formula decomposition was not possible to conduct. No corresponding signal was observed in the negative mode and no chemical formula (and *a fortiori* no chemical structure) could be hypothesized. The identification level remained 5.

3.1.1.4. Relative intensities of the identified cyclic oligoesters

Figure 3-13 shows the cumulative areas calculated on the basis of the intensities of three major adducts ($[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$) for the cyclic oligoesters observed in the positive mode and according to a logarithmic scale. It can be seen that the two combinations c(2TCDDM+2PA) and the newly identified c(2TCDDM+2AA) are the most intense, with 45% and 28% of the total cumulative surface of the 14 combinations, respectively. The linear combinations and the unknown 8 accounts for only 2.1% and 0.2% of this total, respectively.

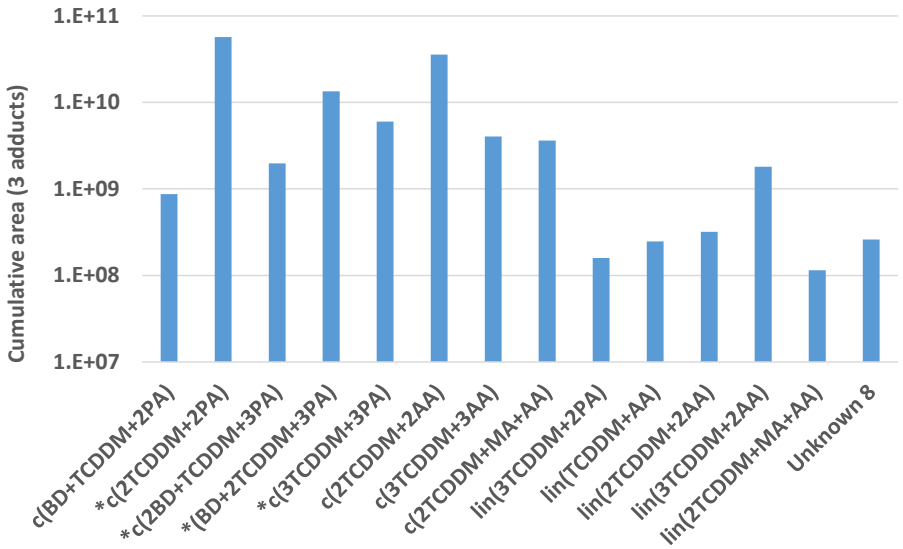

Figure 3 - 13. Cumulative surface areas for the 14 identified oligoesters at various identification levels (*: originally identified in Chapter 2)

3.1.2. Negative mode

Figure 3-14 shows the total ion chromatogram obtained for the CA_{mix} in the negative mode. The chromatogram shows a hump signal between about 16 and 20 minutes, also observed in the blank.

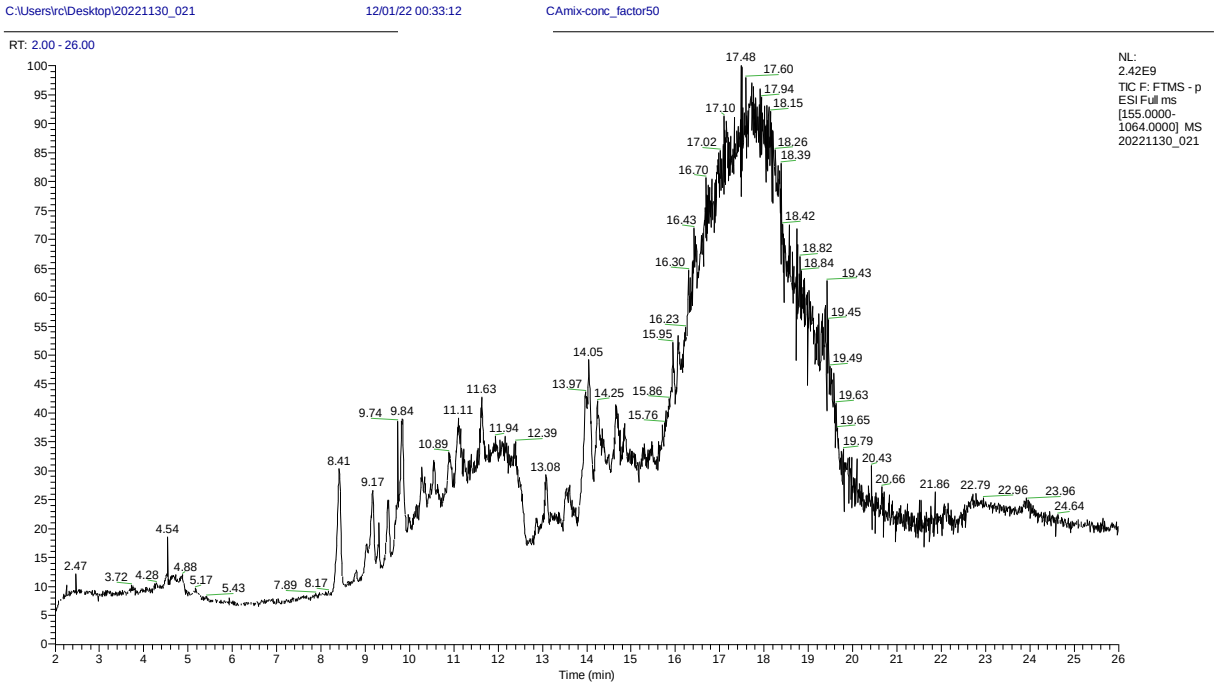

Figure 3 - 14. Total ion chromatogram obtained for the concentrated CA_{mix} in the negative mode

3.1.2.1. Non-targeted analysis

In total, the 99 PC groups were considered specific to the extract (Table 3-15). Cumulative intensities were ten times lower compared to the positive mode.

Table 3 - 15. Summary of the completion of the manual review of the signals obtained by LC-ESI(-)-HRMS in the CA_{mix} extract, after removal of signals lower than 10 times the blank level

Number of signals	386
Cumulative intensity	1.3E+11
Contribution of 99 annotated PCgroups	17.0%

Chemical formula could be annotated for 59 out of the 99 PC groups. Data mining and literature searches were performed for most of them, but their identification level remained at 4. Fragmentation and/or comparison with acquired authentic standards would help to level up the confidence level.

Deduced neutral masses were searched in the NIAS-db 1.0 database. Hits were returned for c(2TCDDM+2PA) and lin(TCDDM+AA), which were in accordance with the observed signals in the positive mode. Another hit was returned for c(TCDDM+PA), which was not observed in the positive mode. This result should be considered carefully since the neutral mass could not be confirmed by a second adduct ion. Last, 9 additional hits were returned but none was considered plausible considering the proposed monomers.

Deduced neutral masses were searched in the FCCdb database as well (Groh *et al.*, 2017). This database lists about 10,000 substances that are on positive lists of regulations or guidelines for FCMs, among them half with a defined chemical formula. Eighteen hits were returned. Among those hits, TCDDM was likely observed at a relatively high intensity. Unlike the other monomers, TCDDM mass was in the *m/z* range. It was not observed in the positive mode. Isophorone diisocyanate isocyanurate (IPDI trimer; $C_{36}H_{54}N_6O_6$; CAS 67873-91-0) was likely observed as well, as $[M + Cl]^-$ and $[M + CH_2COOH - H]^-$ adducts at a fairly high intensity. TCDDM and IPDI trimer are starting substances of the formulation. Last, a hit returned 2-undecylbenzene-1-sulphonic acid at relatively high intensity, which could be a surfactant.

3.1.2.2. Fragmentation results and identification level

Detailed information on the MS^1 and MS^2 assay results for 4 detected compounds is provided in the Annex 3. A summary of the confidence levels is provided in Table 3-16.

The fragmentation of a single cyclic combination of TCDDM and PA identified in negative mode allowed the confidence level to be improved to 2b.

Table 3 - 16. Summary of the oligoesters identified in the polyester-based coating by ESI(-) only, with associated confidence levels and their cumulative surface area in the studied extract

Confidence level	Compound	Most intense adduct	Cumulative area
2b	c(TCDDM+PA)	$[M - H]^-$?	3,55E+07
4	TCDDM	$[M - H]^-$?	1,97E+09
	IPDI trimer	$[M + CH_3COOH - H]^-$	4,86E+09
	2-undecylbenzene-1-sulfonic acid	[M – H]-	3,61E+09

3.2. *In vitro* digestion of CA_{mix} and identification of transformation product

3.2.1. Esterase activity determination of intestinal fluid

Absorbance increased during incubation time when reference substance p-NPB was incubated with intestinal fluid but not when incubated with blank intestinal fluid which is pancreatin-free meaning that there was no non-enzymatic hydrolysis during incubation time (Figure 3-15a).

The Michaelis Menten parameters were calculated from Lineweaver Burk plot. Michaelis Menten constant (K_M) of the intestinal fluid was 227.2 µmol/L and the maximum reaction rate (V_{max}) was 6.1 µmol/(L×min) (Figure 3-15b). The specific esterase activity was 6.1 U/g.

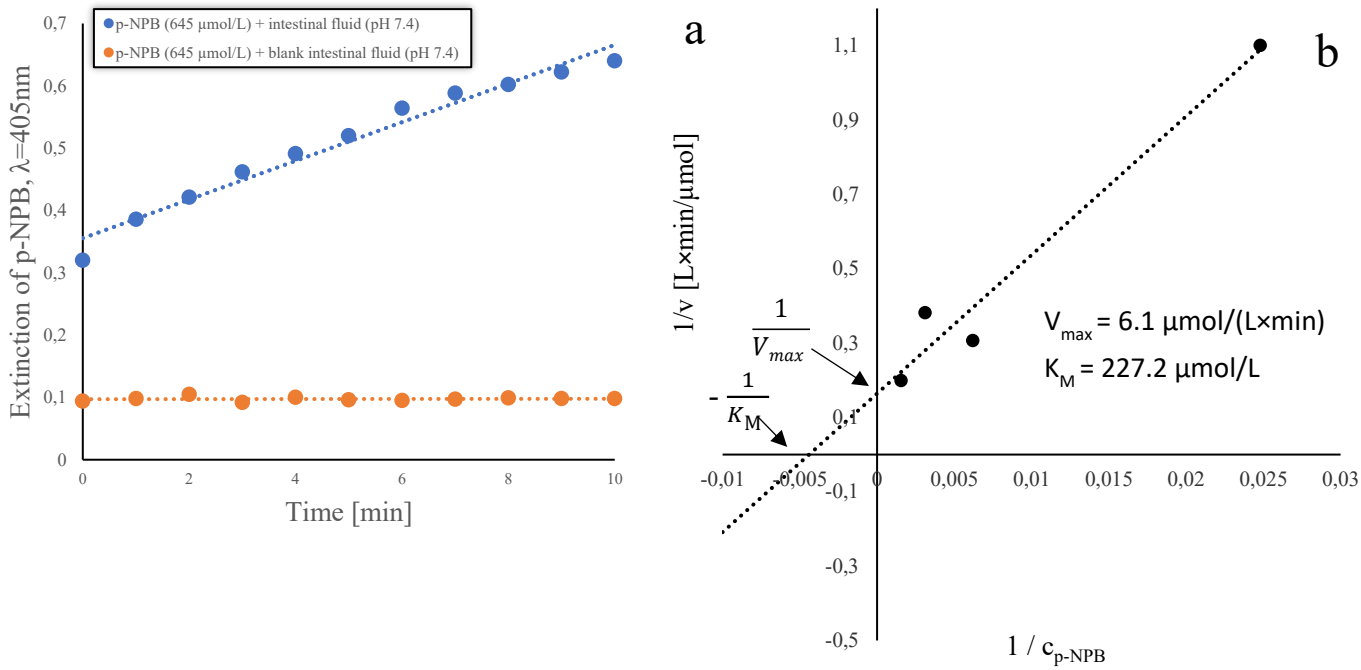

Figure 3 - 15. Esterase activity determination of intestinal fluid. (a) Comparison between the extinction of p-NPB by time incubated with intestinal and blank intestinal fluid; (b) Lineweaver Burk plot for the determination of the Michaelis Menten parameters (KM and V_{max})

3.2.2. Total ion chromatograms

The total ion chromatograms of the digestion assays after 4 h incubation is shown in Figure 3-16. The chromatograms look almost overloaded. In the positive ionization mode, they look similar, showing that most of the signal comes from the intestinal fluid. On the opposite, in the negative ionization mode, many intense peaks appear specific from the presence of pancreatin, regardless the presence of CA_{mix}.

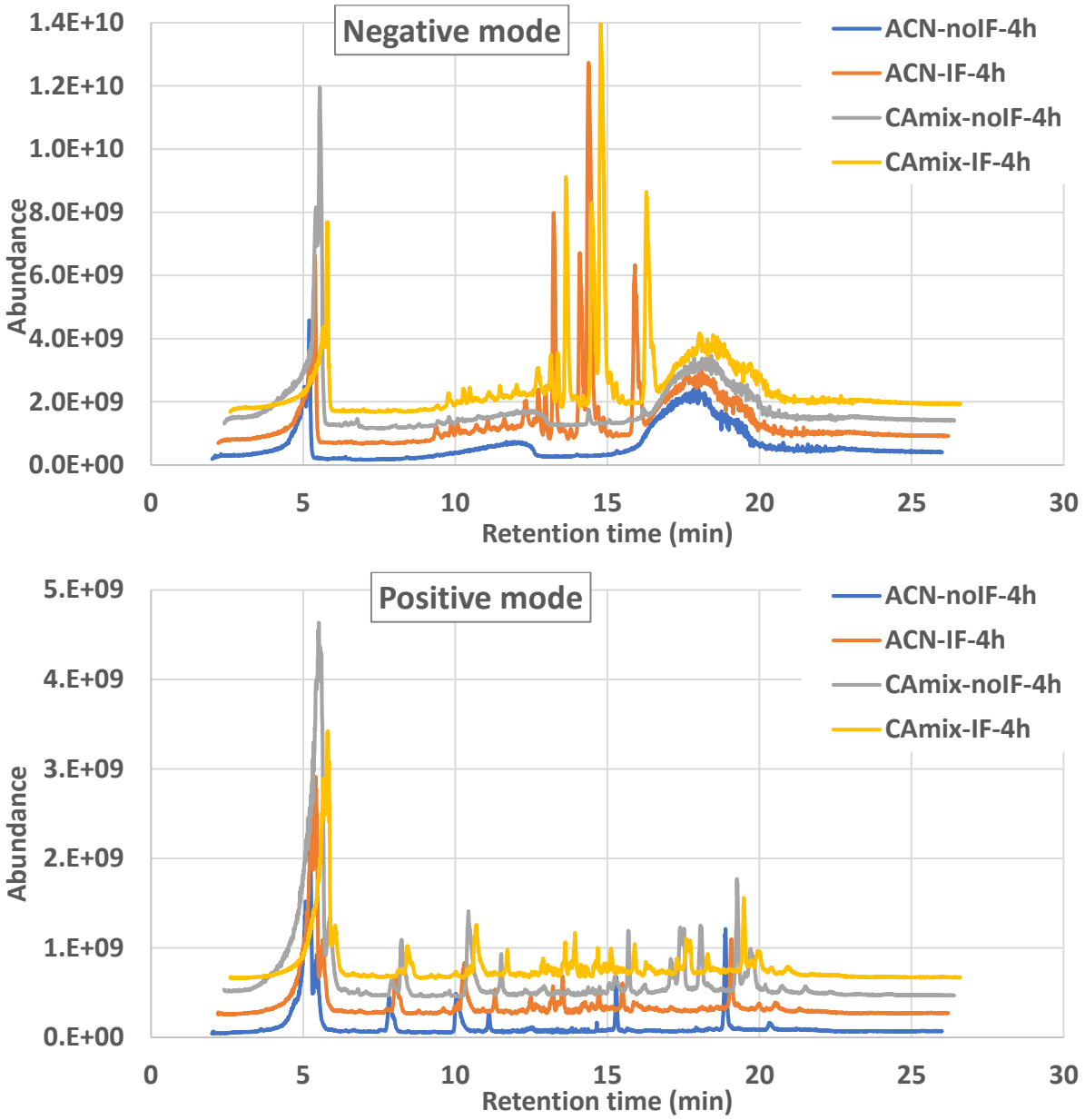

Figure 3 - 16. Total ion chromatogram of the digestion assays after 4 h incubation obtained in the positive (top) and in the negative (bottom) ionization modes

3.3. QA/QC

The deuterated standards, observed in the positive ionization mode, show fairly stable signals along the sequences. Their relative SDs are comprised between 41% and 56%, showing that some matrix effect and ion suppression occur. Indeed, the applied sample preparation did not get rid of the intestinal fluid, which brings a significant amount of dried matter. Thus, the results should be considered carefully in terms of relative variations between modalities.

However, comparing injections without and with pancreatin ("noIF" and "IF"), it appeared that there was no significant signal suppression of the deuterated standards.

3.4. Parent compounds

Signal areas corresponding to the 18 parent compounds in the digestion assays were normalized with their corresponding matrix assay and with a selected deuterium-labelled standard. Results were interpreted according to the combination type.

Due to the number of replicates (n=1), no statistical test was performed to determine whether there was a significantly difference between results without or with enzyme. However, tendencies appeared.

3.4.1. TCDDM-PA ester bond

Figure 3-17 shows results for the 4 parent compounds that are exclusively based on TCDDM and PA monomers. Considering the analytical variability, there is no indication of any degradation. The bond between TCDDM and PA might be insensitive to pancreatin.

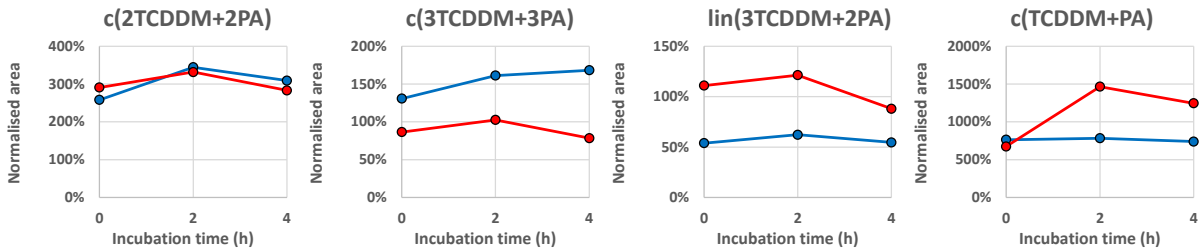

Figure 3 - 17. Normalised areas of parent oligoesters involving TCDDM and PA only as monomers, according to incubation time without (blue) or with (red) pancreatin. Normalisation with corresponding matrix assay and with D4 c(2NPG+2iPA) or D4 lin(2NPG+2iPA)

3.4.2. BD-PA and TCDDM-PA ester bonds

Figure 3-18 shows results for the 3 parent compounds that involve BD, TCDDM and PA monomers. Results at t = 0 appear consistent without and with pancreatin. A major and continuous degradation seems to occur with time, suggesting that such combination type is sensitive to pancreatin. Considering the results described above, it could be hypothesised that the BD-PA ester bond is more sensitive than the TCDDM-PA bond.

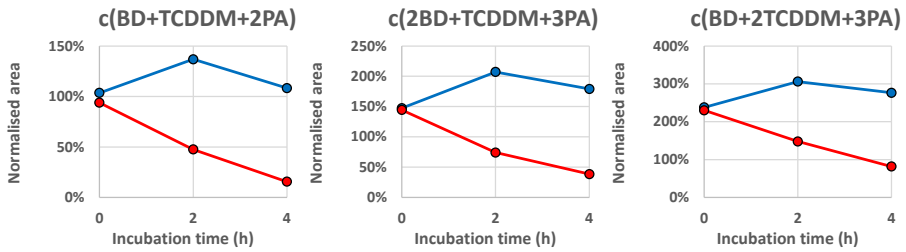

Figure 3 - 18. Normalised areas of parent oligoesters involving BD, TCDDM and PA as monomers, according to incubation time without (blue) or with (red) pancreatin. Normalisation with corresponding matrix assay and with D4 c(2NPG+2iPA)

3.4.3. TCDDM-AA ester bonds

Figures 3-19 and 3-20 show results for the 2 cyclic and 3 linear parent compounds that are exclusively based on TCDDM and AA monomers. A major and relatively fast degradation seems to occur with time for 4 out of these 5 combinations, suggesting that such combination type is sensitive to pancreatin.

On the opposite, lin(TCDDM+AA) seems to increase up to 2 h and then slowly decrease. Thus, although present in the original extracts, this small linear combination likely appeared to be a degradation product of c(2TCDDM+2AA), c(3TCDDM+3AA), lin(2TCDDM+2AA), lin(3TCDDM+2AA), c(2TCDDM+MA+AA) and/or lin(2TCDDM+MA+AA). It could also be further degraded, in accordance with kinetics concepts.

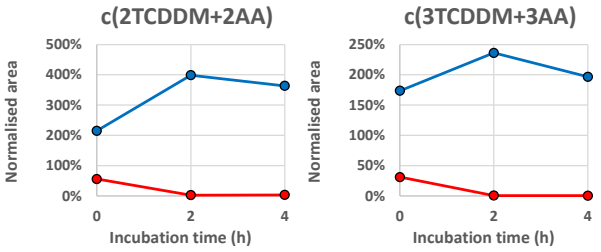

Figure 3 - 19. Normalised areas of cyclic parent oligoesters involving TCDDM and AA only as monomers, according to incubation time without (blue) or with (red) pancreatin. Normalisation with corresponding matrix assay and with D4 c(2NPG+2iPA)

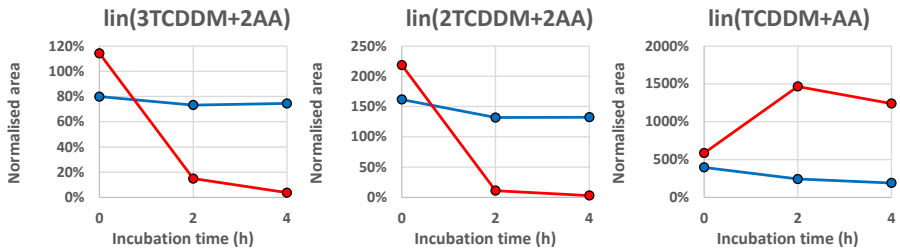

Figure 3 - 20. Normalised areas of linear parent oligoesters involving TCDDM and AA only as monomers, according to incubation time without (blue) or with (red) pancreatin. Normalisation with corresponding matrix assay and with D4 lin(2NPG+2iPA)

3.4.4. TCDDM-MA and TCDDM-AA ester bonds

Figure 3-21 shows results for the 2 parent compounds that involve TCDDM, MA and AA monomers. Results at t = 0 appear consistent without and with pancreatin. A major and relatively fast degradation seems to occur with time for these 2 combinations, suggesting that such combination type is sensitive to pancreatin. Considering the results described above, it cannot be hypothesised whether the TCDDM-MA bond would be sensitive to pancreatin.

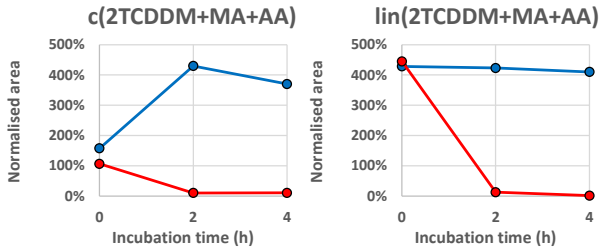

Figure 3 - 21. Normalised areas of parent oligoesters involving TCDDM, MA and AA as monomers, according to incubation time without (blue) or with (red) pancreatin. Normalisation with corresponding matrix assay and with D4 c(2NPG+2iPA) or D4 lin(2NPG+2iPA)

3.4.5. TCDDM

The manual review of TCDDM in the data did not reveal consistent peaks in the assays. The parent compound was likely not concentrated enough. Although such results should be considered carefully, it is likely not a final degradation product of oligoesters.

3.4.6. Unknown PCgroup 8

Figure 3-22 shows results for the unknown 8 parent compound. A major and relatively fast degradation seems to occur with time, corroborating the ester nature of this compound (probable stearic, palmitic, and myristic moieties).

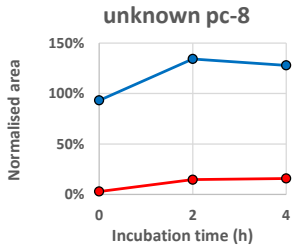

Figure 3 - 22. Normalized areas of unknown PCgroup 8, according to incubation time without (blue) or with (red) pancreatin. Normalization with corresponding matrix assay and with D8 c(4NPG+4iPA)

3.4.7. IPDI trimer

Although highly reactive, IPDI trimer likely did not react in the presence of intestinal fluid without pancreatin. However, it seemed readily reacted in the presence of pancreatin, even if ACN-degraded.

3.4.8. 2-undecylbenzene-1-sulfonic acid

This compound is present in all injections, suggesting that it was falsely identified as belonging to the CA_{mix}. It is rather a procedural contaminant.

3.5. Expected degradation products and their identification levels

Signals corresponding to 8 linear oligoesters as plausible degradation products were observed. Signals were normalized with a selected deuterium-labelled standard only.

The fragmentation experiment increased the confidence level for 6 out of 8 combinations. Thus, the identification level remained at 4 for two combinations: lin(2BD+TCDDM+3PA) and lin(2TCDDM+3PA), for which fragmentation data could not be acquired due to low intensities. The linear combination lin(BD+2TCDDM+3PA) was identified at level 2b and the other 5 degradation products were identified at level 2a.

Results were interpreted according to the combination type. Due to the number of replicates (n=1), no statistical test was performed to determine whether there was a significant difference between results without or with the enzyme. However, tendencies appeared.

Table 3-17 sum up all identified parent compounds and their degradation products with their confidence level of identification.

Table 3 - 17. Summary of chemicals identified in the polyester-based coating before and after *in vitro* intestinal digestion of CA_{mix}

Origin of compound	Number	Confidence level	Compound
Parent compounds	1	1	*c(2TCDDM+2PA)
	2	2a	c(BD+TCDDM+2PA)
	3		*c(2BD+TCDDM+3PA)
	4		*c(BD+2TCDDM+3PA)
	5		*c(3TCDDM+3PA)
	6		c(2TCDDM+2AA)
	7		c(3TCDDM+3AA)
	8		lin(TCDDM+AA)
	9		lin(2TCDDM+2AA)
	10		lin(3TCDDM+2AA)
	11		lin(2TCDDM+MA+AA)
	12	2b	c(2TCDDM+MA+AA)
	13	4	lin(3TCDDM+2PA)
	14	5	Unknown 8
	15	2b	c(TCDDM+PA)
	16	4	TCDDM
	17	4	IPDI trimer
	18	4	2-undecylbenzene-1-sulfonic acid
Degradation products	1	2a	lin(3TCDDM+3PA)
	2	2a	lin(2TCDDM+2PA)
	3	2a	lin(TCDDM+PA)
	4	2a	lin(2BD+2PA)
	5	2a	lin(BD+TCDDM+2PA)
	6	2b	lin(BD+2TCDDM+3PA)
	7	4	lin(2TCDDM+3PA)
	8	4	lin(2BD+TCDDM+3PA)

*: originally identified in the Chapter 2

Identified in negative mode

Identified in positive mode

Identified in positive and negative modes

3.5.1. TCDDM-PA ester bond

Figure 3-23 shows results for 4 linear combinations that are exclusively based on TCDDM and PA monomers. Lin(3TCDDM+3PA) seemed to be at higher level in the presence of pancreatin. Lin(2TCDDM+3PA), lin(2TCDDM+2PA), and lin(3TCDDM+3PA) could be a degradation product but it is not in line with observations on the potential parent compounds. Lin(TCDDM+PA) could be a degradation product of parent compounds involving other monomers.

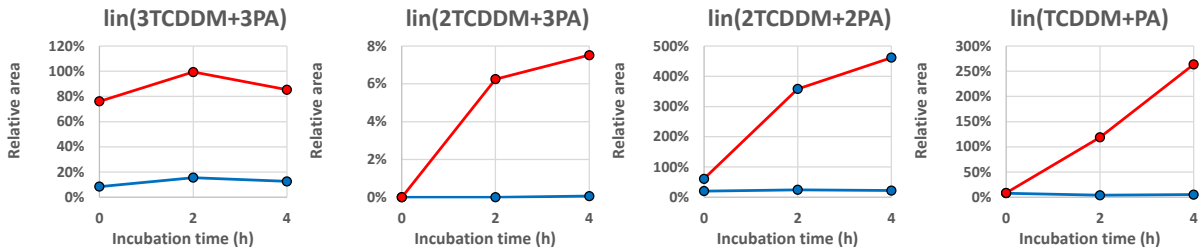

Figure 3 - 23. Normalized areas of suspected degradation oligoesters involving TCDDM and PA only as monomers, according to incubation time without (blue) or with (red) pancreatin. Normalization with D4 lin(2NPG+2iPA)

3.5.2. BD-PA and TCDDM-PA ester bonds

Figure 3-24 shows results for 4 linear combinations that are based on BD, TCDDM, and PA monomers. Negligible in the controls without pancreatin, they seem to increase and then decrease. These linear combinations likely appeared to be degradation products that further degraded, in accordance with kinetics concepts.

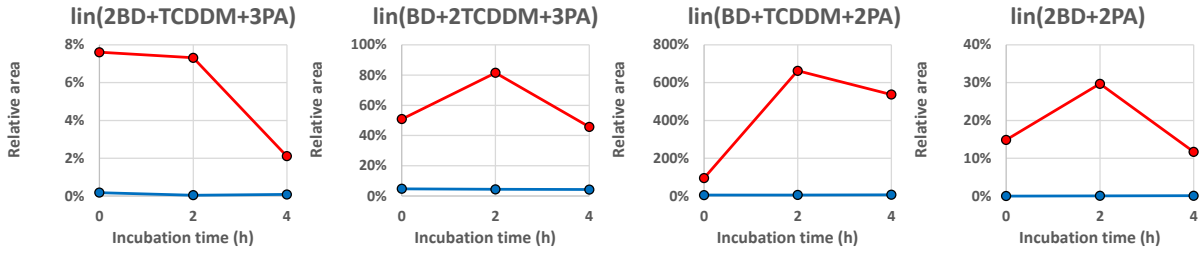

Figure 3 - 24. Normalized areas of suspected degradation oligoesters involving BD, TCDDM, and PA as monomers, according to incubation time without (blue) or with (red) pancreatin. Normalization with D4 lin(2NPG+2iPA)

4. Discussion

In this study, ACN extraction was conducted on previously described polyester coatings to identify non-volatile NIAS and to investigate their gastrointestinal fate. Using a non-targeted LC/ESI-HRMS approach in the positive and negative ionization modes, the presence of 18 substances (parent compounds) were identified in the ACN extract (concentrated 50 times to detect as much substances present at the trace quantities as possible) with varying levels of confidence.

As regards oligoesters, 9 cyclic combinations in accordance with the known starting monomers were confirmed in the ACN extract (level 1 for one combination, level 2a for 6 combinations, level 2b for 2 combinations). Five linear combinations were hypothesized as

well: 4 compounds at level 2a and 1 combination at confidence level 4. In addition, the TCDDM monomer was directly observed (confidence level 4). Potential presence of MA in such oligoesters was also observed for the first time. IPDI trimer, another starting substance, was also observed at level 4 confidence.

The present work regarding identification of parent compounds cannot be considered exhaustive due to several limitations. Firstly, the instrumental choices were restricted to specific parameters. Other chromatographic or ionisation parameters might be tested to expand the chemical space covered by the analytical strategy. Secondly, it is acknowledged that relevant information is possibly lost during the post-acquisition pre-processing steps or disregarded during the manual review of the output data. In particular, data mining could be deepened as regards the FCCdb hits. QA/QC criteria might be included to better define the performance of the workflow. Lastly, further work is needed in order to level up the identification confidence level of all hypothesized compounds of interest (e.g., MS^2 experiments, comparison with authentic standards…).

Esterase activity of the intestinal-fluid simulant was investigated to assess the enzymatic activity of pancreatin enzyme. Non-enzymatic hydrolysis was not observed during incubation as absorbance increased during incubation when p-NPB was incubated with intestinal fluid but not when incubated with blank intestinal fluid. In our study, Michaelis Menten constant (K_M) of the intestinal fluid was 227.2 µmol/L and the maximum reaction rate (V_{max}) was 6.1 µmol/(L×min). The specific esterase activity was 6.1 U/g.

Similar results were obtained in the literature. For example in the study of Eckardt *et al.* (2019) K_M of the intestinal fluid was 86 µmol/L, V_{max} was 6.2 µmol/(L×min), and specific esterase activity was 6.2 U/g. Data indicate that enzymatic activity of pancreatin enzyme included in the composition of the intestinal fluid simulant was acceptable for conducting *in vitro* intestinal digestion assay for ACN extract of polyester coatings.

After *in vitro* intestinal digestion of ACN extract (non-concentrated), previously identified parent compounds and their potential degradation products were monitored by LC/ESI-HRMS. Monitoring of parent compounds showed that oligoesters based on only TCDDM and PA seemed not to be sensitive to pancreatin. On the contrary, parent oligoesters containing BD-PA and TCDDM-AA ester bonds seemed readily hydrolyzed in the presence of pancreatin.

In total 8 degradation products (all linear oligoesters) were identified (level 2a for 5 combinations, level 2b for one combination, and level 4 for 2 combinations) with different confidence level. Suspected linear oligoesters expected to arise from the parent oligoesters showed increasing values in the presence of pancreatin. Some of them were possibly further degraded. Smaller degradation products could be expected, but the chromatographic equipment used and the performance of the analytical methods did not allow them to be detected.

Among the monomers, only the suspected TCDDM (parent compound) could be monitored. It showed no increase, so that TCDDM is likely not the final degradation product. The analysis of all monomers would be useful to determine whether the digestion goes down to them.

Unknown parent compound PC group 8 was corroborated as an esterified compound. Parent compound 2-undecylbenzene-1-sulfonic acid was refuted as a compound of interest, since likely originating from the procedure rather than from the polyester-based lacquer.

Figure 3-25 shows the possible hydrolysis pathway of cyclic parent oligoesters identified in this study. The figure shows that out of 30 potential degradation products, only 8 linear degradation products have been identified in this study using both positive and negative ionization modes.

Overall, this preliminary study demonstrates that oligoesters are partly degraded to linear oligoesters. This raises questions about the degradation products that are absorbed through the intestinal barrier.

Various authors have demonstrated that cyclic oligoesters hydrolyze into their linear forms, which exhibit lower toxicity. For instance, previously identified cyclic oligoesters were classified as Cramer class III substances with high toxicity level, whereas their linear counterparts were classified as Cramer class I substances, indicating lower toxicity level (Lestido-Cardama *et al.,* 2022b; Eckardt *et al.*, 2020, 2019).

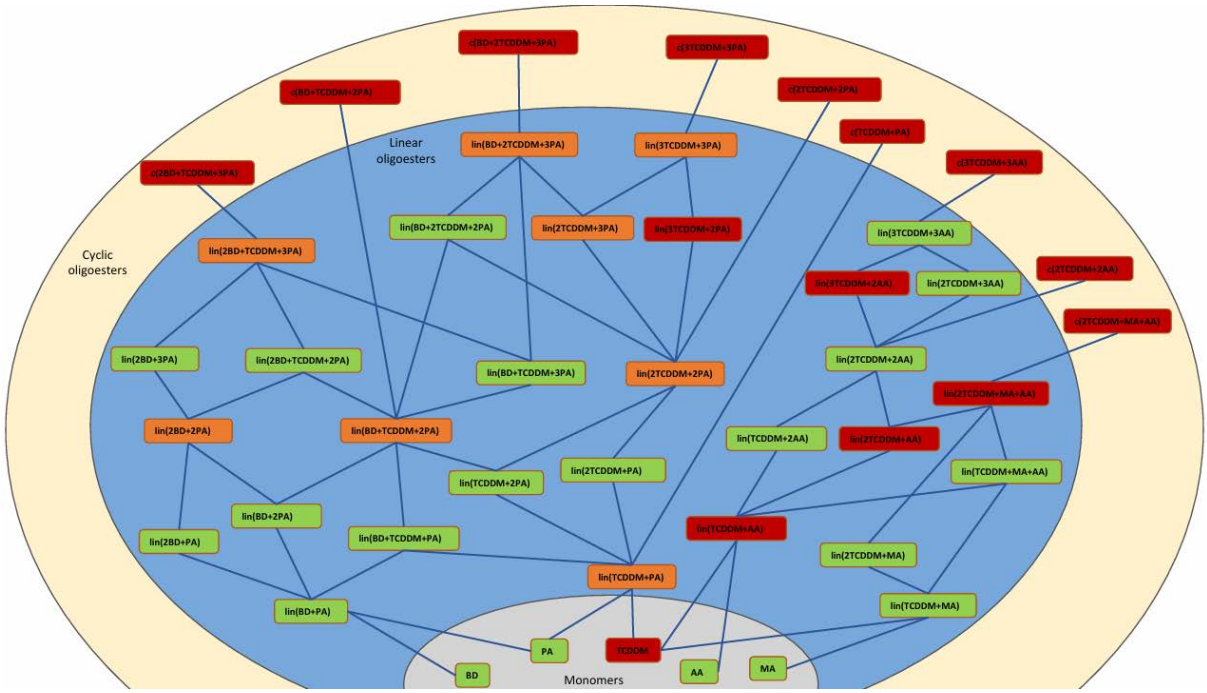

Figure 3 - 25. Illustration of possible hydrolysis pathway of cyclic parent oligoesters (parent compounds framed in red, degradation products identified in this study framed in orange, and other possible degradation products framed in green (personal interpretation)

The obtained findings align with those reported in the literature. Eckardt *et al.* (2019) investigated the likelihood of digestive degradation of three cyclic polyester oligomers (one dimer and two trimers) from common aromatic polyesters under intestinal conditions to evaluate the associated risk. In their study, oligomers with a molecular weight of less than 1000 g/mol were extracted, isolated, and analyzed for their degradation behavior under *in vitro* intestinal conditions. Rapid cleavage of all three cyclic oligomers into their linear forms (≥75% of the initial amount) was observed within the first hour of *in vitro* intestinal incubation. Further hydrolysis to shorter linear oligomers was noted, particularly for one cyclic trimer, although complete degradation to the monomer level was not achieved (Eckardt *et al*., 2019). In another study, conducted by Ubeda *et al*. (2020) bioaccessibility of two cyclic oligoesters of polyurethane was evaluated by studying gastric and intestinal digestion. The concentration of the compounds decreased during digestion and that their hydrolysed molecules increased.

The preliminary results obtained in this study for the gastrointestinal fate of identified oligoesters are promising, however, due to the number of replicates (n=1), no statistical test was performed to determine whether there was a significant difference between results without or with enzyme. The lack of replicates remains the biggest limitation of this study and

further research should be addressed to assure the reliability of the data including at least three replicates.

For the identified parent compounds and their potential degradation products, further toxicological analysis is necessary. It is crucial to evaluate the absorption potential of these molecules by the human body and their possible toxic effects. Assessing the absorption potential is a critical first step in the safety evaluation of chemicals. Due to the lack of standards (except for c(2TCDDM+2PA) which was synthesized in the frame of the ANR project (ANR-21-CE21-0005_OLIGO) by LABERCA) evaluating the toxicity of these identified parent compounds and their degradation products using *in vitro* or *in vivo* bioassays is challenging and costly because it requires their synthesis.

Toxicity prediction of these molecules using *in silico* QSAR tools can serve as an initial approach to prioritize the identified molecules based on their hazard. If positive alerts are detected, synthesizing oligoesters will allow for the confirmation of these *in silico* alerts *via* testing them in different bioassays and enable their proper quantification for risk assessment.

5. Conclusion

This study conducted ACN extraction on polyester coatings to identify non-volatile NIAS and investigate their gastrointestinal fate using LC/ESI-HRMS. A total of 18 parent compounds and 8 degradation products were identified. Some parent compounds, such as oligoesters based on TCDDM and PA, were likely resistant to *in vitro* digestion, while others, like oligoesters containing BD-PA and TCDDM-AA ester bonds, were sensitive to pancreatin. Linear oligoesters arising from parent oligoesters increased in the presence of pancreatin, and some may have further be degraded, although smaller degradation products could not be adequately identified due to chromatographic limitations.

To improve data reliability, future research should include at least three replicates. Additionally, toxicity prediction using *in silico* QSAR tools can help prioritize molecules based on health hazard.

Chapter 6

Inter-laboratory study of NanoAmes test, a proposed miniaturized version of the Ames test - Preliminary data

Summary

Introduction

In earlier chapters, we reported negative MMT test results for ACN extracts and EtOH migrates from polyester coatings. A likely reason is the limited biological sensitivity of the assay, as NIAS in FCMs are often present at very low concentrations. The standard Ames test (OECD TG 471) lacks sufficient sensitivity to detect genotoxic substances at these levels. To address this, various miniaturized Ames test formats (e.g., Ames MPF, MMT) have been developed to enhance sensitivity and assess the genotoxic potential of FCMs, particularly for unpredictable NIAS (see Bibliographic Report). However, none of these variants are yet validated as alternatives to the OECD standard (OECD, 2022).

The NanoAmes test, a highly miniaturized version developed by GenEvolution, aims to detect low-level genotoxicants in complex matrices like FCM/FCA extracts and migrates. This chapter presents an inter-laboratory validation of the NanoAmes test conducted by CTM INSERM-UMR1231 (“PackTox” platform) and GenEvolution.

Materials and Methods

Fourteen reference chemicals (genotoxic and non-genotoxic), along with solvent controls, were tested using *S. typhimurium* TA98 and TA100 strains, with and without S9 metabolic activation. The goal was to evaluate the repeatability and reproducibility of NanoAmes. Lowest effective concentrations (LECs) and dose-response relationships were determined and results from both labs were compared.

Results

Negative and positive control data

Negative control values were consistent across labs for both strains, with or without S9. Positive controls without S9 (2-Nitrofluorene (2NF) for TA98, 4-Nitroquinoline 1-oxide (4NQO) for TA100) also showed similar responses. However, with S9, 2-aminoanthracen (2AA)

responses were lower in PackTox than in GenEvolution, with concentration discrepancies noted particularly for TA100.

LEC determination

TA98 Strain: Negative results for 4-Aminophenol (4AP), D-Mannitol, and Melamine were consistent across labs. Positive controls 2NF, 4NQO, 2AA, benzo-α-pyrene (BaP), N-Nitroso-N-Nethylurea (ENU), and ICR 191 acridine mutagen hydrochloride (ICR) yielded expected results in both labs. In contrast, methyl methanesulfonate (MMS)/-S9, cyclophosphamide monohydrate (CPA)/+S9, dimethylbenzanthracene (DMBA)/-S9, and aflatoxin B1 (AFB1)/-S9 did not give expected responses in PackTox laboratory.

TA100 Strain: Similar negative results were obtained for 4AP, D-Mannitol, and Melamine. Positive results for 4NQO, 2AA, BaP, CPA, ENU, and ICR were consistent across labs. PackTox occasionally detected MMS/-S9 positive only at very high concentrations (2500–5000 ng/well), whereas GenEvolution detected it positive at 5 ng/well. DMBA/-S9 and AFB1/-S9 also failed to respond as expected in PackTox.

Dose-Response Relationship

Clear dose-responses were observed for some substances (e.g., 2NF, 4NQO, BaP) in PackTox, but other compounds did not show consistent patterns.

Repeatability

Repeatability issues were observed in both labs, especially in PackTox. For TA98, ENU, DMBA, and AFB1 showed variability. For TA100, sodium azide (SA), MMS, ENU, DMBA, and AFB1 were problematic.

Discussion

Inter-laboratory validation results indicate that the NanoAmes test is a promising tool for detecting genotoxicity at low chemical concentrations. Historical negative controls were consistent between two labs, with and without S9 mix.

For the TA100 strain, results were generally aligned across labs for most test chemicals. However, significant discrepancies (>10-fold in LEC values) were found for AFB1 /+S9 and SA /-S9. Notably, MMS showed mutagenic activity at vastly different concentrations: 5 ng/well in GenEvolution vs. 2500–5000 ng/well in PackTox. Changing the MMS batch did not alter this outcome.

For the TA98 strain, results were again consistent for most chemicals. However, MMS and CPA showed lab-specific differences: both were negative in PackTox (up to 6000 ng/well) but positive in GenEvolution. Batch testing ruled out chemical lot differences.

Interestingly, DMBA and AFB1 without S9 gave positive results in both labs in both strains, suggesting metabolic activation may occur during extended pre-incubation.

Some positive controls (e.g., 2NF, 4NQO, BaP) exhibited clear dose-response trends in both strains, but further testing is needed for other chemicals.

Strain sensitivity observations:

- TA98 showed higher sensitivity to 2AA/+S9, BaP/+S9, DMBA/+S9, and ICR.
- TA100 was more sensitive to 4NQO/-S9 and CPA/+S9.
- Sensitivity to ENU and AFB1 varied and lacked consistency between labs and strains.

When compared to the standard Ames and Ames MPF tests, NanoAmes showed higher biological sensitivity for most chemicals, except for AFB1 (likely due to low S9 content and batch differences), and showed similar sensitivity for 4NQO, MMS, and 2AA.

To sum up NanoAmes is a sensitive and promising genotoxicity assay to detect chemicals at low concentrations. However, reproducibility issues—particularly in the PackTox lab—highlight the need to standardize factors such as bacterial culture density, S9 mix quality, equipment calibration, and operator technique to enhance reliability.

ARTICLE

1. Introduction

The bacterial reverse mutation or Ames test (Ames *et al*., 1975) performed according the OCDE guideline 471 (OECD, 2020) is a regulatory genotoxicity bioassay. The principle of this bioassay is to detect gene mutations of chemicals by using several histidine-dependent *Salmonella typhimurium* (TA1535, TA1537, TA97a, TA97, TA98, TA100, TA102) and tryptophan-dependent *Escherichia coli* (WP2 uvrA, or WP2 uvrA (pKM101)) strains (OECD, 2020). This bioassay is recommended by EFSA to be used in combination with *in vitro* micronucleus assay as the first step in mutagenicity testing of numerous chemicals as these two bioassays can cover all modes of action of mutagenic substances (EFSA, 2011). It has been used to test genotoxicity of several substance categories; FCMs additives, food additives, cosmetics, etc.

For general screening purposes, *S. typhimurium* TA98 and TA100 strains with and without metabolic activation can be used first as it has been shown that these two strains together are capable of detecting up to 89% of mutagens because of the ability to detect frameshift mutations in case of TA98 and base substitutions in case of TA100 (Zeiger *et al.*, 1985).

NIAS are found in FCMs extracts or migrate at very low concentrations (e.g., parts per billion range). Assessing the genotoxicity of these substances is challenging for researchers, risk assessors, and managers due to the lack of commercial standards. Implementing the standard Ames test, as outlined in the OECD TG 471 guideline is difficult when there are many samples to test or when sample quantities are limited. This method is time-consuming, labor-intensive, and requires substantial amounts of the test sample.

EU Regulation 10/2011 sets detection limit of 0.01 mg/kg (10 ppb) for non-regulated substances (excluding mutagenic, carcinogenic, reproductive toxins, or nanomaterials) migrating from functional barrier of multilayer materials into food (EU, 2011). Rainer *et al*. (2018) studied the suitability of the Ames test to detect genotoxicity of FCMs migrates. They showed that only highly potent genotoxins such as AFB1, BaP, 4NQO, can theoretically be detected at a level of 0.01 mg/kg in migrates or food. Furthermore, only a small fraction (10%) of genotoxic chemicals, such as glycidol (2,3-epoxypropanol), 5-chloro-2-methyl-4-isothiazol-3-one, m-phenylenediamine, associated with FCMs can be detected at or below this level. These findings suggest that the current Ames test is not enough sensitive for detecting the

presence of genotoxic substances in FCMs migrates at low levels. Therefore, there is a need to develop alternative methods with improved biological sensitivity to assess the genotoxicity of FCMs, especially to detect unpredictable NIAS present in these mixtures.

Several miniaturized versions of the Ames test based on different formats (e.g., Ames MPF, MMT) (refer to Bibliographic report- Miniaturized variants of Ames test) were developed to cope with the above-mentioned problems, however, up to date, none of them is validated as an alternative to the regulatory standard OECD TG 471 bacterial reverse mutation test (OECD, 2022).

NanoAmes test, an even more miniaturized methods of the Ames test developed by GenEvolution laboratory, an expert Contract Research Organization dedicated to *in vitro* genetic toxicology and toxicology expertise, to detect the genotoxicity of chemicals at low levels in complex matrices such as FCMs or FCA extracts/migrates, as an alternative to the standard method.

In this bioassay, sample requirements can be reduced from the highest concentration of 5000 µg/plate for standard test to 2.5 µg/well for NanoAmes.

Data of this bioassay demonstrated good sensitivity, specificity, and global accuracy according to GenEvolution laboratory when using reference substances. The sensitivity, specificity and concordance of the standard Ames test and NanoAmes test are shown in Table 3-18.

For confidentiality reasons, specific details will not be provided in this manuscript.

Table 3 - 18. Sensitivity, specificity, and concordance (%) values of standard Ames test and NanoAmes test from personal communication of GenEvolution laboratory

	Ames [1]	**Ames [2]**	**NanoAmes**
Sensitivity %	58.8	49.4	94.1% (lower confidence interval: 80.3%)
Specificity %	73.9	80.3	92.3% (lower confidence interval: 64.0%)
Concordance %	62.5	62.9	93.6% (lower limit: 82.5%)

[1]Kirkland *et al.* (2005)

[2]Matthews *et al.* (2006)

The next step of this research development involves implementing this assay in another laboratory for conducting an inter-laboratory validation study.

This chapter is dedicated to inter-laboratory validation of the NanoAmes test by two laboratories: CTM INSERM-UMR1231, platform "PackTox", and GenEvolution laboratory.
For an initial validation process of NanoAmes, several genotoxic and non-genotoxic reference chemicals (Table 3-19) together with their negative solvent controls were tested with and without a metabolic activation system or S9 mix on *S. typhimurium* TA98 and TA100 strains to study the repeatability and reproducibility of this bioassay. Results obtained from the two laboratories were compared in order to assess the relevance of this bioassay in detecting genotoxic chemicals present at low levels.

2. Materials and methods

2.1. Test substances, chemicals and reagents

Chemicals used to perform NanoAmes test were purchased from Sigma-Aldrich (St. Louis, MO, USA). Beta-naphthoflavone and phenobarbital-induced S9 was obtained from Biopredic International (Saint-Grégoire, France). Fourteen chemicals were used in the initial inter-laboratory validation of the method. Chemicals were dissolved and diluted either in water or DMSO. Information about the identity, Ames positive and negative status, mode of action, requirements for S9 mix, as well as the solvent in which they were dissolved are listed in Table 3-19.

Table 3 - 19. List of substances used for inter-laboratory validation of NanoAmes (Kirkland *et al*., 2016)

Chemical	CAS number	Ames +/-	Mode of action	With or without S9 mix	Solvent
Aflatoxin B1 (AFB1)	1162-65-8	+	Activated by CYP3A4, which is not highly expressed in rats compared with humans. Forms various adducts	with	DMSO
2-Aminoanthracen (2AA)	613-13-8	+	Cytochrome P450 induction and mutagenicity	with	DMSO
4-Aminophenol (4AP)	123-30-8	-			DMSO
Benzo[a]pyrene (BaP)	50-32-8	+	Requires metabolic activation (CYP 1A1, 1B1, epoxide hydrolase), forms bulky adducts	with	DMSO
Cyclophosphamide monohydrate (CPA)	6055-19-6	+	Requires metabolic activation (CYP2B6)	with	H_2O
7,12-Dimethylbenzanthracene (DMBA)	57-97-6	+	Requires metabolic activation (CYP1B1), forms bulky adducts	with	DMSO

ICR 191 acridine mutagen hydrochloride (ICR)	17070-45-0	+	Causes frameshift mutation	without	DMSO
D-Mannitol	68-65-8	-			H_2O
Melamine	108-78-1	-	Ames negative, causes bladder and ureteral carcinomas		DMSO
Methyl methanesulfonate (MMS)	66-27-3	+	Strong clastogen (N7 alkylation)	without	H_2O
N-Nitroso-N-Nethylurea (ENU)	759-73-9	+	Strong gene mutagen (O6 alkylation)	without	H_2O
2-Nitrofluorene (2NF)	607-57-8	+	Formation of DNA adduct	without	DMSO
4-Nitroquinoline 1-oxide (4NQO)	156-57-7	+	Causes primarily mutations of the base-substitution type although frameshift mutations are also induced	without	DMSO
Sodium azide (SA)	26628-22-8	+	Interacts with DNA and creates point mutation in the genome	without	H_2O

Ames-positive *in vivo* genotoxins/carcinogens used for inter-laboratory validation were mainly selected from EURL ECVAM list of recommended chemicals (Kirkland *et al*., 2016). Additional substances, which are not part of the EURL ECVAM list were also included, e.g., ICR. Standard positive control substances (e.g., 2-AA, 2-NF, SA) were also added.

Melamine, D-Mannitol, and 4AP were chosen as negative controls for the NanoAmes test, as D-mannitol and 4AP are considered non-genotoxic showing negative *in vivo* genotoxicity data and melamine is a non-genotoxic carcinogen. Melamine and D-Mannitol are listed in EURL ECVAM list (Kirkland *et al*., 2016).

Due to confidentiality reasons the solutions (reagents) used in the NanoAmes test will not be disclosed.

2.2. Bacteria

Two strains of *S. typhimurium* (TA98, TA100) were first used for inter-laboratory validation of the method. The strains were obtained from Trinova Biochem (Giessen, Germany). After amplification of bacteria from the bacterial disc and phenotype characterization, strains were maintained in frozen stocks (− 80 °C, DMSO culture).

2.3. Fresh culture preparation

Cryotube was thawed and 140 µL of bacterial suspension was introduce in 7mL of nutritive broth supplemented with ampicillin (25 µg/mL). The cryotube was discarded after the use.

The caps were slightly unscrewed, and tubes were incubated overnight at 37° C under the shaking.

2.4. Pre-incubation stage

Several concentrations of each chemical listed in Table 3-19 were tested with and without S9 mix on two strains of *S. typhimurium*. Test chemicals or their corresponding negative solvent controls were transferred (1.2 µL) in one well of 96-well microplate (one well for one condition), then 6 µL of Solution A or Solution B, 15 µL of bacteria, 150 µL of Solution C were added. Remaining wells of 96-well microplate were filled with 150 µL of Solution C. Plates were surrounded by a film (parafilm type) and incubated at least overnight at 37 ° C under shaking.

2.5. Incubation stage

Sterile 10 mL glass tubes were placed on hot plates and held at around 45 °C. For each strain and for each concentration of product to be tested or for each solvent control with or without metabolic activation system, several components were successively added to a tube: 3 mL of supercooled overlay agar, 720 µL of Solution A, the content of the appropriate well after homogenization. Then, 650 µL of the mixture was distributed 5 times in 5 well of a 25-well plate containing 2mL of minimal glucose agar. After solidification of the agar, the dishes were turned over and incubated at 37 ° C for 72 hours.

2.6. Cytotoxicity

Cytotoxicity was judged when reduction in the number of revertant colonies, clearing or diminution of the bacterial background lawn, and presence of microcolonies were observed. Precipitation due to sample in the final plates was also estimated. Figure 3-26 illustrates both the normal lawn, and the lawn observed under conditions of cytotoxicity and precipitation.

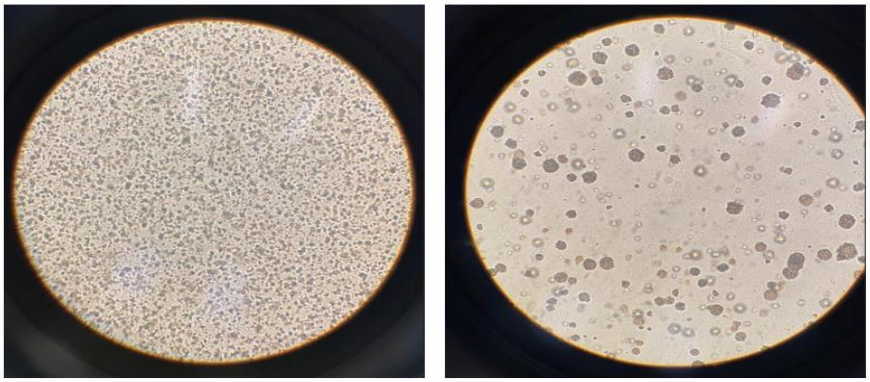

Figure 3 - 26. Illustration of the normal lawn and lawn observed during cytotoxicity (with microcolonies) in NanoAmes test (from personal communication of GenEvolution laboratory)

At least three experiments were conducted to follow the repeatability of the bioassay, however, for most of chemicals, number of repetitions were more than three.

2.7. Lowest effective concentration and dose response determination

At the end of the incubation, revertant colonies were counted manually in each well. The mean and SD of 5 wells were calculated. If the mean of revertant colonies in the test sample divided by the mean of the revertant colonies in the negative solvent control was equal or higher than 3 than positive response was concluded. LEC was defined as the first concentration of tested chemical with a ratio close to 3-fold background. The number of revertant colonies was plotted as a function of different concentrations of the tested chemical to observe the dose-response relationship.

Illustration of NanoAmes protocol is shown in Figure 3-27.

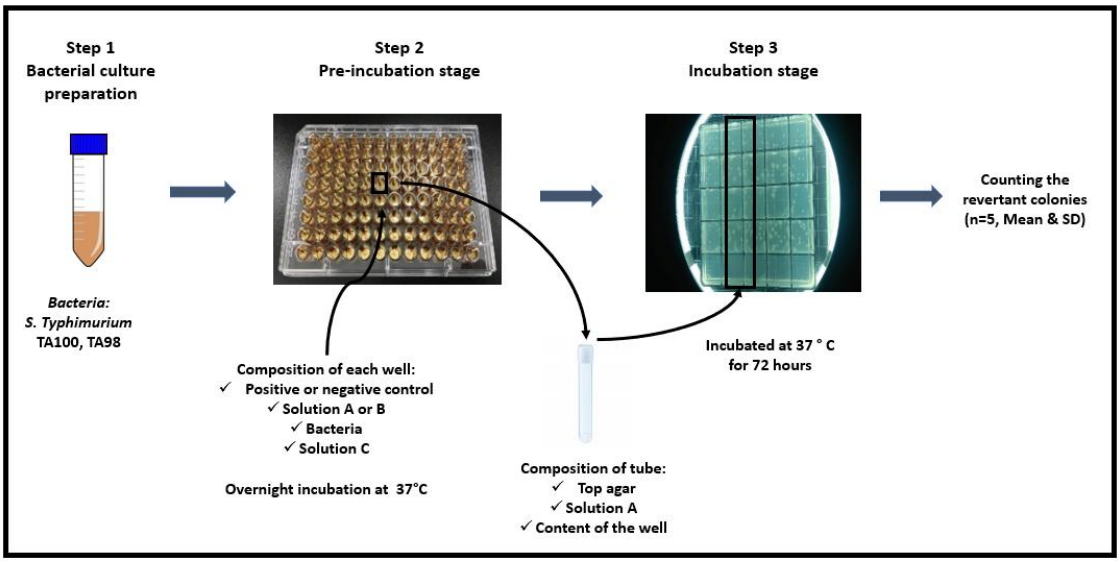

Figure 3 - 27. Illustration of NanoAmes protocol (personal interpretation)

3. Results

3.1. Historical data

Negative control data

Historical negative (solvent) control values with and without the S9 mix obtained from PackTox and GenEvolution laboratories were similar in the two laboratories for both strains (Tale 3-20).

Table 3 - 20. Historical negative (solvent) control values of two laboratories for *S. typhimurium* TA98 and TA100 strains

Strain	*S. typhimurium* TA98				*S. typhimurium* TA100			
Laboratory	GenEvolution		PackTox		GenEvolution		PackTox	
Solvent control	DMSO -S9	DMSO +S9	DMSO -S9	DMSO +S9	DMSO -S9	DMSO +S9	DMSO -S9	DMSO +S9
Mean of revertants	1.60	1.60	1.52	1.86	9.30	9.40	10.29	10.70
SD	1.19	1.14	1.67	2.13	3.29	3.05	4.28	4.00
Number of wells	105	85	200	155	115	120	245	247

Positive control data

Table 3-21 presents historical positive control values obtained from the PackTox team and the GenEvolution laboratory. The response to 2NF without S9 mix for the TA98 strain was similar in both laboratories. However, the response to 2AA with S9 mix in the PackTox laboratory was approximately four times lower than in the GenEvolution laboratory for the same strain.

For the TA100 strain, the concentration of the 4NQO positive control (without S9 mix) used in the PackTox laboratory was reduced by half (5 ng/well instead of 10 ng/well) due to occasional cytotoxicity at higher concentrations. Despite this reduction, a similar response was observed.

In the case of the 2AA positive control with S9 mix, the concentration used in the PackTox laboratory was 2.5 times higher (50 ng/well instead of 20 ng/well) than in the GenEvolution laboratory due to issues with assay repeatability. Even with the increased concentration, the mean number of revertant colonies in the PackTox laboratory was still approximately 1.7 times lower compared to the GenEvolution laboratory. These discrepancies may be due to variations in the bacterial strains, chemical batches, or S9 used.

Table 3 - 21. Historical positive control values of two laboratories for *S. typhimurium* TA 98 and TA100 strain

Strain	*S. typhimurium* TA98				*S. typhimurium* TA100			
Laboratory	GenEvolution		PackTox		GenEvolution		PackTox	
Solvent control	2NF (5ng/well) -S9	2AA (20ng/well) +S9	2NF (5ng/well) -S9	2AA (20ng/well) +S9	4NQO (10ng/well) -S9	2AA (20ng/well) +S9	4NQO (5ng/well) -S9	2AA (50ng/well) +S9
Mean of revertants	15.60	180.70	17.02	43.28	89.20	144.80	91.48	87.05
SD	8.86	111.66	9.60	29.18	52.75	131.11	67.41	42.06
Number of wells	46	30	85	25	35	40	25	40

The concentration of positive controls varies between laboratories

3.2. LEC determination

- ***S. typhimurium* TA98 strain**

The mean LEC values (calculated from individual experiments which are presented in Annex 4) of tested chemicals obtained from two laboratories for the *S. typhimurium* TA98 strain are shown in Table 3-22.

The inter-laboratory study showed that for *4AP, D-Mannitol,* and *Melamine,* negative results were obtained as expected with and without the metabolic activation system until the highest tested concentration (2500 ng/well) in both laboratories. For DMBA without S9, a positive

response was obtained from the two laboratories which was not expected as DMBA requires a metabolic activation system. In the PackTox laboratory, MMS yielded a negative response up to the highest tested dose (6000 ng/well), whereas in the GenEvolution laboratory, MMS gave a positive response as expected. AFB1, which requires metabolic activation, showed a positive response in the PackTox laboratory without the S9 mix, but it was negative in the GenEvolution laboratory, as expected. CPA with the S9 mix was negative in the PackTox laboratory up to 6000 ng/well, while it was positive in the GenEvolution laboratory as expected. Remaining positive controls (2NF, 4NQO, 2AA, BaP, ENU, ICR) were all positive in both laboratories as expected. Although LEC values varied, with the PackTox laboratory generally showing higher mean LEC values for most of the chemicals suggesting a lower sensitivity. To sum up, MMS/-S9, CPA/+S9, DMBA/-S9, AFB1/-S9 did not give expected responses in PackTox laboratory.

Table 3 - 22. The lowest effective concentration values obtained from two laboratories for the *S. typhimurium* TA98 strain

References	Test article	GenEvolution		PackTox	
		Mean (ng/well)	SD	Mean (ng/well)	SD
Positive References	2NF (-S9)	1.88	0.68	1.75	1.13
	4NQO (-S9)	1.10	0.82	6.12	5.31
	MMS (-S9)	1.25	1.06	n, 6000	
	2AA (+S9)	2.33	1.47	3.13	1.79
	B[a]P (+S9)	12.42	15.38	14.29	10.48
	CPA (+S9)	220.00	0.00	n, 6000	
	ENU (-S9)	3500.00	3535.53	3500.00	0.00
	ENU (+S9)	1500.00	707.11	3464.00	1805.84
	DMBA (-S9)	200.00	0.00	866.67	870.34
	DMBA (+S9)	50.00	0.00	333.33	457.89
	AFB1 (-S9)	n		58.57	31.85
	AFB1 (+S9)	1.30	2.33	2.80	4.82
	ICR (-S9)	0.25	0.00	0.70	0.27
	ICR (+S9)	1.67	0.58	3.38	4.46
Negative References	D-Mannitol (-S9)	n, 2500		n, 2500	
	D-Mannitol (+S9)	n, 2500		n, 2500	
	Melamine (-S9)	n, 2500		n, 2500	
	Melamine (+S9)	n, 2500		n, 2500	
	4AP (-S9)	n, 2500		n, 2500	
	4AP(+S9)	n, 2500		n, 2500	



Negative (n)

Positive

- ***S. typhimurium* TA100 strain**

The mean LEC values (calculated from individual experiments which are presented in Annex 4) of tested chemicals obtained from two laboratories for the *S. typhimurium* TA100 strain are presented in Table 3-23.

Inter-laboratory results showed that for *4AP, D-Mannitol, and Melamine,* results were negative with and without the metabolic activation system at the highest tested concentration (2500 ng/well) in both laboratories as expected. For DMBA and AFB1 without the S9 mix, positive results were obtained in the two laboratories, however, a negative response was expected as these two chemicals require a metabolic activation system. For the rest of the

positive controls (4NQO, 2AA, BaP, CPA, ENU, ICR), both laboratories obtained positive responses with LEC values varying from different laboratories. For some chemicals (AFB1/+S9, SA/-S9) the difference between LEC values between two laboratories was more than 10-fold. Positive results for MMS/-S9 were occasionally observed at very high concentrations (ranging from 2500 ng/well to 5000 ng/well) in the PackTox laboratory. In contrast, GenEvolution laboratory detected positive results at a much lower concentration of 5 ng/well. To investigate whether these differences were due to the chemical batch used, the MMS batch was changed, however, no difference in response was observed.

Table 3 - 23. The lowest effective concentration values obtained from two laboratories for the *S. typhimurium* TA100 strain

References	Test article	GenEvolution		PackTox	
		Mean	SD	Mean	SD
Positive References	SA (-S9)	21.88	22.49	227.14	182.64
	4NQO (-S9)	0.70	0.27	1.50	1.55
	MMS (-S9)	5.00	0.00	3916.67	1020.62
	2AA (+S9)	11.67	6.83	16.10	17.76
	B[a]P (+S9)	18.75	19.20	15.00	8.66
	CPA (+S9)	121.00	140.01	110.00	105.36
	ENU (-S9)	3333.33	2309.40	2583.33	1428.87
	ENU (+S9)	2000.00	0.00	2831.43	1496.30
	DMBA (-S9)	400.00	163.30	600.00	0.00
	DMBA (+S9)	100.00	0.00	557.14	878.65
	AFB1 (-S9)	100.00	0.00	51.67	40.41
	AFB1 (+S9)	48.10	45.91	1.93	1.57
	ICR (-S9)	4.17	1.44	1.80	0.97
	ICR (+S9)	12.50	3.54	9.70	9.51
Negative References	D-Mannitol (-S9)	n, 2500		n, 2500	
	D-Mannitol(+S9)	n, 2500		n, 2500	
	Melamine (-S9)	n, 2500		n, 2500	
	Melamine (+S9)	n, 2500		n, 2500	
	4AP (-S9)	n, 2500		n, 2500	
	4AP(+S9)	n, 2500		n, 2500	



Negative

Positive

3.3. Dose-response relationship

Dose-response relationship was analyzed with concentrations of chemicals tested on *S. typhimurium TA98 and TA100* strains with and without the S9 mix. For some positive controls (2NF, 4NQO, BaP), a dose-response relationship was observed in both strains in Packtox laboratory, however, for the rest of the chemicals this relationship was not always consistently seen. Examples of dose-response relationships for certain chemicals (from Packtox laboratory) are illustrated in Figure 3-28.

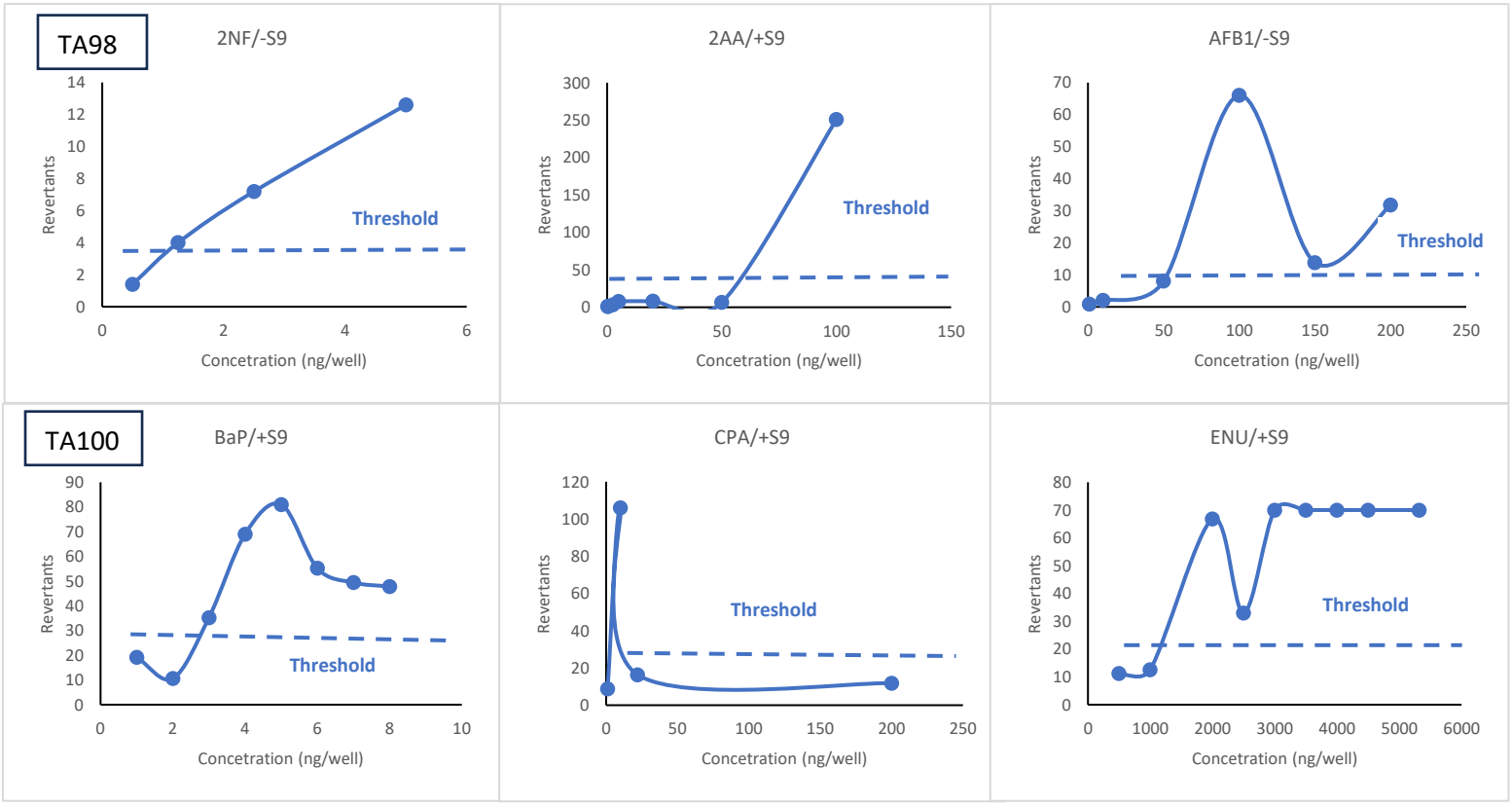

Figure 3 - 28. Dose-response relationship of some chemicals tested on *S. typhimurium* TA98 and TA100 strains obtained by the PackTox team

3.4. Repeata bility of NanoAmes test in Packtox laboratory

After comparing the results from individual assays of negative and positive controls tested with and without metabolic activation system on *S. typhimurium* TA98 and TA100 strains from two different laboratories, it was concluded that NanoAmes tests exhibit repeatability issues for certain chemicals in both laboratories, with these tendencies being particularly evident in

the Packtox laboratory for both strains (Annex 4). This repeatability issue is linked to the high SD observed in Packtox laboratory for the LEC of some chemicals (Tables 3-22 and 3-23), which represents the mean of LEC obtained from individual experiments (Annex 4).

For the TA98 strain, the repeatability problem was particularly noticeable for ENU, DMBA, and AFB1. Similarly, for the TA100 strain, repeatability issues were observed for SA, MMS, ENU, DMBA, and AFB1.

4. Discussion

Miniaturized versions of the Ames test can detect genotoxic chemicals at low concentrations and offer promising alternatives to the standard Ames test specially to assess NIAS at low levels. However, before these methods can be accepted as regulatory bioassays, their intra- and inter-laboratory reproducibility, as well as performance based on testing reference chemicals, must be demonstrated (OECD, 2005).

Several genotoxic (n=11) and non-genotoxic reference chemicals (n=3) along with their negative solvent controls, were tested with and without the S9 mix on *S. typhimurium* TA98 and TA100 strains to evaluate the repeatability and reproducibility of this bioassay. Results from the two laboratories were compared to assess the bioassay's relevance for detecting genotoxic chemicals at low levels.

The results of inter-laboratory validation showed that NanoAmes test is a promising bioassay to test the genotoxicity of chemicals at low quantities. Sample requirements were reduced from 5000 µg/plate for the standard test to 2.5 µg/well for NanoAmes decreasing the quantity of substances needed.

Results of historical negative control values were similar from two different laboratories with and without a metabolic activation system.

For the **TA100 strain**, results from the two laboratories were generally similar for most chemicals (AP, D-Mannitol, Melamine, 4NQO, 2AA, BaP, CPA, ENU, ICR). However, significant discrepancies were noted for AFB1/+S9 and SA/-S9, with difference in LEC values between the laboratories exceeding 10-fold or more. Positive results for MMS/-S9 were noted at very high concentrations (2500-5000 ng/well) in the PackTox lab, while GenEvolution detected mutagenic activity only at just 5 ng/well. Changing the MMS batch did not change this response indicating that this is not the affecting parameter.

Additionally, DMBA and AFB1 without the S9 mix gave positive results in both laboratories for this strain, although these chemicals typically require metabolic activation.

For the **TA98 strain** also, results from the two laboratories were generally similar for most chemicals (AP, D-Mannitol, Melamine, 2NF, 4NQO, 2AA, BaP, ENU, ICR). DMBA without S9 also showed a positive response in both laboratories. For TA98 strain, AFB1 yielded a positive response in the PackTox laboratory without the S9 mix which was not expected. These results suggest that DMBA and AFB1 may be metabolized into genotoxic compounds (as it was maybe the case with the TA100) during the extended pre-incubation period, potentially explaining the positive results without S9. Further research and analysis are needed to identify the stability and/or possible derived metabolites in the experimental conditions and assess their genotoxic potential.

For the TA98 strain, MMS/-S9 and CPA/+S9 were negative in the PackTox laboratory up to the highest tested dose (6000 ng/well), but positive in the GenEvolution laboratory, as expected. Then, different batches of these chemicals and S9 mix were tested again by PackTox, but similar responses were observed suggesting that differences were not due to the batches.

To sum up, the LEC of positive controls which represents an indicator of sensitivity varies between experiments and laboratories for almost all chemicals. In addition, for some positive controls (2NF, 4NQO, BaP), a dose-response relationship was observed in both strains, with or without the metabolic activation system. For the rest of chemicals, dose response needs to be reproduced with other concentrations.

Further investigation into other factors, such as bacterial origin and initial bacterial culture concentration, is recommended to understand the differences between the two laboratories' results.

Based on these preliminary results, the TA98 strain shows higher biological sensitivity compared to the TA100 strain for 2AA/+S9, BaP/+S9, DMBA/+S9, and ICR/±S9 chemicals. For 4NQO/-S9 and CPA/+S9 (CPA was negative in the PackTox laboratory for both strains), the TA100 strain exhibited higher biological sensitivity. Due to high variability in SD with and without S9 mix, it was not possible to determine which strain was more sensitive to ENU. The response to AFB1/+S9 varied between strains and laboratories.

The data presented in this manuscript are preliminary, and additional analysis is needed to make a final assessment of the bioassay's repeatability and reproducibility in order to further validate it.

The LEC of the NanoAmes test was compared with that of the standard Ames test and the Ames MPF test (Table 3-24) (Flückiger-Isler and Kamber, 2014).

Table 3 - 24. The lowest effective concentration of several chemicals in different versions of the Ames test

Substance	S9[a]	Standard Ames test			Ames MPF			NanoAmes	
		LEC [mg L^{-1}]	Strain[b]	Source	LEC [mg L^{-1}]	Strain[b]	Source	LEC [mg L^{-1}] From PackTox	Strain[c]
2AA	+	NA	NF	NF	0.01	TA98	Rainer *et al*. (2021)	0.005	TA98
AFB1	+	0.0014	TA98, TA100	Kenyon *et al.* (2007)	0.0008	TA98	Rainer *et al*. (2021)	0.004	TA98
BaP	+	0.21	TA100	Kenyon *et al.* (2007)	0.20	TA98	Rainer *et al*. (2019)	0.022	TA98
CPA	+	0.74	TA1535	Eliopoulos *et al.* (1995)	1	TA100	Rainer *et al*. (2021)	0.169	TA100
DMBA	+	9.26	TA100	Kaden *et al.* (1979)	6.25	TA100	Rainer *et al*. (2019)	0.513	TA98
ENU	-	12.2	TA98, TA100	Zeiger *et al.* (1992)	25	TA98, TA100	Rainer *et al*. (2021)	3.974	TA100
MMS	-	7.04	WP2	Kenyon et al. (2007)	10	TA100	Rainer *et al*. (2019)	6.026	TA100
4NQO	-	0.004	TA100	Blahová *et al.* (1997)	0.003	TA100	Rainer *et al*. (2019)	0.002	TA100
2NF	-	0.21	TA98	Schilter et al. (2019)	1	TA98	Rainer *et al*. (2021)	0.003	TA98
SA	-	1.2	TA100	Rainer *et al*. (2021)	NF	NF	NF	0.349	TA100

[a]This column shows whether an external metabolic activation system (S9) was used to achieve the positive result

[b]Tester strain/s for which the LEC was found

[c]Tester strain for which LEC was the lowest

NF: Not found

To compare the performance of NanoAmes test, LECs of the standard Ames test and Ames MPF were divided into LECs of the NanoAmes test (Table 3-25). The results demonstrated that the NanoAmes test generally exhibited higher biological sensitivity for most of the tested chemicals compared to the standard Ames test and Ames MPF showing higher ratios. NanoAmes is less sensitive for AFB1 than both other formats, and showed similar sensitivity

for 4NQO, MMS, and 2AA. For AFB1, low sensitivity may be related to the small amount of used S9 in the NanoAmes test and the differences between S9 batches.

Table 3 - 25. The ratio between lowest effective concentration (LEC) of different Ames protocols (LECs values used for the calculations were taken from Table 3-24 for the indicated strains)

Substance	LEC standard Ames/ LEC NanoAmes (PackTox)	LEC Ames MPF/ LEC NanoAmes (PackTox)
AFB1/+S9	0.35	0.20
BaP/+S9	9.55	9.09
CPA/+S9	4.38	5.92
DMBA/+S9	18.05	12.18
2AA/+S9	-	2.00
ENU/-S9	3.07	6.29
MMS/-S9	1.17	1.66
4NQO/-S9	2.00	1.50
2NF/-S9	70.00	333.33
SA/-S9	3.44	-

To sum up the results of inter-laboratory validation study suggest that NanoAmes is a promising bioassay for assessing the genotoxicity of chemicals present at low concentrations. However, it shows repeatability issues for certain chemicals across both laboratories, with these issues being especially pronounced in the PackTox laboratory for both strains. In the case of the TA98 strain, the repeatability problem was particularly evident with ENU, DMBA, and AFB1 chemicals. Similarly, for the TA100 strain, repeatability issues were observed with SA, MMS, ENU, DMBA, and AFB1 chemicals. Repeatability issues may be associated with several factors, including the concentration of the initial bacterial culture (highlighting the need to correlate optical density with bacterial concentration) and variability in S9 batch, equipment differences (e.g., water baths for uniform temperature), strain origin, and differences between operators. Addressing these factors is crucial for improving the consistency and reliability of the NanoAmes test.

Further investigation is then necessary to gain a better understanding of these parameters.

5. Conclusion

In this study NanoAmes test was implemented in PackTox laboratory. Issues with repeatability and reproducibility were observed between experiments conducted within and across

laboratories. Repeatability issues for certain chemicals were observed especially in the PackTox laboratory for both strains. Issues were observed with MMS/-S9 for both strains. Additionally, DMBA and AFB1, which typically require a metabolic activation system, produced positive responses in PackTox for both strains even without the S9 mix. Notably, for the TA98 strain, CPA/+S9 yielded a negative result.

The NanoAmes test is a promising alternative for assessing the genotoxicity of NIAS in FCMs extracts and migrates, showing higher biological sensitivity in comparison with standard Ames test and Ames MPF for most of the tested positive reference chemicals, except for 4NQO/-S9, MMS/-S9, 2AA/+S9, and AFB1/+S9.

IV. DISCUSSION

NIAS from polyester coatings can migrate into food representing a potential risk of consumer's exposure to toxic chemicals. FCMs extracts and migrates are complex mixtures containing numerous chemicals, most of them present at very low concentrations. Even at low concentrations, some of these substances may exhibit toxic effects, such as genotoxicity and endocrine disruption. Therefore, it is crucial to identify, quantify, and assess their hazards to ensure accurate risk assessment and protect consumer health.

In this study migration tests (using EtOH 95%) and extractions (using ACN) on polyester coatings were performed and samples were analyzed by LC-HRMS for non-volatile NIAS, and GC-HRMS for semi-volatile and volatile compounds.

Regarding semi-volatile and volatile NIAS, 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionic acid, also known as fenozan, was identified and its presence was confirmed using the reference standard in all samples by GC-HRMS. Fenozan was already reported as a degradation product of different sterically hindered phenolic antioxidants, such as Irganox 1010, and Irganox 1024 (Ji *et al*., 2024; Liu and Mabury, 2021; Bertoldo and Ciardelli, 2004a; Haider and Karlsson, 2002).

Regarding non-volatile NIAS, one cyclic tetramer and three cyclic hexamers (c(BD+2TCDDM+3tPA), c(3TCDDM+3tPA), c(2BD + TCDDM+3tPA), c(2TCDDM+2tPA)) were identified (at high confidence level 2) by LC-HRMS in all samples. Oligoesters are common predictable NIAS from coatings, as their formation can be anticipated based on the composition of the coatings.

It is worth noting that comprehensive analysis, including the identification of all NIAS, remains challenging due to analytical limitations and the absence of commercial reference standards.

This work focuses on the hazard assessment of identified NIAS with molecular weight below 1000 Da as according to EFSA they may have the potential to be absorbed by the human body (EFSA, 2008). First, the toxicity (carcinogenicity, mutagenicity, developmental/reproductive toxicity, organ toxicity, and endocrine-disrupting activities) of identified NIAS (fenozan and four identified oligoesters) were predicted using different *in silico* QSAR tools before performing *in vitro* bioassays for identified NIAS with available commercial standard. Using *in silico* QSAR tools is effective for predicting the toxicity of chemicals. Identified alerts can be further validated through *in vitro* bioassays or *in vivo* experiments. However, QSAR tools have also some limitations: they require complete chemical structures, provide only predictions

based on structure, and they cannot be applied to mixtures with unknown substances, where different interactions like synergism, potentiation, or antagonism may occur.
In addition, Log $K_{o/w}$ values were used to predict chemicals accumulation in the organism. Lipinski's 'rule of 5' (refer to Chapter 2) was applied to assess absorption and permeability of the identified substances when absorption data were lacking in the literature.

To our best knowledge, the toxicity of **fenozan** is not completely evaluated to date with data being proprietary and not accessible to the public. In the literature, Log $K_{o/w}$ value of fenozan was found to be equal to 4.7. The bioavailability of fenozan varied from 46.8 -57.8%, suggesting this molecule may be rapidly absorbed by the human body and slowly eliminated from it (Prokopov *et al*., 2006a, Prokopov *et al*., 2006b).
The predictive hazard assessment of fenozan revealed positive alerts for mutagenicity, carcinogenicity, developmental toxicity, endocrine disruption (ER α binding, thyroperoxidase inhibition, sodium/iodide symporter (NIS1), pregnane X receptor binding, constitutive androstane receptor), and organ toxicity (hepatotoxicity and thyroid toxicity). To validate these QSAR predictions, *in vitro* bioassays, the Ames test and micronucleus assay using flow cytometric reading were conducted following EFSA's recommendations for testing mutagenic chemicals (EFSA, 2019a). Both bioassays yielded negative results indicating the absence of genotoxicity of fenozan. Notably, some studies have shown that fenozan may have anti-carcinogenic effects (Bespalov *et al*., 2016, Erokhin *et al*., 2007).
Additionally, the iodide uptake assay was performed to assess potential thyroid disruption. Fenozan showed a weak inhibitory effect in the iodide uptake assay (with an IC50 value 3,840 times higher than that of perchlorate, a potent iodide uptake inhibitor) using rat thyroid FRTL-5 cells. These results suggest a potential anti-thyroid mode of action of fenozan.
ANSES indicates that BHT, which is an analogue of fenozan showed changes to thyroid physiology in several rat studies (ANSES, 2016a). However, the rat model is not the most suitable for studying human thyroid disrupting activity due to species sensitivity variability (ANSES, 2016b). The *in vivo* LAGDA, which highlights adverse effects, is preferred over fish testing since BHT affects the thyroid and has low androgenic or estrogenic activity. The HSE UK agency, Danish EPA, and AGES Austrian agency agree that the long-term fish test per OECD 234 is not relevant, while the Danish EPA and AGES support the LAGDA assay as the most

relevant test (ANSES, 2016b). Then, to better understand the potential consequences of sodium/iodide symporter (NIS1) inhibition by fenozan and its possible role in thyroid toxicity, LAGDA could be performed (OECD, 2015).

Additionally, a TPO assay can be conducted to verify the *in silico* alert for thyroperoxidase inhibition.

It is important to note that various QSAR tools indicated a positive prediction for the developmental toxicity of fenozan. Disruption of thyroid hormone synthesis by fenozan may represent a potential pathway for neurodevelopmental effects in the fetus following maternal exposure, warranting further investigation to elucidate the link between these two toxicological endpoints (Mughal *et al*., 2018).

Steric bulk of BHT may prevent direct activation of estrogenic and androgenic receptors but *in vitro* data suggest that BHT can bind these receptors, raising concerns about its influence on these pathways (ANSES, 2016b). Therefore, assessing fenozan's ER and AR activity is another important consideration, which can be verified using OECD-validated TA (refer to the paragraph 'Transactivation *in vitro* assays to detect ER and AR agonists and antagonists').

To address other *in silico* alerts identified for fenozan, various bioassays can be conducted to verify specific endpoints—for example, a CTA can be performed to assess potential carcinogenic activity, human and mouse pregnane X receptor reporter gene assay can be conducted to verify pregnane X receptor binding, and human CAR-3 assay can be conducted to verify positive alert for constitutive androstane receptor.

Regarding risk assessment, the TTC approach is not applicable because fenozan exhibits endocrine-disrupting activity (EFSA, 2019b).

Regarding identified four **oligoesters**, all possible structural isomers of these molecules were designed considering monomers specified by the manufacturer in the coating's formulation and those without SMLs in ResAP(2004)) and tested in different QSAR tools.

All oligoesters were predicted to have Log $k_{o/w}$ values above 10, suggesting that these molecules may have a potential for accumulation in the human body, with a Log $k_{o/w}$ of 3 requiring further investigation according to EFSA (EFSA, 2008). Based on the Lipinski's 'rule of 5', only c (2TCDDM+2tPA) may show poor absorption and limited permeability, though experimental data are needed to confirm this (Lipinski *et al*., 2001).

Regarding identified oligoesters, they must be synthesized, a process that is both costly and time-consuming. Once synthesized, they can be individually tested using various bioassays, however, only *in silico* toxicity prediction of these molecules was conducted as they are not commercially available. *In silico* QSAR tools showed negative prediction for mutagenicity but positive prediction for carcinogenicity for the four identified selected oligoesters, suggesting tumor-promoting properties of these chemicals. In addition, positive prediction was obtained for developmental toxicity/ teratogenicity, and endocrine-disrupting activity (aromatase, ER binding activity) for several isomers of the four oligoesters.

No toxicological data were available for the four identified cyclic oligoesters; however, *in silico* analyses indicated an absence of genotoxicity, and extracts and migrates containing these molecules were also negative *in vitro*. This combination of evidence allowed the application of the TTC approach for the risk assessment of these compounds. The four identified cyclic oligoesters were classified as Cramer Class III substances in this study, with estimated exposures around 0.77-0.84 µg/kg bw per day for c(BD+2TCDDM+3tPA), c(3TCDDM+3tPA), c(2TCDDM+2tPA) and 0.05 µg/kg bw per day for c(2BD+TCDDM+3tPA) (calculated for the French population) falling below the TTC value of 1.5 µg/kg b.w./day, indicating no safety concern. It is important to further investigate the potential risks associated with the metabolites of these oligoesters, as metabolic transformation may alter their toxicological profiles.

FCM extracts and migrates often contain numerous NIAS, many of which remaining unidentified due to their unknown chemical nature and presence at very low concentrations—below the detection limits of conventional analytical chemistry tools. Consequently, assessing only the identified NIAS does not ensure complete consumer safety, as unidentified NIAS within these complex mixtures may still pose health risks even at very low concentrations (e.g., genotoxic or endocrine disrupting chemicals). Bioassays provide valuable insights into the presence of unidentified genotoxic NIAS in the mixture that may not be detected or identified by analytical chemistry methods due to their limited capabilities. They allow overall toxicity assessment of complex mixtures like extracts and migrates of FCMs. In addition, bioassays offer several advantages over *in silico* methods, as they take into account the interactions among substances in a mixture and can detect the so-called "cocktail" or "mixture" effects.

For this reason, genotoxicity and potential carcinogenicity of ACN extracts and EtOH 95% migrates were tested using *in vitro* bioassays as a whole approach to study their overall toxicity.

MMT and DDR tests were conducted to assess the genotoxicity of ACN extracts and EtOH 95% migrates of polyester coatings as complex mixtures. Miniaturized assays like MMT require smaller sample quantities, making this assay relevant. Samples were also tested in the DDR test to explore different mutagenic modes of action while measuring various biomarkers associated with clastogenicity, aneuploidy, and genotoxicity. This bioassay demonstrates high sensitivity (92.1%) and specificity (96.2%) (Bryce *et al*., 2017).

All ACN extracts and EtOH 95% migrates were negative in both the MMT and DDR assays, indicating no mutagenicity concerns with the extracts and migrates from polyester-coated cans under tested conditions. Spiking experiments with the MMT and DDR assays were also conducted to ensure that bioassays could respond to known reference substances and identify potential matrix effects that might mask positive results (e.g., due to quenching). No matrix effects were observed in either bioassay for any of the extracts and migrates.

The potential carcinogenicity of ACN extracts and EtOH 95% migrates of polyester coatings was assessed using the *in vitro* CTA to check *in silico* predictions of tumor-promoting activity of the four identified oligomers. Since this bioassay was not routinely used, it has to be first implemented in the PackTox lab, then the first crucial step was also to generate historical data on positive and negative controls. Our data regarding historical controls in PackTox confirmed the bioassays' proper performance. Negative results were obtained in both initiation and promotion assays for all samples in the CTA. These findings suggest that the potential carcinogenic alert predicted with those FCM extracts and migrates was not confirmed regarding a promotion mode of action assessed in the CTA. To our knowledge, this is the first time that the potential carcinogenicity of FCMs extracts and migrates is evaluated using the CTA assay. It has been shown by Ohmori and Kawamura (2009) that two contaminants (abietic and dehydroabietic acids) from virgin and recycled paper products showed no initiation potential, but they exhibited tumor-promoting effects at concentrations of 20–60 nmol/mL. These results indicate that certain contaminants from FCMs may exhibit tumor-promoting activity, underscoring the need for further investigation and toxicological assessment.

The negative responses observed for ACN extracts and EtOH 95% migrates of polyester coatings in the MMT, DDR, and CTA bioassays may be attributed either to the absence of genotoxic or potentially carcinogenic compounds in these extracts or to the presence of such compounds at concentrations too low to elicit a positive response in the applied bioassays. In this context, the biological sensitivity of the bioassays is critical for accurately evaluating the hazard potential of complex mixtures containing NIAS. A negative result does not necessarily imply safety, especially if the substances of concern are present below the detection threshold of the assay. Additionally, factors such as sample dilution during bioassay must be considered. Following extraction and migration tests, it is essential to concentrate the samples prior to testing in order to reflect realistic exposure scenarios associated with chemical migration from food packaging into food.

It is important to emphasize that, while the CTA remains a valuable screening tool for identifying potential carcinogenic chemicals, it cannot capture all modes of action of tumor promoters. This limitation arises because the assay lacks the complexity of the *in vivo* environment, and tumor promotion involves multiple key processes that no single *in vitro* test can fully detect (Smith *et al*., 2016).

One potential approach to address this limitation is to combine CTA with toxicogenomic profiling and epigenetic biomarkers, which can help reveal the underlying mechanisms of carcinogenic substances (Coppedè, 2023; Guichard *et al.*, 2023; Kirsch *et al.*, 2020; Mascolo et *al*., 2018; Pillo *et al*., 2024).

Our results are in compliance with the literature results. The genotoxicity of polyester and other type of coatings has been examined by different authors using multiple *in vitro* bioassays (Ames II-Test, Ames MPF, reversed phases-HPTLC-UV/Vis/FLD-SOS-Umu-C, Ames test (OECD 471), *in vitro* micronucleus assay (OECD 487)). However, the results consistently showed no genotoxic effects under conditions tested (Meyer, 2020 ; Rainer *et al.*, 2019 ; Séverin *et al*., 2016; Mittag *et al*., 2006). It should be noted that this study did not assess the endocrine-disrupting potential of the extracts and migrants. Given that endocrine disruptors may exert effects at very low concentrations, further investigation of this endpoint is essential to ensure consumer safety.

Assessing only the toxicity of identified NIAS or the overall toxicity of FCMs is not sufficient to ensure consumer safety, as ingested food—and any migrating substances—undergoes biochemical transformations in the gastrointestinal tract due to exposure to various digestive enzymes and conditions. Hydrolysis and metabolism can occur following oral ingestion, potentially breaking down the parent compounds into metabolites with different absorption properties and toxicological profiles. Therefore, it is essential to investigate the gastrointestinal fate of identified NIAS (i.e., parent compounds) and evaluate the toxicological profiles of any secondary metabolites. For this reason, the four identified polyesters oligomers were also studied in terms of stability in the gastrointestinal tract. To investigate the gastrointestinal fate of four identified cyclic oligoesters, another ACN extraction was conducted by grating polyester coatings. This new extraction aimed to identify new NIAS not identified previously and track their gastrointestinal fate. In total 18 parent compounds, TCDDM, MA, IPDI trimer, and another starting substance were identified in ACN extract. Then, the ACN extract subjected to *in vitro* intestinal digestion with an intestinal-fluid simulant and 18 identified parent compounds and their potential degradation products were monitored using LC/ESI-HRMS.

Monitoring revealed that some parent oligoesters based on TCDDM and PA resisted pancreatin, while others containing BD-PA and TCDDM-AA ester bonds were hydrolyzed, producing eight linear oligoesters. Degradation increased with pancreatin, though smaller products could not be confirmed due to chromatographic limits. The study suggests partial oligoester degradation into linear oligoesters, raising questions about the absorption of degradation products through the intestinal barrier.

Our results are in line with the literature results. For example, Eckardt *et al*. (2019) found that three cyclic polyester oligomers hydrolyzed (≥75%) into linear forms within an hour of *in vitro* digestion, though complete degradation to monomers was not observed. Ubeda *et al*. (2020) showed that cyclic polyurethane oligoesters decreased in concentration during digestion as hydrolyzed molecules increased.

These degradation products may have completely different absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiles compared to their parent compounds. For example, cyclic oligoesters have been shown to hydrolyze into their linear forms, which are considered less toxic, as some are classified under Cramer Class I, while cyclic oligoesters

are categorized under the more toxic Cramer Class III (Lestido-Cardama *et al*., 2022b; Eckardt *et al*., 2020, 2019).

Further investigation with additional replicates is necessary to fully understand the gastrointestinal fate of these oligoesters.

In addition, if confirmed, further toxicological analysis is needed to be performed on degradation products of parent compounds, however, lack of commercial standards makes *in vitro* or *in vivo* toxicity evaluation challenging.

In this study polyester coatings extracts and migrates were all negative in the MMT and DDR tests. Given that NIAS are present in FCMs extracts or migrate at very low concentrations, it can be assumed that one of possible reasons of the negative results is the limited biological sensitivity of the assay. GenEvolution laboratory has developed a miniaturized version of the regulatory genotoxicity Ames test, known as the NanoAmes test, to address this issue.

That is why this thesis also includes an inter-laboratory pre-validation of the NanoAmes test. Then, GenEvolution and Packtox tested 14 genotoxic and non-genotoxic chemicals, including solvent controls, with and without metabolic activation (S9 mix), on *S. typhimurium* TA98 and TA100 strains to evaluate the bioassay's repeatability and reproducibility. This bioassay showed consistent negative control results across both labs.

For most chemicals, similar results were observed in both laboratories across two strains. However, chemicals like AFB1 and DMBA unexpectedly produced positive results without S9 in both strains at the Packtox lab, one explanation could be possible metabolism into genotoxic compounds during pre-incubation or instability of the substance under the experimental conditions. Preliminary data are promising as the NanoAmes test showed lower LECs compared to the standard Ames and Ames MPF tests for all chemicals, except for AFB1/+S9, indicating a higher biological sensitivity. However, for both strains, repeatability issues were observed across both laboratories, particularly at PackTox. In the TA98 strain, repeatability problems were notable with ENU, DMBA, and AFB1, while for TA100, issues were related to SA, MMS, ENU, DMBA, and AFB1. These inconsistencies may come from bacterial culture and its concentration, S9 batch variability, equipment differences, and operator variability.

It is important to note that to date researchers are working on the development of more sensitive genotoxicity tests to detect genotoxic substances in FCMs extract or migrate. For instance, a HPTLC-SOS-Umu-C assay has been developed using a *Salmonella* strain to identify genotoxic chemicals in food packaging. This assay has demonstrated very high biological sensitivity to standard tested mutagens. In addition, seven out of ten genotoxic chemicals could be detected using this assay at the regulatory limit of 10 ppb, and two out of ten could be identified at the TTC level for adults. The authors emphasize that with further development, HPTLC-based bioassays could become a leading tool in the safety assessment of genotoxic chemicals in food packaging (Bergmann *et al*., 2023; Debon *et al*., 2022; Meyer, 2020).

V. CONCLUSION

Migration tests using food simulant EtOH 95% and extractions with ACN were conducted on polyester coatings in migration cells. Chemical analysis of samples revealed the presence of four cyclic oligoesters (identified with high confidence level 2) and fenozan (confirmed with a reference standard), a well-known degradation product of antioxidants.

No significant differences were observed regarding the quantity of oligoesters and fenozan in different solvents.

The concentration (semi-quantification) of c(BD+2TCDDM+3tPA), c(3TCDDM+3tPA), c(2TCDDM+2tPA) in different metal sheets and in both solvents was in a range of 45.4–85.0 µg/dm^2 (272.4–510 µg/kg food) and for c(2BD+TCDDM+3tPA) around 3.1–6.1 µg/dm^2 (18.6–36.6 µg/kg food).

The toxicity prediction using QSAR tools of 147 structural isomers of the four oligoesters were negative for mutagenicity but positive for carcinogenicity. In addition, positive prediction was obtained for developmental toxicity/ teratogenicity, and endocrine-disrupting activity (aromatase, ER binding activity) for several isomers of four oligoesters.

The concentration of fenozan was around 0.2 µg/dm^2 (1.2 µg/kg food) after a rough semi-quantification. QSAR tools predicted some toxicities of fenozan for mutagenicity, carcinogenicity, endocrine disrupting activity and organ toxicity. Fenozan was not genotoxic in the Ames test (*S. typhimurium* TA 98, TA 100, TA 1535, and *E. coli* WP2 strains) nor clastogenic or aneugenic in the micronucleus assay using HepG2 cells.

In contrast, fenozan inhibited the iodide uptake assay using rat thyroid FRTL-5 cells (IC50 of 1152 µM) suggesting a potential anti-thyroid activity *in vitro.*

Polyester coating's extracts and migrates (n=10) were also tested in different *in vitro* bioassays as a global screening approach to test overall toxicity of these complex mixtures. All samples were negative regarding genotoxicity in the MMT and DDR test.

The CTA was implemented in the Packtox laboratory, and historical values were established to demonstrate the performance of the bioassays. Extracts and migrates of polyesters coatings were negative in CTA using Bhas 42 cells in both initiation and promotion assays indicating the absence of carcinogenicity under tested conditions.

To follow gastrointestinal hydrolysis of other NIAS, a new ACN extraction was conducted by grating the polyester coatings to extract as much as possible NIAS. In total, 18 parent

compounds (9 cyclic and 5 linear oligoesters, and some monomers) were identified (in 50 times concentrated extract) using LC/ESI-HRMS.

In vitro digestion assay was conducted using intestinal fluid on polyester coatings ACN extracts to investigate gastrointestinal fate of NIAS from polyester coatings.

Data revealed that some parent oligoesters based on TCDDM and PA are resistant to pancreatin, while others containing BD-PA and TCDDM-AA ester bonds degrade into linear oligoesters. Eight linear oligoesters were identified as degradation products, though smaller products were undetectable due to chromatographic limitations.

An inter-laboratory pre-validation of the NanoAmes test, a miniaturized version of the regulatory genotoxicity Ames test developed by GenEvolution laboratory was performed. Fourteen Ames-positive and -negative chemicals were tested by GenEvolution and Packtox laboratories, with and without the metabolic activation system (S9 mix), on *S. typhimurium* TA98 and TA100 strains to assess repeatability and reproducibility of this bioassay.

Results were consistent between laboratories for negative controls and for most of the positive chemicals in both strains. However, some discrepancies were observed with certain chemicals.

In addition, issues with repeatability and reproducibility were observed between experiments conducted within and across laboratories.

A dose-response relationship was observed for only some positive controls (e.g., 2NF, 4NQO, BaP) in both strains at the Packtox laboratory. LECs of positive controls were lower in the NanoAmes compared with the Ames test and Ames MFP for most of them (except for AFB1/+S9).

VI. PERSPECTIVES

This thesis addresses multiple aspects related to the safety assessment of polyester coatings. It offers a comprehensive analysis of NIAS identification and characterization from polyester coatings. However, further research is still necessary to fully understand the safety of the polyester coatings evaluated in this study. The key areas of focus are presented hereafter.

- To comprehensively assess the overall toxicity of polyester coating extracts and migrates, endocrine-disrupting activity (e.g., ER and AR activity, steroidogenesis interference, aromatase activity) of these mixtures need to be verified. If positive results are obtained, the extracts and migrates can be fractionated and tested again to identify the substances of concern.
- To optimize analytical parameters to improve NIAS identification: in LC, changing the column or mobile phase composition can explore additional chemical space, while in GC, less energetic ionization methods like APCI preserve the molecular ion and simplify signal annotation.
- To apply QSAR modeling approaches to predict the toxicity of chemical mixtures, as demonstrated by Belfield *et al*. (2023), enabling a more comprehensive assessment of potential combined effects.
- To perform docking studies to better understand the mechanism of action (but the chemical structure should be known) (Belfield *et al*., 2023).
- To further investigate the toxicity of the four identified cyclic oligoesters that have migrated from polyester coating extracts and migrates hydrolysis can be performed to identify their exact monomer composition. To date, there remains a need for the development of methods for monomer analysis. One promising approach is pyrolysis-GC-MS, in which a dry extract is heated in the injector in the presence of reactants. The resulting pyrolysis products (monomers) are readily derivatized within the injector and subsequently analyzed by GC-MS.
- For the toxicity assessment of fenozan, it is recommended to test this chemical on various bioassays to verify *in silico* alerts obtained for other toxicological endpoints (carcinogenicity, developmental toxicity, endocrine disruption mode of action, and organ toxicity), for example, the TPO assay can be used to study thyroid disruption, while the CTA can be applied to assess the potential carcinogenicity of these molecules. In addition, performing the *in vivo* LAGDA would be relevant to study the

potential consequences of sodium/iodide symporter (NIS1) inhibition by fenozan and its involvement in thyroid toxicity (OECD, 2015).

- For *in vitro* digestion assays of polyester coating ACN extracts, it is essential to include at least three replicates to ensure data reliability. Additionally, predicting the toxicity of parent molecules and their degradation products using *in silico* QSAR tools could be performed. An exposure assessment for degradation products could be conducted, and, if possible, a risk assessment as well. In addition to intestinal enzymes, gastric and salivary enzymes could be tested. An absorption study across the intestinal barrier can also be performed.
- For the inter-laboratory validation of the NanoAmes test, other OECD-recommended strains to complete the required battery of strains to detect all genotoxic chemicals need to be investigated. Further investigation into the parameters causing variations is essential to improve the bioassay's repeatability and reproducibility. Statistical analysis of the results is also necessary to improve data interpretation. Including additional laboratories in the validation process is highly recommended to enhance study reliability.
- Future studies should challenge the NanoAmes test regarding its reliability to evaluate genotoxicity of FCMs extracts and migrates. The focus could shift towards testing the genotoxicity of various polyester coating formulations in order to retain the highest safe formulation.

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ANNEX

Annex 1

Appendix A. Supplementary data of the article "Identification of non-volatile non-intentionally added substances from polyester food contact coatings and genotoxicity assessment of polyester coating's migrates" (included in part III. RESULTS- Chapter 2)

Supplementary information

MATERIALS AND METHODS

Chromatographic separation

Data acquisition was performed on an Orbitrap Q Exactive instrument fitted with a heated electrospray source (Thermo Fisher Scientific, San José, CA, USA) according to Cariou *et al*. (2022). Briefly, chromatographic separation was performed on a C18-like analytical column (100 mm × 2.1 mm, 1.9 µm) fitted with a pre-column (10 mm × 2.1 mm, 3 µm) (Hypersil Gold, Thermo Fisher Scientific) and maintained at 40 °C. The mobile phase was composed of 10 mM ammonium acetate and 20 µM sodium acetate in both water (A) and ACN/water 99:1 (*v/v*, B). The gradient began with (A/B) 80:20 (*v/v*, 1 min), ramped linearly to 0:100 over 16 min, to be maintained for 9 min before reverting in 2 min to initial conditions. The flow rate was set at 0.4 mL.min-1. The ionization parameters were as follows: sheath gas flow, 50 arbitrary units (AU); auxiliary gas flow, 10 AU; capillary temperature, 350 °C; heater temperature, 300 °C; spray voltage, 3.5 kV; S-lens radio frequency, 70 AU.

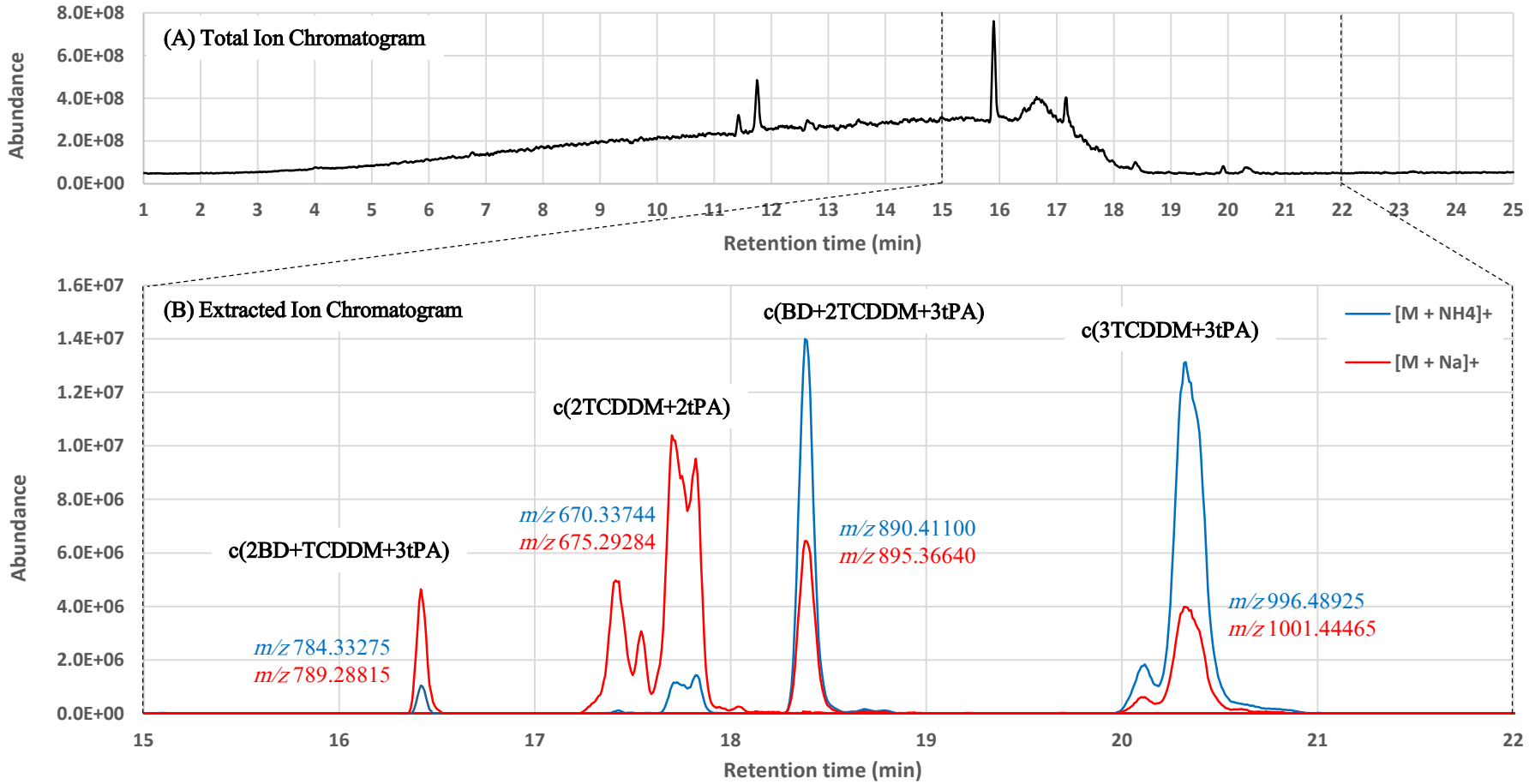

Figure S1. Representative total ion chromatogram (TIC, A) and overlaid extracted ion chromatograms (EICs, ±3 ppm, B) obtained for an ACN extract by LC-ESI(+)-HRMS.

Table S1. Results of MMT for spiked ACN and EtOH 95% migrates tested on TA100 strain using pre-incubation method.

Sample	Without S9			With S9		
	Pre-incubation/ Spiked			Pre-incubation/ Spiked		
	Mean of revertants[1]	SR (%) [2]	SR (%) [3]	Mean of revertants	SR (%)[2]	SR (%)[3]
DMSO						
ACN	56.00±6.75					
EtOH 95%	65.67±5.67					
AB	62.33±6.42	110	**100**	Cytotox.		
A0	62.33±6.98	110	100	Cytotox.		
A1	68.33±1.63	120.59	109.63	Cytotox.		
A2	73.67±6.98	130	118.18	Cytotox.		
A3	65.00±4.64	114.71	104.28	Cytotox.		
A4	66±2.45	116.47	105.88	Cytotox.		
A5	59.33±4.49	104.71	95.19	Cytotox.		
EB	69±6.36	121.76	**100**	Cytotox.		
E0	58.67±8.20	103.53	85.02	Cytotox.		
E1	65.67±10.21	115.88	95.17	Cytotox.		
E2	82.67±8.26	145.88	119.81	Cytotox.		
E3	68.00±7.07	120.00	98.55	Cytotox.		
E4	79.67±9.23	140.59	115.46	Cytotox.		
E5	74.33±2.16	131.18	107.73	Cytotox.		
SA	56.67±2.48	**100**		Cytotox.		
2AA	109±9.42			Cytotox		

[1]The means of revertants and standard deviation calculated for three wells.

[2]SR: spike recovery based on positive control: the mean of revertant colonies in the sample wells was compared to the mean of revertant colonies in positive control wells (2NF for the assay without S9 mix, and 2AA for the assay with S9 mix).

[3]SR: spike recovery based on spiked solvent blank: the mean of revertant colonies in the sample wells was compared to the mean of revertant colonies in spiked solvent blank (spiked AB for ACN migrates, and spiked EB for EtOH 95% migrates.

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Annex 2

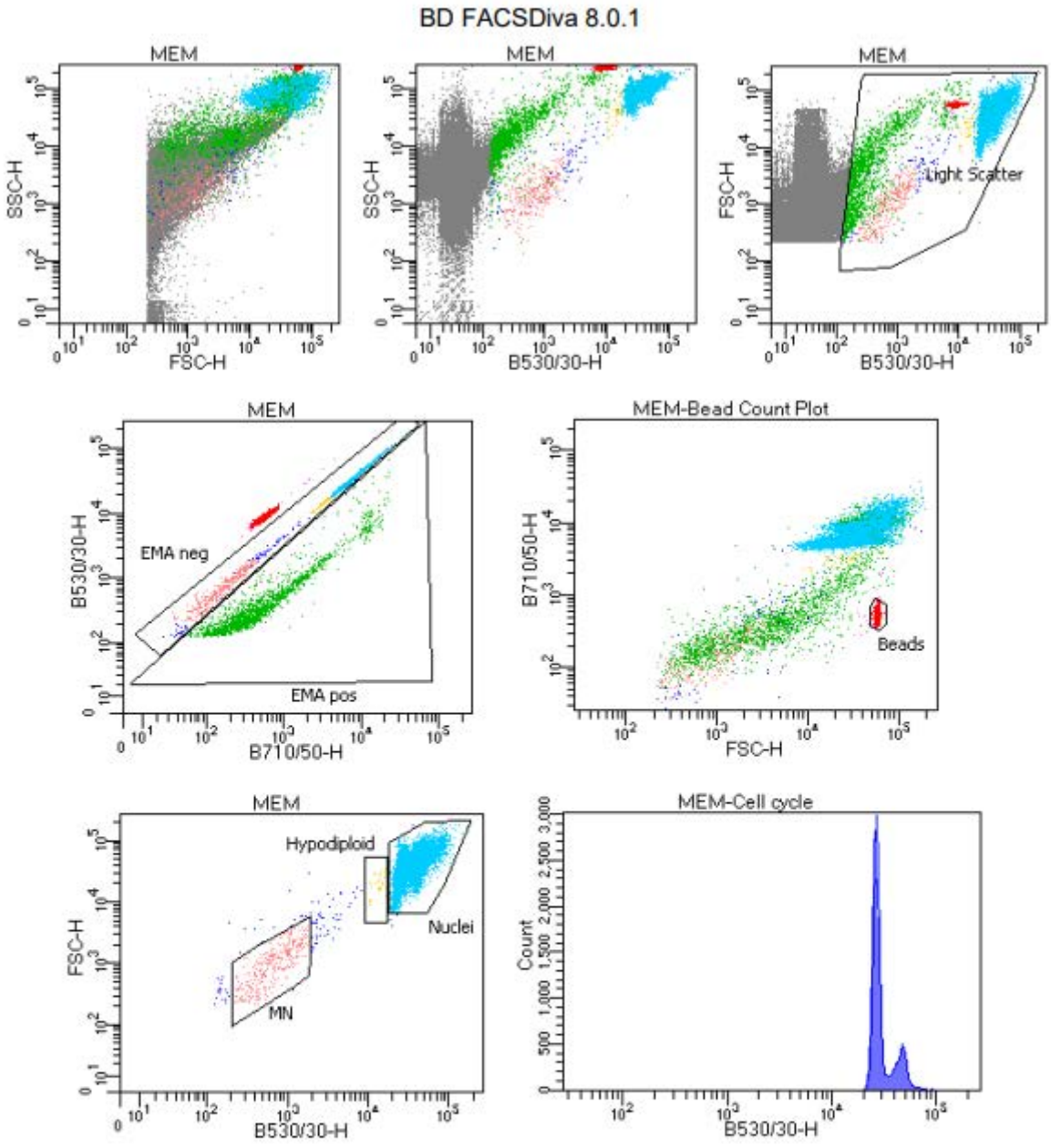
Supplementary data of the article "Hazard assessment of fenozan, a released non intentionally added substance from polyester based can coating" (included in part III. RESULTS- Chapter 3)

Table S1. Summary of tR, intensities and the NIST research results for the 23 *unknowns*.

Name	tR	AB	A0	A1-A5	EB	E0	E1-E5	Monoisotopic mass	Molecular formula	NIST search	Match score
Unknown 79	24,46	0	0	1,4E+09	0	7,6E+06	2,8E+09	382,3236	$C_{27}H_{42}O$		47,34
Unknown 80	21,39	0	0	7,2E+08	2,7E+06	1,8E+09	4,6E+08	360,1573	$C_{20}H_{24}O_6$		67,25
Unknown 87	12,75	0	0	5,3E+07	4,6E+07	1,6E+07	4,0E+08	286,1933	$C_{19}H_{26}O_2$		43,55
Unknown 89	12,98	0	0	4,5E+07	3,0E+07	0	3,3E+08	238,0841	$C_{12}H_{14}O_5$		47,41
Unknown 36	18,63	4,5E+07	2,0E+08	3,9E+08	0	6,6E+08	3,1E+08	316,2250	$C_{17}H_{32}O_5$		10,78
Unknown 71	14,66	0	2,6E+07	1,1E+08	0	0	2,7E+08	278,1882	$C_{17}H_{26}O_3$	3,5-di-tert- Butyl- 4 -hydroxyphenyl propionic acid	86,02
Unknown 81	28,41	0	0	4,2E+08	0	0	1,7E+08	180,0423	$C_9H_8O_4$		8,02
Unknown 84	22,08	0	0	9,5E+07	0	0	1,3E+08	384,3392	$C_{27}H_{44}O$	Cholesta-4,6-dien-3-ol, (3á)	89,49
Unknown 97	16,63	0	0	2,2E+07	0	7,2E+07	1,3E+08	213,0790	$C_{13}H_{11}NO_2$		18,25
Unknown 82	17,67	0	0	1,8E+08	0	0	1,3E+08	284,0119	$C_{12}H_{10}Cl_2N_2O_2$		29,50
Unknown 83	14,44	0	0	1,6E+08	0	0	1,2E+08	256,2402	$C_{16}H_{32}O_2$		60,30
Unknown 86	10,41	0	0	8,3E+07	1,1E+08	1,3E+08	1,1E+08	149,0477	$C_8H_7NO_2$		39,74
Unknown 54	23,79	3,2E+06	0	4,8E+07	1,9E+06	9,0E+06	6,6E+07	386,3185	$C_{26}H_{42}O_2$		39,58
Unknown 85	16,39	0	0	9,2E+07	0	0	4,1E+07	284,2715	$C_{18}H_{36}O_2$		39,39

	12,01	0	0	2,9E+07	0	1,5E+07	3,2E+07	321,9122		4,4'-Dihydroxy-2,2',6,6'-tetrachlorobiphenyl	86,07
Unknown 93									$C_{12}H_6Cl_4O_2$		
Unknown 88	19,08	0	0	5,2E+07	1,1E+06	4,6E+08	2,2E+07	354,1912	$C_{17}H_{36}Cl_2OSi$		37,54
Unknown 95	22,22	0	0	2,6E+07	0	0	1,9E+07	48,3108	$C_{28}H_{45}ClO_2$		42,87
Unknown 91	26,51	0	0	3,7E+07	0	0	1,8E+07	400,3341	$C_{27}H_{44}O_2$		52,33
Unknown 110	7,66	0	0	2,9E+06	8,4E+05	0	1,7E+07	346,2144	$C_{21}H_{30}O_4$		43,08
Unknown 99	26,29	0	0	1,4E+07	0	0	7,5E+06	268,0922	$C_{17}H_{16}OS$		9,79
Unknown 100	16,97	0	0	1,1E+07	0	0	4,8E+06	362,2457	$C_{22}H_{34}O_4$		16,16
Unknown 92	11,90	0	0	3,3E+07	2,9E+07	2,5E+07	0	222,0892	$C_{12}H_{14}O_4$		18,49
Unknown 101	12,37	0	0	8,3E+06	8,7E+06	1,1E+07	0	219,1120	$C_{10}H_{13}N_5O$		12,39

Figure S1. Gating strategy of *in vitro* micronucleus assay



Tube: MEM

Population	#Events	%Parent	%Total
All Events	493,773	####	100.0
Light Scatter	24,251	4.9	4.9
EMA neg	20,000	82.5	4.1
MN	327	1.6	0.1
Nuclei	19,549	97.7	4.0
Hypodiploid	34	0.2	0.0
EMA pos	1,814	7.5	0.4
Beads	2,412	9.9	0.5

Annex 3

Identification information obtained for each chemical belonging to the CA_{mix} extract and listed in Table 3-17 (included in the Part III. Results- Chapter 3)

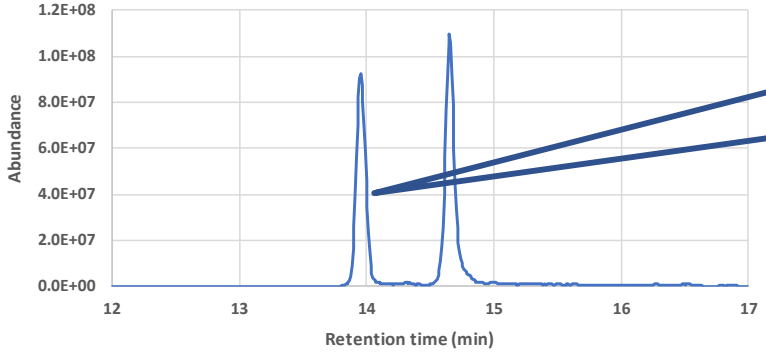
IDENTIFICATION			
PC-group	N/A		
Category	Oligoester	**Substance**	**c(BD+TCDDM+2PA)**
Chemical formula	$C_{32}H_{34}O_8$	**Monoisotopic mass**	546.2254
Apex t_R (min)	13.95	**Δppm**	+0,01
Deduced neutral mass	546.2254	**ΔmDa**	+0
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 564.2592

Annotated adduct ion $[M+NH_4]^+$

Most intense coating



Observed mass spectrum

m/z	Relative area (%)	Annotated ion
547.2327	18%	$[M+H]^+$
564.2592	100%	$[M+NH_4]^+$
569.2146	59%	$[M+Na]^+$
585.1885	1%	$[M+K]^+$
-	-	$[M+NH_4+ACN]$

Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

ONE-DIMENTIONAL DATA

positive mode	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
564.26	-	2	-	**Precursor $[M+NH4]^+$**		
547.2328	27	-	-	$[M+H]^+$		
387.1101	-	1	-	$[C_{20}H_{19}O_8]^+$	BD+2PA fragment	
161.1326	100	100	100	$[C_{12}H_{17}]^+$	TCDDM fragment	
159.1167	-	-	3	$[C_{12}H_{15}]^+$	TCDDM fragment	
133.1014	-	21	60	$[C_{10}H_{13}]^+$	TCDDM fragment	
119.0859	-	10	41	$[C_9H_{11}]^+$	TCDDM fragment	
105.0704	1	11	41	$[C_8H_9]^+$	TCDDM fragment	
95.0861	-	2	47	$[C_7H_{11}]^+$		
93.0705	-	1	19	$[C_7H_9]^+$		
91.0548	-	6	52	$[C_7H_7]^+$	Tropilium cation	
81.0706	1	18	61	$[C_6H_9]^+$		
79.0550	-	3	17	$[C_6H_7]^+$		
67.0551	-	1	18	$[C_5H_7]^+$		
59.0613	1	1	3	$[NH_4+ACN]^+$		

IDENTIFICATION

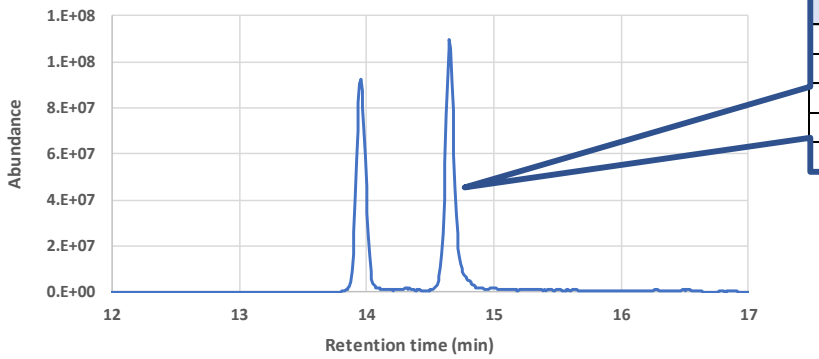
PC-group	N/A		
Category	Oligoester	**Substance**	**c(BD+TCDDM+2PA)**
Chemical formula	$C_{32}H_{34}O_8$	**Monoisotopic mass**	546.2254
Apex t_R (min)	14.64	**Δppm**	+0,01
Deduced neutral mass	546.2254	**ΔmDa**	+0
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 564.2592

Annotated adduct ion $[M+NH_4]^+$

Most intense coating



Observed mass spectrum

m/z	Relative area (%)	Annotated ion
547.2327	9%	$[M+H]^+$
564.2592	100%	$[M+NH_4]^+$
569.2146	63%	$[M+Na]^+$
585.1885	1%	$[M+K]^+$
-	-	$[M+NH_4+ACN]$

Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

ONE-DIMENTIONAL DATA

positive mode	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
564.26	28	-	-	**Precursor $[M+NH4]^+$**		
547.2329	68	-	-	$[M+H]^+$		
493.1861	2	-	-	$[C_{28}H_{29}O_8]^+$	BD loss	
387.1078	5	6	-	$[C_{20}H_{19}O_8]^+$	TCDDM loss	
221.0810	0	1	-	$[C_{12}H_{13}O_4]^+$	BD+PA fragment	
161.1326	100	100	100	$[C_{12}H_{17}]^+$	TCDDM fragment	
159.1170	0	2	5	$[C_{12}H_{15}]^+$	TCDDM fragment	
149.0235	-	1	40	$[C_8H_5O_3]^+$	Phthalic anhydride ion	
133.1013	2	24	86	$[C_{10}H_{13}]^+$	TCDDM fragment	
131.0857	-	-	3	$[C_{10}H_{11}]^+$		
119.0859	1	13	74	$[C_9H_{11}]^+$	TCDDM fragment	
107.0860	-	0	4	$[C_8H_{11}]^+$		
105.0704	1	12	76	$[C_8H_9]^+$	TCDDM fragment	
95.0861	-	4	49	$[C_7H_{11}]^+$		
93.0705	-	2	25	$[C_7H_9]^+$		
91.0549	-	6	88	$[C_7H_7]^+$	Tropilium cation	
83.0863	-	1	3	$[C_6H_{11}]^+$		
81.0706	-	9	75	$[C_6H_9]^+$		
79.0550	-	5	62	$[C_6H_7]^+$		
67.0551	-	3	41	$[C_5H_7]^+$		

IDENTIFICATION

PC-group	N/A		
Category	Oligoester	**Substance**	**c(2TCDDM+2PA)**
Chemical formula	$C_{40}H_{44}O_8$	**Monoisotopic mass**	652.3036
Apex t_R (min)	17.03	**Δppm**	+0,21
Deduced neutral mass	652.3038	**ΔmDa**	+0,14
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 670.3376

Annotated adduct ion $[M+NH_4]^+$

Most intense coating

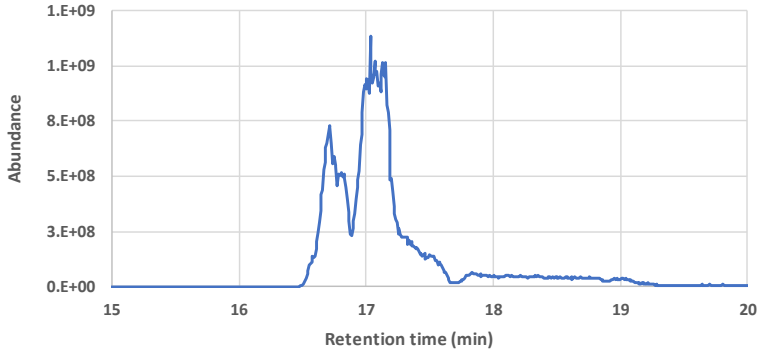


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
653.30977	11%	$[M+H]^+$
670.33758	100%	$[M+NH_4]^+$
675.29127	5%	$[M+Na]^+$
-	-	$[M+K]^+$
-	-	$[M+NH_4+ACN]^+$
698.36622	2%	?

negative mode

669.3075	8%	$[M+H_2O-H]^-$
711.3175	100%	$[M+CH_3COOH-H]^-$

ONE-DIMENTIONAL DATA

positive mode

m/z	Relative area (%) NCE 10%	NCE 30%	NCE 50%	Ion formula	Annotation	*Comment*
670.34	29			**Precursor $[M+NH_4]^+$**		
653.3109	72			$[M+H]^+$		
493.1861	6	1		$[C_{28}H_{29}O_8]^+$	2TCDDM+PA loss	
161.1325	100	100	100	$[C_{12}H_{17}]^+$	TCDDM fragment	
149.0235		0	11	$[C_8H_5O_3]^+$	Phthalic anhydride ion	
133.1013	2	23	77	$[C_{10}H_{13}]^+$	TCDDM fragment	
119.0859	1	12	63	$[C_9H_{11}]^+$	TCDDM fragment	
105.0704	1	11	62	$[C_8H_9]^+$	TCDDM fragment	
95.0861		4	44	$[C_7H_{11}]^+$		
93.0705		2	21	$[C_7H_9]^+$		
91.0548	0	6	76	$[C_7H_7]^+$	Tropilium cation	
81.0706	1	10	68	$[C_6H_9]^+$		
79.0550		5	52	$[C_6H_7]^+$		
67.0551		3	34	$[C_5H_7]^+$		

IDENTIFICATION			
PC-group	N/A	*Analytical standard synthesised within project ANR OLIGO in 2023*	
Category	Oligoester	**Substance**	**Std_c(2TCDDM+2iPA)**
Chemical formula	$C_{40}H_{44}O_8$	**Monoisotopic mass**	652.3036
Apex t_R (min)	17.80	**Δppm**	-0.11
Deduced neutral mass	652.3035	**ΔmDa**	-0.07
Confidence level	1		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 670.3375

Annotated adduct ion $[M+NH_4]^+$

Most intense coating

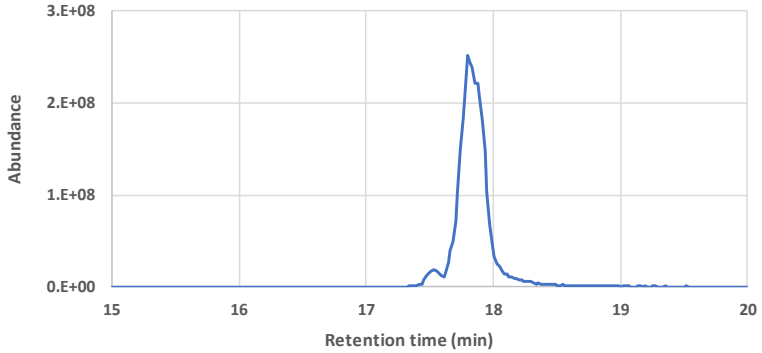


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
653.31027	3%	$[M+H]^+$
670.33737	100%	$[M+NH_4]^+$
675.29262	15%	$[M+Na]^+$
-	-	$[M+K]^+$
711.36392	16%	$[M+NH_4+ACN]^+$

ONE-DIMENTIONAL DATA

positive mode

	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
670.3374	100	-	-	**Precursor $[M+NH_4]^+$**		
653.3109	89	-	-	$[M+H]^+$		
493.1861	3	-	-	$[C_{28}H_{29}O_8]^+$	TCDDM+2iPA fragment	
161.1325	51	100	100	$[C_{12}H_{17}]^+$	TCDDM fragment	
159.1169	-	1	3	$[C_{12}H_{15}]^+$	TCDDM fragment	
149.0235	-	1	6	$[C_8H_5O_3]^+$	Phthalic anhydride ion	
133.1013	1	21	77	$[C_{10}H_{13}]^+$	TCDDM fragment	
119.0858	0	10	61	$[C_9H_{11}]^+$	TCDDM fragment	
107.0860	-	0	3	$[C_8H_{11}]^+$		
105.0704	0	10	59	$[C_8H_9]^+$	TCDDM fragment	
95.0861	-	3	38	$[C_7H_{11}]^+$		
93.0705	-	2	18	$[C_7H_9]^+$		
91.0548	-	5	71	$[C_7H_7]^+$	Tropilium cation	
81.0706	0	9	66	$[C_6H_9]^+$		
79.0550	-	3	47	$[C_6H_7]^+$		
67.0551	-	2	28	$[C_5H_7]^+$		

IDENTIFICATION

PC-group	N/A		
Category	Oligoester	**Substance**	**c(TCDDM+2BD+3PA)**
Chemical formula	$C_{44}H_{46}O_{12}$	**Monoisotopic mass**	766.2989
Apex t_R (min)	15.86	**Δppm**	-0.20
Deduced neutral mass	766.298774	**ΔmDa**	-0.16
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 784.3326

Annotated adduct ion $[M+NH4]^+$

Most intense coating

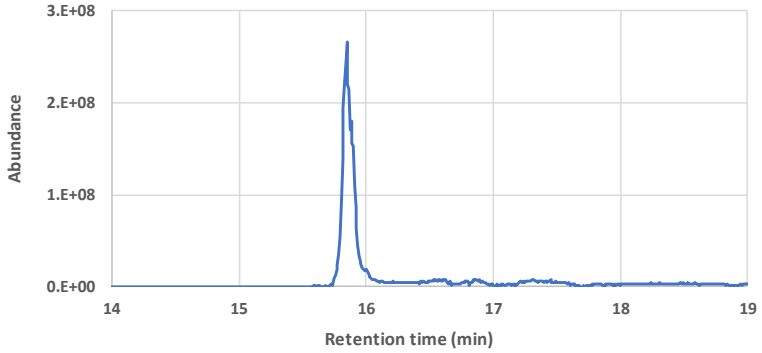


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
767.3067	1%	$[M+H]^+$
784.3326	100%	$[M+NH_4]^+$
789.2882	25%	$[M+Na]^+$
-	-	$[M+K]^+$
825.3585	3%	$[M+NH_4+ACN]$

ONE-DIMENTIONAL DATA

positive mode

	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
784.33	100	-	-	**Precursor $[M+NH_4]^+$**		
767.3061	87	-	-	$[M+H]^+$		
749.2962	0	-	-	$[C_{44}H_{45}O_{11}]^+$	H_2O loss	
695.2494	1	-	-	$[C_{40}H_{39}O_{11}]^+$	BD loss	
607.1811	3	-	-	$[C_{32}H_{31}O_{12}]^+$	TCDDM loss	
589.1709	4	-	-	$[C_{32}H_{29}O_{11}]^+$	TCDDM loss	
529.2227	2	-	-	$[C_{32}H_{33}O_7]^+$	TCDDM+BD+2PA fragment	
441.1551	0	-	-	$[C_{24}H_{25}O_8]^+$	2BD+2PA fragment	
387.1078	1	7	-	$[C_{20}H_{19}O_8]^+$	BD+2PA fragment	
161.1326	79	100	86	$[C_7H_{11}]^+$	TCDDM fragment	
159.1170	2	4	7	$[C_{12}H_{15}]^+$	TCDDM fragment	
149.0235	5	25	86	$[C_8H_5O_3]^+$	Phthalic anhydride ion	
133.1014	2	30	78	$[C_{10}H_{13}]^+$	TCDDM fragment	
131.0857	-	0	4	$[C_{10}H_{11}]^+$		
119.0859	1	17	70	$[C_9H_{11}]^+$	TCDDM fragment	
105.0704	1	15	68	$[C_8H_9]^+$	TCDDM fragment	
95.0861	-	5	46	$[C_7H_{11}]^+$		
93.0705	-	3	24	$[C_7H_9]^+$		
91.0549	-	10	100	$[C_7H_7]^+$	Tropilium cation	
81.0706	1	17	81	$[C_6H_9]^+$		
79.0550	-	7	54	$[C_6H_7]^+$		
67.0551	-	4	36	$[C_5H_7]^+$		

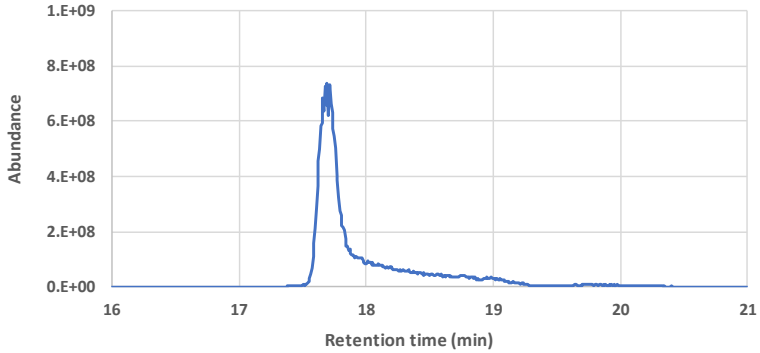
IDENTIFICATION			
PC-group	N/A		
Category	Oligoester	**Substance**	**c(BD+2TCDDM+3PA)**
Chemical formula	$C_{52}H_{56}O_{12}$	**Monoisotopic mass**	872.3772
Apex t_R (min)	17.70	**Δppm**	-2.43
Deduced neutral mass	872.3751	**ΔmDa**	-2.12
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 890.4089

Annotated adduct ion $[M+NH_4]^+$

Most intense coating



Observed mass spectrum

m/z	Relative area (%)	Annotated ion
-	-	$[M+H]^+$
890.4089	100%	$[M+NH_4]^+$
895.3664	0.6%	$[M+Na]^+$
-	-	$[M+K]^+$
931.4371	0.5%	$[M+NH_4+ACN]^+$

Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

ONE-DIMENTIONAL DATA

positive mode

	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
890.41	100	-	-	**Precursor $[M+NH_4]^+$**		
873.3840	73	-	-	$[M+H]^+$		
713.2591	4	-	-	$[M+C_{12}H_{17}]^+$	TCDDM loss	
387.1075	1	3	-	$[C_{20}H_{19}O_8]^+$	BD+2PA fragment	
161.1325	74	100	100	$[C_{12}H_{17}]^+$	TCDDM fragment	
159.1169	2	4	8	$[C_{12}H_{15}]^+$	TCDDM fragment	
149.0234	1	5	29	$[C_8H_5O_3]^+$	Phthalic anhydride ion	
133.1013	2	27	82	$[C_{10}H_{13}]^+$	TCDDM fragment	
119.0858	1	15	70	$[C_9H_{11}]^+$	TCDDM fragment	
105.0703	1	14	70	$[C_8H_9]^+$	TCDDM fragment	
95.0861	-	5	47	$[C_7H_{11}]^+$		
93.0705	-	3	23	$[C_7H_9]^+$		
91.0548	-	9	95	$[C_7H_7]^+$	Tropilium cation	
81.0706	1	15	81	$[C_6H_9]^+$		
79.0549	-	6	54	$[C_6H_7]^+$		
67.0551	-	3	33	$[C_5H_7]^+$		

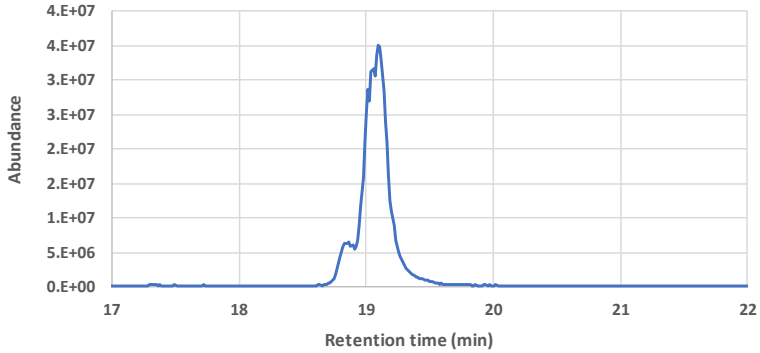
IDENTIFICATION			
PC-group	N/A		
Category	Oligoester	**Substance**	**c(3TCDDM+3PA)**
Chemical formula	$C_{60}H_{66}O_{12}$	**Monoisotopic mass**	978.4554
Apex t_R (min)	19.10	**Δppm**	+0,37
Deduced neutral mass	978.4558	**ΔmDa**	+0,36
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 996.4896

Annotated adduct ion $[M+NH_4]^+$

Most intense coating



Observed mass spectrum

m/z	Relative area (%)	Annotated ion
-	-	$[M+H]^+$
996.4896	100%	$[M+NH_4]^+$
-	-	$[M+Na]^+$
-	-	$[M+K]^+$
-	-	$[M+NH_4+ACN]^+$

Figure Total ion chromatogram obtained for the MS2 experiment at 10% NCE.

ONE-DIMENTIONAL DATA

positive mode

	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
996.49	100	-	-	Precursor $[M+NH_4]^+$		
979.4624	80	-	-	$[M+H]^+$		
961.4522	1	-	-	$[C_{60}H_{65}O_{11}]^+$	H_2O loss	
819.3375	6	-	-	$[C_{28}H_{29}O_8]^+$	TCDDM loss	
801.3271	6	-	-	$[C_{28}H_{29}O_8]^+$	TCDDM loss	
653.3112	1	-	-	$[C_{20}H_{24}O_5]^+$	2TCDDM+2PA fragment	
635.3005	2	0	-	$[C_{20}H_{22}O_4]^+$	2TCDDM+2PA fragment	
493.1861	2	1	-	$[C_{28}H_{29}O_8]^+$	2TCDDM+PA loss	
161.1326	62	100	100	$[C_{12}H_{17}]^+$	TCDDM fragment	
159.1170	2	4	7	$[C_{12}H_{15}]^+$	TCDDM fragment	
149.0235	0	3	17	$[C_8H_5O_3]^+$	Phthalic anhydride ion	
133.1014	2	26	78	$[C_{10}H_{13}]^+$	TCDDM fragment	
119.0859	1	15	65	$[C_9H_{11}]^+$	TCDDM fragment	
105.0704	1	13	68	$[C_8H_9]^+$	TCDDM fragment	
95.0861	-	5	46	$[C_7H_{11}]^+$		
93.0705	-	3	21	$[C_7H_9]^+$		
91.0549	-	11	95	$[C_7H_7]^+$	Tropilium cation	
81.0706	1	14	69	$[C_6H_9]^+$		
79.0550	-	6	52	$[C_6H_7]^+$		

IDENTIFICATION

PC-group	N/A		
Category	Oligoester	**Substance**	**c(2TCDDM+2AA)**
Chemical formula	$C_{36}H_{52}O_8$	**Monoisotopic mass**	612.3661
Apex t_R (min)	14.85	**Δppm**	+0,74
Deduced neutral mass	612.3665	**ΔmDa**	+0,45
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 630.4004

Annotated adduct ion $[M+NH_4]^+$

Most intense coating

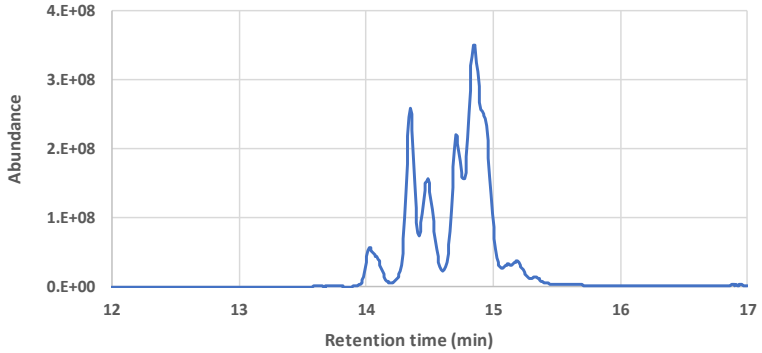


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
-	-	$[M+H]^+$
630.4004	74%	$[M+NH_4]^+$
635.3553	100%	$[M+Na]^+$
-	-	$[M+K]^+$
-	-	$[M+NH_4+ACN]$

ONE-DIMENTIONAL DATA

positive mode

	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
630.40	100	-	-	**Precursor $[M+NH_4]^+$**		
613.3738	26	-	-	$[M+H]^+$		
595.3633	1	-	-	$[C_{36}H_{51}O_7]^+$	H_2O loss	
453.2489	1	-	-	$[C_{24}H_{37}O_8]^+$	TCDDM loss	
435.2383	1	-	-	$[C_{24}H_{35}O_7]^+$	TCDDM loss	
307.1906	-	1	-	$[C_{18}H_{27}O_4]^+$	TCDDM+AA fragment	
161.1326	7	100	100	$[C_{12}H_{17}]^+$	TCDDM fragment	
159.1170	-	0.5	2	$[C_{12}H_{15}]^+$	TCDDM fragment	
147.0653	-	2	-	$[C_6H_{11}O_4]^+$	AA fragment	
133.1014	0.2	13	56	$[C_{10}H_{13}]^+$	TCDDM fragment	
129.0549	-	2	2	$[C_6H_9O_3]^+$	AA fragment	
119.0859	-	6	39	$[C_9H_{11}]^+$	TCDDM fragment	
111.0445	-	1	3	$[C_6H_7O_2]^+$	AA fragment	
107.0860	-	-	2	$[C_8H_{11}]^+$		
105.0704	0.1	6	37	$[C_8H_9]^+$	TCDDM fragment	
101.0603	-	0.5	5	$[C_5H_9O_2]^+$		
95.0861	-	1	21	$[C_7H_{11}]^+$		
95.0498	-		1	$[C_6H_7O]^+$		
93.0705	-	1	10	$[C_7H_9]^+$		
91.0549	-	2	38	$[C_7H_7]^+$	Tropilium cation	
83.0863	-	0.4	2	$[C_6H_{11}]^+$		
83.0499	-	-	1	$[C_5H_7O]^+$		
81.0706	0.1	8	47	$[C_6H_9]^+$		
79.0550	-	2	24	$[C_6H_7]^+$		
69.0707	-	-	1	$[C_5H_9]^+$		
67.0551	-	1	16	$[C_5H_7]^+$		
55.0552	-	-	1	$[C_4H_7]^+$		

IDENTIFICATION

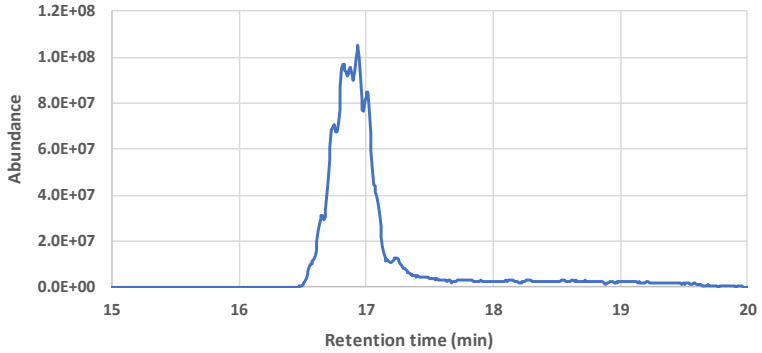
PC-group	N/A		
Category	Oligoester	**Substance**	**c(3TCDDM+3AA)**
Chemical formula	$C_{54}H_{78}O_{12}$	**Monoisotopic mass**	918.5497
Apex t_R (min)	16.94	**Δppm**	-1.42
Deduced neutral mass	918.5484	**ΔmDa**	-1.31
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 936.5822

Annotated adduct ion $[M+NH_4]^+$

Most intense coating



Observed mass spectrum

m/z	Relative area (%)	Annotated ion
-	-	$[M+H]^+$
936.5822	100%	$[M+NH_4]^+$
941.5389	72%	$[M+Na]^+$
-	-	$[M+K]^+$
-	-	$[M+NH_4+ACN]$

Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

ONE-DIMENTIONAL DATA

positive mode

	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
936.58	100	-	-	**Precursor $[M+NH_4]^+$**		
919.5562	9	-	-	$[M+H]^+$		
901.5459	1	-	-	$[C_{54}H_{77}O_{11}]^+$	H_2O loss	
759.4318	1	-	-	$[C_{42}H_{63}O_{12}]^+$	TCDDM loss	
741.4213	1	-	-	$[C_{42}H_{61}O_{11}]^+$	TCDDM loss	
613.3742	0.5	-	-	$[C_{36}H_{53}O_8]^+$	2TCDDM+2AA fragment	
435.2388	0.4	-	-	$[C_{24}H_{35}O_7]^+$	TCDDM+2AA fragment	
307.1907	-	1	-	$[C_{18}H_{27}O_4]^+$	TCDDM+AA fragment	
289.1801	-	1	-	$[C_{18}H_{25}O_3]^+$	TCDDM+AA fragment	
271.1695	-	1	-	$[C_{18}H_{23}O_2]^+$	TCDDM+AA fragment	
161.1326	4	100	100	$[C_{12}H_{17}]^+$	TCDDM fragment	
159.1170	-	1	2	$[C_{12}H_{15}]^+$	TCDDM fragment	
147.0653	-	2	-	$[C_6H_{11}O_4]^+$	AA fragment	
133.1014	-	14	56	$[C_{10}H_{13}]^+$	TCDDM fragment	
131.0857	-	-	1	$[C_{10}H_{11}]^+$		
129.0548	-	2	2	$[C_6H_9O_3]^+$	AA fragment	
119.0859	-	7	42	$[C_9H_{11}]^+$	TCDDM fragment	
111.0445	-	1	2	$[C_6H_7O_2]^+$	AA fragment	
107.0860	-		2	$[C_8H_{11}]^+$		
105.0704	-	6	36	$[C_8H_9]^+$	TCDDM fragment	
101.0602	-	0.4	4	$[C_5H_9O_2]^+$		
95.0861	-	1.8	24	$[C_7H_{11}]^+$		
95.0497	-	-	2	$[C_6H_7O]^+$		
93.0705	-	1	11	$[C_7H_9]^+$		
91.0549	-	3	48	$[C_7H_7]^+$	Tropilium cation	
83.0862	-	0.3	2	$[C_6H_{11}]^+$		
81.0706	-	8	41	$[C_6H_9]^+$		
79.0550	-	2	24	$[C_6H_7]^+$		
67.0551	-	1	15	$[C_5H_7]^+$		

IDENTIFICATION

PC-group	N/A		
Category	Oligoester	**Substance**	**lin(TCDDM+AA)**
Chemical formula	$C_{18}H_{28}O_5$	**Monoisotopic mass**	324.1937
Apex t_R (min)	4.52	**Δppm**	+0
Deduced neutral mass	324.1937	**ΔmDa**	+0
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z*	325.20	323.19
Annotated adduct ion	$[M+H]^+$	$[M-H]^-$
Most intense coating		

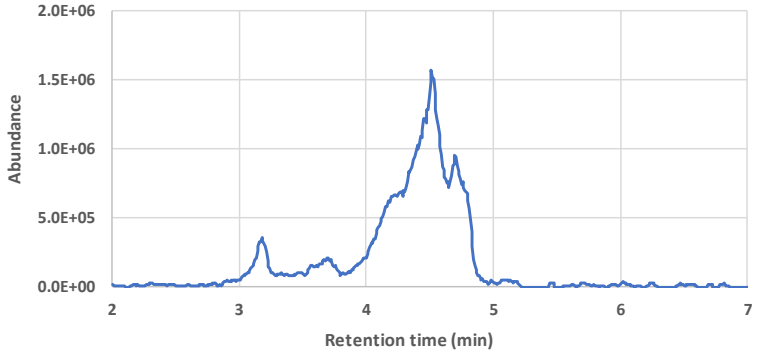


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating,

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
325.2010	78%	$[M+H]^+$
342.2275	99%	$[M+NH_4]^+$
347.1829	100%	$[M+Na]^+$
-	-	$[M+K]^+$
383.2541	19%	$[M+NH_4+ACN]$

negative mode

323.1864	100%	$[M-H]^-$
497.1514	5%	$[M+H_6Si_2O_7-H]^-$

ONE-DIMENTIONAL DATA

positive mode

m/z	Relative area (%) NCE 10%	NCE 30%	NCE 50%	Ion formula	Annotation	*Comment*
325.20	4.86	-	-	**Precursor $[M+H]^+$**		
307.1905	6	-	-	$[C_{18}H_{27}O_4]^+$	H_2O loss	
179.1431	10	2.9	-	$[C_{12}H_{19}O]^+$	TCDDM fragment	
161.1325	100	100	100	$[C_{12}H_{17}]^+$	TCDDM fragment	
147.0652	22	15	8	$[C_6H_{11}O_4]^+$	AA fragment	
135.1170	-	1	5	$[C_{10}H_{15}]^+$		
133.1013	6	28	100	$[C_{10}H_{13}]^+$	TCDDM fragment	
129.0548	12	21	23	$[C_6H_9O_3]^+$	AA fragment	
121.1014	0	2	5	$[C_6H_{13}]^+$		
119.0858	2	10	66	$[C_9H_{11}]^+$	TCDDM fragment	
111.0444	2	8	32	$[C_6H_7O_2]^+$	AA fragment	
105.0703	2	10	66	$[C_8H_9]^+$	TCDDM fragment	
101.0602	0	6	62	$[C_5H_9O_2]^+$		
95.0861	0	4	48	$[C_7H_{11}]^+$		
91.0548	0	5	87	$[C_7H_7]^+$	Tropilium cation	
83.0498	-	1	15	$[C_5H_7O]^+$		
81.0706	1	9	70	$[C_6H_9]^+$		
79.0549	0	4	51	$[C_6H_7]^+$		
67.0550	-	2	34	$[C_5H_7]^+$		
55.0551	-	-	12	$[C_4H_7]^+$		

negative mode

m/z	Relative area (%) NCE 10%	NCE 30%	NCE 50%	Ion formula	Annotation	*Comment*
323.1869	100	33	-	**Precursor [M-H]-**		
193.1236	-	0	3	$[C_{12}H_{17}O_2]^-$	TCDDM fragment	
145.0508	1	4	0	$[C_6H_9O_4]^-$	AA fragment	
127.0403	2	11	2	$[C_6H_7O_3]^-$	AA fragment	
83.0499	9	100	100	$[C_5H_7O]^-$		
81.0342	0	4	21	$[C_5H_5O]^-$		

IDENTIFICATION

PC-group	N/A		
Category	Oligoester	**Substance**	**lin(2TCDDM+2AA)**
Chemical formula	$C_{36}H_{54}O_9$	**Monoisotopic mass**	630.3768
Apex t_R (min)	10.92	**Δppm**	+0
Deduced neutral mass	630.3768	**ΔmDa**	+0
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z*	648.41	629.37
Annotated adduct ion	$[M+NH_4]^+$	$[M-H]^-$
Most intense coating		

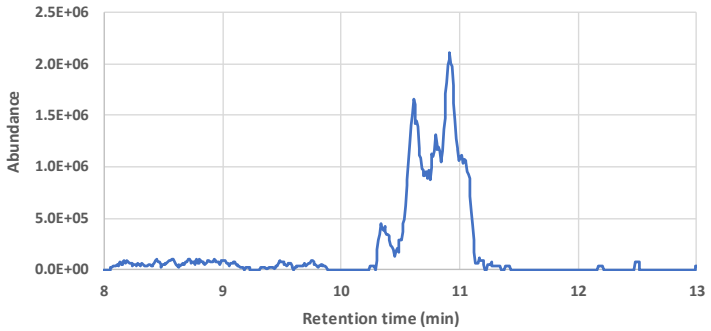


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
631.3841	23%	$[M+H]^+$
648.4106	100%	$[M+NH_4]^+$
653.3660	31%	$[M+Na]^+$
	-	$[M+K]^+$
	-	$[M+NH_4+ACN]$

ONE-DIMENTIONAL DATA

positive mode

m/z	Relative area (%) NCE 10%	NCE 30%	NCE 50%	Ion formula	Annotation	*Comment*
648.4111	26	-	-	**Precursor $[M+NH_4]^+$**		
631.3841	100	-	-	$[M+H]^+$		
613.3738	9	-	-	$[C_{36}H_{53}O_8]^+$	2TCDDM+2AA fragment	
503.3370	1	-	-	$[C_{30}H_{47}O_6]^+$	2TCDDM+AA fragment	
453.2484	2	-	-	$[C_{24}H_{37}O_8]^+$	TCDDM+2AA fragment	
435.2380	3	-	-	$[C_{24}H_{35}O_7]^+$	TCDDM+2AA fragment	H_2O loss
325.2012	2	-	-	$[C_{18}H_{29}O_5]^+$	TCDDM+AA fragment	
307.1906	1	1	-	$[C_{18}H_{27}O_4]^+$	TCDDM+AA fragment	H_2O loss
179.1431	0	3	-	$[C_{12}H_{19}O]^+$	TCDDM fragment	
161.1325	9	100	100	$[C_{12}H_{17}]^+$	TCDDM fragment	
147.0652	0	5	3	$[C_6H_{11}O_4]^+$	AA fragment	
133.1013	0	11	53	$[C_{10}H_{13}]^+$	TCDDM fragment	
129.0548	-	4	6	$[C_6H_9O_3]^+$	AA fragment	
119.0858	-	5	33	$[C_9H_{11}]^+$	TCDDM fragment	
111.0444	-	1	5	$[C_6H_7O_2]^+$	AA fragment	
105.0703	-	5	33	$[C_8H_9]^+$	TCDDM fragment	
101.0602	-	1	9	$[C_5H_9O_2]^+$		
95.0861	-	1	20	$[C_7H_{11}]^+$		
93.0704	-	1	9	$[C_7H_9]^+$		
91.0548	-	2	32	$[C_7H_7]^+$	Tropilium cation	
81.0706	-	6	37	$[C_6H_9]^+$		
79.0549	-	1	20	$[C_6H_7]^+$		
67.0550	-	1	13	$[C_5H_7]^+$		
59.0613	1	1	3	$[NH_4+ACN]^+$		

negative mode

m/z	Relative area (%) NCE 10%	NCE 30%	NCE 50%	Ion formula	Annotation	*Comment*
629.3701	100	11	-	**Precursor [M-H]-**		
451.2346	-	3	-	$[C_{24}H_{35}O_8]^-$	TCDDM loss	
433.2238	-	3	-	$[C_{24}H_{33}O_7]^-$	TCDDM loss - H_2O	
389.2341	0	3	-	$[C_{23}H_{33}O_5]^-$		
323.1869	1	85	-	$[C_{18}H_{27}O_5]^-$	TCDDM+AA fragment	
145.0508	-	5	1	$[C_6H_9O_4]^-$	AA fragment	
127.0402	0	11	3	$[C_6H_7O_3]^-$	AA fragment	
109.0296	-	0	2	$[C_6H_5O_2]^-$		
83.0499	0	100	100	$[C_5H_7O]^-$		
81.0342	-	6	25	$[C_5H_5O]^-$		

IDENTIFICATION

PC-group	N/A		
Category	Oligoester	**Substance**	**lin(3TCDDM+2AA)**
Chemical formula	$C_{48}H_{72}O_{10}$	**Monoisotopic mass**	808.5125
Apex t_R (min)	14.24	**Δppm**	+0,01
Deduced neutral mass	808.5126	**ΔmDa**	+0,01
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 826.55

Annotated adduct ion $[M+NH_4]^+$

Most intense coating

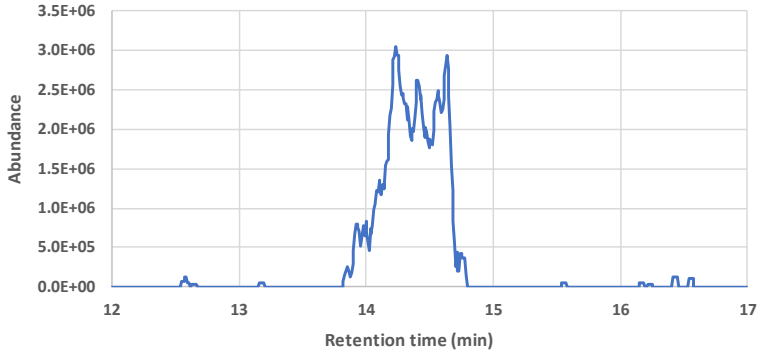


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
809.5198	8%	$[M+H]^+$
826.5464	57%	$[M+NH_4]^+$
831.5018	100%	$[M+Na]^+$
847.4757	27%	$[M+K]^+$
867.5729	1%	$[M+NH_4+ACN]$

ONE-DIMENTIONAL DATA

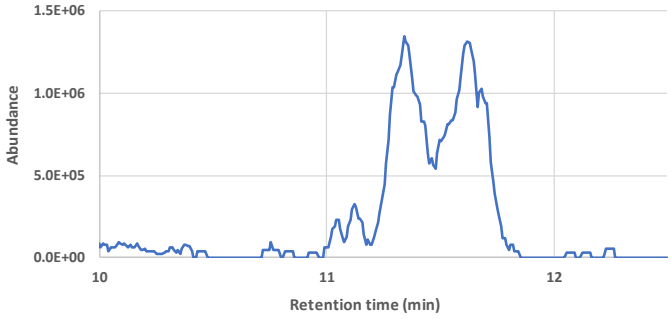
positive mode	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
826.5463	36	-	-	**Precursor $[M+NH_4]^+$**		
809.5196	100	-	-	$[C_{48}H_{73}O_{10}]^+$	$[M+H]^+$	
631.3837	2	-	-	$[C_{36}H_{55}O_9]^+$	TCDDM loss	
283.2632	1	5	2	$[C_{18}H_{35}O_2]^+$	TCDDM+AA fragment	
179.1431	-	3	-	$[C_{12}H_{19}O]^+$	TCDDM fragment	
161.1325	3	100	100	$[C_{12}H_{17}]^+$	TCDDM fragment	
147.0652	-	4	1	$[C_6H_{11}O_4]^+$	AA fragment	
133.1013	-	13	56	$[C_{10}H_{13}]^+$	TCDDM fragment	
129.0548	-	3	4	$[C_6H_9O_3]^+$	AA fragment	
119.0858	-	5	35	$[C_9H_{11}]^+$	TCDDM fragment	
105.0703	-	6	35	$[C_8H_9]^+$	TCDDM fragment	
95.0861	-	1	21	$[C_7H_{11}]^+$		
91.0548	-	2	37	$[C_7H_7]^+$	Tropilium cation	
81.0706	-	6	38	$[C_6H_9]^+$		
79.0549	-	1	22	$[C_6H_7]^+$		
67.0550	-	-	16	$[C_5H_7]^+$		

IDENTIFICATION

PC-group	N/A		
Category	Oligoester	**Substance**	lin(2TCDDM+MA+AA)
Chemical formula	$C_{34}H_{48}O_9$	**Monoisotopic mass**	600.3298
Apex t_R (min)	10.84	**Δppm**	0.00
Deduced neutral mass	600.3298	**ΔmDa**	0.00
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z*	601.3400	599.3200
Annotated adduct ion	$[M+H]^+$	$[M-H]^-$
Most intense coating		



Observed mass spectrum

m/z	Relative area (%)	Annotated ion
601.3371	85%	$[M+H]^+$
618.3637	100%	$[M+NH_4]^+$
623.3191	58%	$[M+Na]^+$
-	-	$[M+K]^+$
-	-	$[M+NH_4+ACN]$

Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

ONE-DIMENTIONAL DATA

positive mode

	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
601.3366	100	-	-	**Precursor $[M+H]^+$**		
583.3264	36	-	-	$[C_{34}H_{47}O_8]^+$	H_2O loss	
565.3155	4	-	-	$[C_{34}H_{45}O_7]^+$	H_2O loss	
423.2014	6	-	-	$[C_{22}H_{31}O_8]^+$	TCDDM loss	
307.1904	2	-	-	$[C_{18}H_{27}O_4]^+$	TCDDM+AA fragment	
161.1325	42	100	100	$[C_{12}H_{17}]^+$	TCDDM fragment	
147.0652	2	4	-	$[C_6H_{11}O_4]^+$	AA fragment	
133.1013	0	19	69	$[C_{10}H_{13}]^+$	TCDDM fragment	
129.0548	-	6	6	$[C_6H_9O_3]^+$	AA fragment	
119.0858	-	9	52	$[C_9H_{11}]^+$	TCDDM fragment	
111.0444	-	3	9	$[C_6H_7O_2]^+$	AA fragment	
107.0860	-	-	2	$[C_8H_{11}]^+$		
105.0703	-	9	52	$[C_8H_9]^+$	TCDDM fragment	
101.0602	-	2	16	$[C_5H_9O_2]^+$		
95.0861	-	2	32	$[C_7H_{11}]^+$		
93.0704	-	2	17	$[C_7H_9]^+$		
91.0548	-	4	55	$[C_7H_7]^+$	Tropilium cation	
81.0705	-	9	53	$[C_6H_9]^+$		
79.0549	-	3	35	$[C_6H_7]^+$		
67.0550	-	2	22	$[C_5H_7]^+$		
59.0612	4	5	14	$[NH_4+ACN]^+$		

negative mode

	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
599.3232	100	15	-	**Precursor $[M-H]^-$**		
421.1876	-	3	-	$[C_{22}H_{29}O_8]^-$	TCDDM loss	
359.1870	-	9	-	$[C_{21}H_{27}O_5]^-$		
305.1763	-	6	1	$[C_{18}H_{21}O_4]^-$	TCDDM+AA fragment	
293.1399	2	100	14	$[C_{16}H_{21}O_5]^-$	TCDDM+MA fragment	
247.1343	-	4	15	$[C_{15}H_{19}O_3]^-$		
193.1236	-	3	15	$[C_{12}H_{17}O_2]^-$	TCDDM fragment	
191.1081	-	-	3	$[C_{12}H_{15}O_2]^-$	TCDDM fragment	
175.1130	-	-	7	$[C_{12}H_{15}O]^-$	TCDDM fragment	
127.0403	0	3	3	$[C_6H_7O_3]^-$	AA fragment	
109.0296	0	5	27	$[C_6H_5O_2]^-$	AA fragment	
96.9930	-	5	6	$[C_4HO_3]^-$	MA fragment	
83.0499	0.1	35	87	$[C_5H_7O]^-$		
81.0342	-	3	37	$[C_5H_5O]^-$		
71.0133	-	10	100	$[C_3H_3O_2]$		
55.0182	-	3	21	$[C_3H_3O]$		

IDENTIFICATION

PC-group	N/A		
Category	Oligoester	**Substance**	**c(2TCDDM+MA+AA)**
Chemical formula	$C_{34}H_{46}O_8$	**Monoisotopic mass**	582.3193
Apex t_R (min)	15.11	**Δppm**	-0.35
Deduced neutral mass	582.3191	**ΔmDa**	-0.21
Confidence level	2b No indisputable confirmation of MA in fragmentation		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 600.3529

Annotated adduct ion $[M+NH_4]^+$

Most intense coating

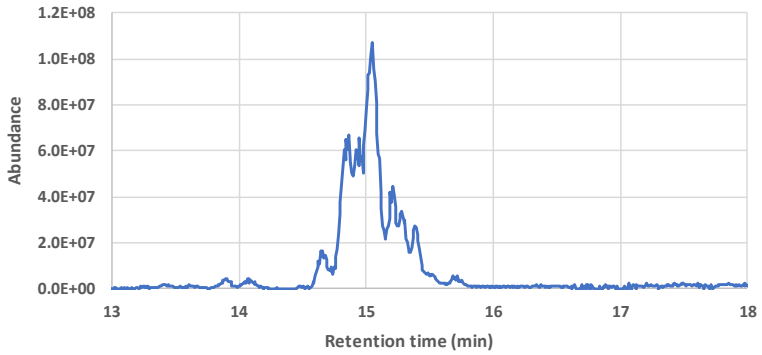


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
-	-	$[M+H]^+$
600.3529	100%	$[M+NH_4]^+$
605.3082	83%	$[M+Na]^+$
-	-	$[M+K]^+$
-	-	$[M+NH_4+ACN]$

ONE-DIMENTIONAL DATA

positive mode

	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
600.35	88	-	-	**Precursor $[M+NH_4]^+$**		
583.3267	100	-	-	$[M+H]^+$		
565.3161	2	0.5	-	$[C_{34}H_{45}O_7]^+$	H_2O loss	
423.2016	1	-	-	$[C_{22}H_{31}O_8]^+$	TCDDM loss	
161.1326	20	100	100	$[C_{12}H_{17}]^+$	TCDDM fragment	
159.1170	-	1	3	$[C_{12}H_{15}]^+$	TCDDM fragment	
147.0653	0.3	2	-	$[C_6H_{11}O_4]^+$	AA fragment	
133.1014	1	18	71	$[C_{10}H_{13}]^+$	TCDDM fragment	
129.0549	0.1	3	3	$[C_6H_9O_3]^+$	AA fragment	
119.0859	0.2	9	54	$[C_9H_{11}]^+$	TCDDM fragment	
111.0445	-	1	4	$[C_6H_7O_2]^+$	AA fragment	
107.0860	-	-	3	$[C_8H_{11}]^+$		
105.0704	0.2	8	54	$[C_8H_9]^+$	TCDDM fragment	
101.0603	-	1	8	$[C_5H_9O_2]^+$		
95.0861	-	2	29	$[C_7H_{11}]^+$		
95.0497	-	-	2	$[C_6H_7O]^+$		
93.0705	-	1	15	$[C_7H_9]^+$		
91.0549	-	3	58	$[C_7H_7]^+$	Tropilium cation	
83.0862	-	1	3	$[C_6H_{11}]^+$		
83.0499	-	-	3	$[C_5H_7O]^+$		
81.0706	0.3	9	59	$[C_6H_9]^+$		
79.0550	-	2	36	$[C_6H_7]^+$		
67.0551	-	1	23	$[C_5H_7]^+$		

IDENTIFICATION

PC-group	N/A		
Category	Oligoester	**Substance**	**lin(3TCDDM+2PA)**
Chemical formula	$C_{52}H_{64}O_{10}$	**Monoisotopic mass**	848.4499
Apex t_R (min)	16.44	**Δppm**	+0,01
Deduced neutral mass	848.4500	**ΔmDa**	+0,01
Confidence level	4		Low intensity No fragmentation data

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z*	866.48	849.46
Annotated adduct ion	$[M+NH_4]^+$	$[M+H]^+$
Most intense coating		

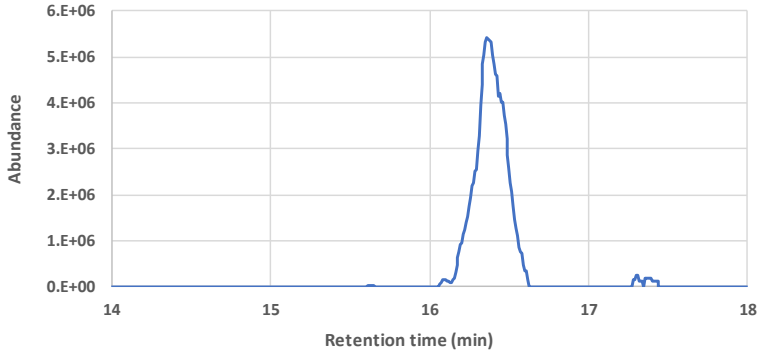


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
849.4572	70%	$[M+H]^+$
866.4838	100%	$[M+NH_4]^+$
871.4392	44%	$[M+Na]^+$
-	-	$[M+K]^+$
-	-	$[M+NH_4+ACN]$

ONE-DIMENTIONAL DATA

positive mode	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
866.48	-	-	-	**Precursor $[M+NH_4]^+$**		
Signals too low for fragmentation						
849.46	-	-	-	**Precursor $[M+H]^+$**		
Signals too low for fragmentation						

IDENTIFICATION			
PC-group	N/A		
Category	N/A	**Substance**	**Unknown 8**
Chemical formula	-	**Monoisotopic mass**	-
Apex t_R (min)	19.70	**Δppm**	-
Deduced neutral mass	-	**ΔmDa**	-
Confidence level	5		No chemical formula hypothesis but informative fragmentation pattern

FRAGMENTATION OF SELECTED ION(S)	
Selected ion *m/z*	940.7284
Annotated adduct ion	?
Most intense coating	

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
940.7284	-	???

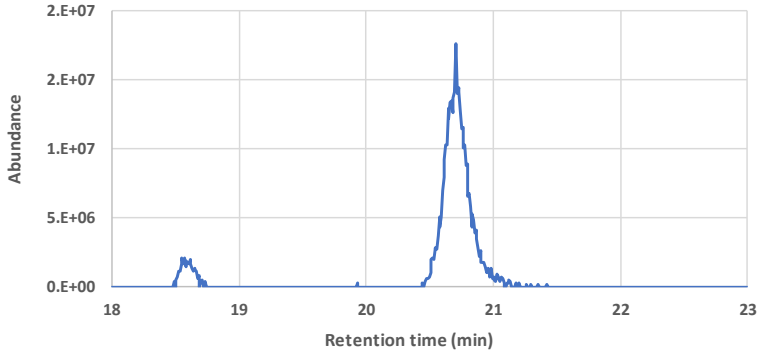


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

ONE-DIMENTIONAL DATA

positive mode

	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
940.73	100	-	-	**Precursor**		
283.2634	1	92	72	$[C_{18}H_{35}O_2]^+$	Stearic acid fragment?	
255.2320	0	57	49	$[C_{16}H_{31}O_2]^+$	Palmitic acid fragment?	
227.2007	1	100	100	$[C_{14}H_{27}O_2]^+$	Myristic acid fragment?	
95.0861	-	2	30	$[C_7H_{11}]^+$		
85.1018	-	1	26	$[C_6H_{13}]^+$		
71.0863	-	1	41	$[C_5H_{11}]^+$		

IDENTIFICATION			
PC-group	N/A		
Category	Oligoester	**Substance**	**c(TCDDM+PA)**
Chemical formula	$C_{20}H_{22}O_4$	**Monoisotopic mass**	326.1518
Apex t_R (min)	7.89	**Δppm**	-0.44
Deduced neutral mass	326.1517	**ΔmDa**	-0.14
Confidence level	2b		

FRAGMENTATION OF SELECTED ION(S)	
Selected ion *m/z*	325.1400
Annotated adduct ion	$[M-H]^-$
Most intense coating	

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
325.1444	100%	$[M-H]^-$

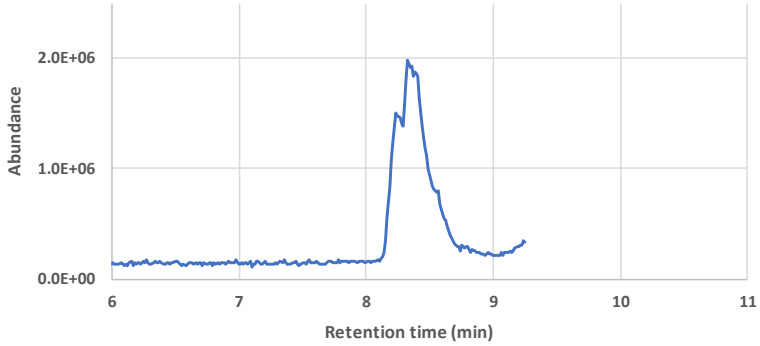


Figure Total ion chromatogram obtained for the MS2 experiment at 10% NCE.

ONE-DIMENTIONAL DATA

negative mode	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
325.1450	100	44	1	**Precursor [M-H]-**		
281.1551	0	5	3	$[C_{19}H_{21}O_2]^-$		
183.0817	-	0	1	$[C_{13}H_{11}O]^-$		
175.1130	-	1	3	$[C_{12}H_{15}O]^-$	TCDDM fragment	
134.0375	-	1	3	$[C_8H_6O_2]^-$	PA fragment	
121.0297	7	100	100	$[C_7H_5O_2]^-$		
120.0218	0	0	9	$[C_7H_4O_2]^-$		

IDENTIFICATION

PC-group	N/A		
Category	Monomer	**Substance**	**TCDDM**
Chemical formula	$C_{12}H_{20}O_2$	**Monoisotopic mass**	196.1463
Apex t_R (min)	5.01	**Δppm**	+0,19
Deduced neutral mass	196.1464	**ΔmDa**	+0,04
Confidence level	4		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z*	195.1391
Annotated adduct ion	$[M-H]^-$?
Most intense coating	

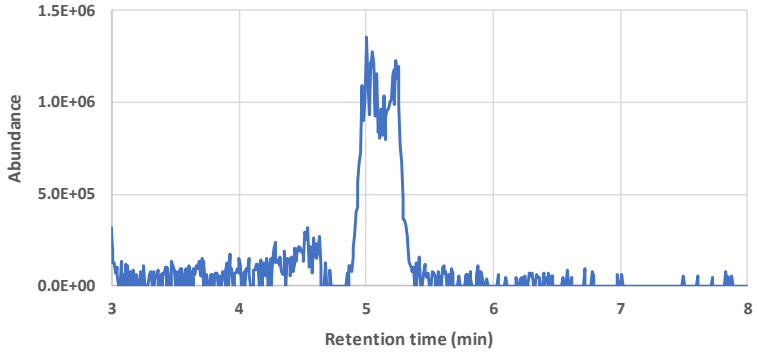


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
195.1391	100%	$[M-H]^-$?

Several isomers

ONE-DIMENTIONAL DATA

No fragmentation assay

IDENTIFICATION

PC-group	N/A		
Category	Cross linker	**Substance**	**IPDI trimer**
Chemical formula	$C_{36}H_{54}N_6O_6$	**Monoisotopic mass**	666.4105
Apex t_R (min)	16.07	**Δppm**	+0,25
Deduced neutral mass	666.4106	**ΔmDa**	+0,16
Confidence level	4		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 725.4245

Annotated adduct ion $[M+CH_3COOH-H]^-$

Most intense coating

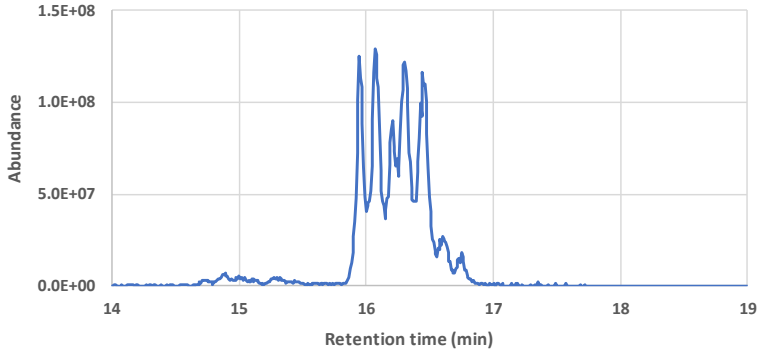


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
-	-	$[M-H]^-$
701.3789	3%	$[M+Cl]^-$
725.4245	100%	$[M+CH_3COOH-H]^-$

ONE-DIMENTIONAL DATA

No fragmentation assay

IDENTIFICATION

PC-group	N/A		
Category	Surfactant?	**Substance**	**2 undecylbenzene 1 sulfonic acid**
Chemical formula	$C_{17}H_{28}O_3S$	**Monoisotopic mass**	312.1759
Apex t_R (min)	9.03	**Δppm**	+0,23
Deduced neutral mass	312.1760	**ΔmDa**	+0,07
Confidence level	4		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 311.1687

Annotated adduct ion $[M-H]^-$

Most intense coating

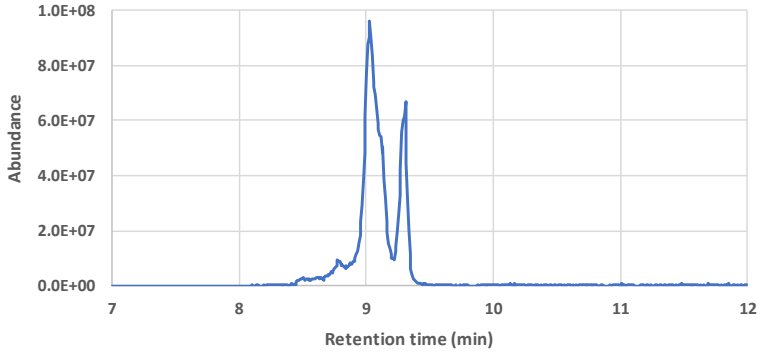


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
311.1687	100%	$[M-H]^-$
-	-	$[M+Cl]^-$
-	-	$[M+CH_3COOH-H]^-$
485.1340	2%	$[M+H_6Si_2O_7-H]^-$

ONE-DIMENTIONAL DATA

No fragmentation assay

IDENTIFICATION

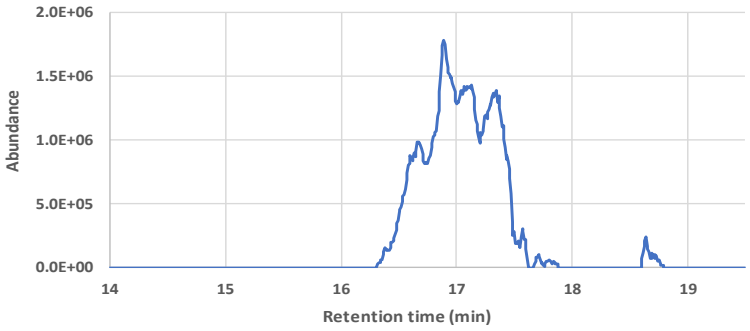
PC-group	N/A		
Category	Oligoester	**Substance**	**lin(3TCDDM+3PA)**
Chemical formula	$C_{60}H_{68}O_{13}$	**Monoisotopic mass**	996.4660
Apex t_R (min)	16.39	**Δppm**	0.000
Deduced neutral mass	996.4660	**ΔmDa**	0
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 1014.5

Annotated adduct ion $[M+NH_4]^+$

Most intense coating



Observed mass spectrum

m/z	Relative area (%)	Annotated ion
997.4733	10%	[M + H]+
1014.4998	100%	[M + NH4]+
1019.4552	42%	[M + Na]+
-	-	$[M+K]^+$
-	-	$[M+NH_4+ACN]$

Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

ONE-DIMENTIONAL DATA

positive mode

	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
1014.5357	98	-	-	**Precursor $[M+NH_4]^+$**		
997.4727	100	-	-	$[M+H]^+$		
979.4548	21	-	-	$[C_{60}H_{67}O_{12}]^+$	H_2O loss	
819.3365	17	-	-	$[C_{28}H_{29}O_8]^+$	TCDDM+2PA fragment	
493.1858	3	0	-	$[C_{28}H_{29}O_8]^+$	TCDDM+2PA fragment	
161.1325	46	100	100	$[C_{12}H_{17}]^+$	TCDDM fragment	
159.1169	5	12	9	$[C_{12}H_{15}]^+$	TCDDM fragment	
149.0234	-	3	21	$[C_8H_5O_3]^+$	Phthalic anhydride ion	
133.1013	-	23	71	$[C_{10}H_{13}]^+$	TCDDM fragment	
119.0858	-	12	59	$[C_9H_{11}]^+$	TCDDM fragment	
105.0703	-	13	65	$[C_8H_9]^+$	TCDDM fragment	
95.0861	-	3	37	$[C_7H_{11}]^+$		
93.0704	-	2	19	$[C_7H_9]^+$		
91.0548	-	8	81	$[C_7H_7]^+$	Tropilium cation	
81.0706	-	13	66	$[C_6H_9]^+$		
79.0549	-	5	46	$[C_6H_7]^+$		

IDENTIFICATION

PC-group	N/A		
Category	Oligoester	**Substance**	**lin(2TCDDM+3PA)**
Chemical formula	$C_{48}H_{50}O_{12}$	**Monoisotopic mass**	818.3302
Apex t_R (min)	10.71	**Δppm**	0.000
Deduced neutral mass	818.3302	**ΔmDa**	0
Confidence level	4		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 817.32

Annotated adduct ion $[M-H]^-$

Most intense coating

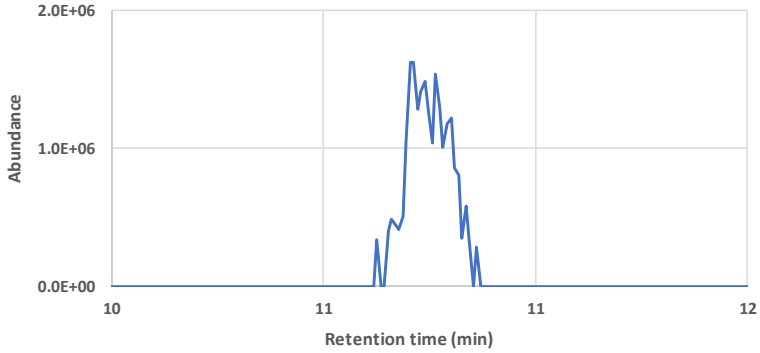


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
817.3230	100%	$[M-H]^-$?

ONE-DIMENTIONAL DATA

negative mode	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
817.3200	-	-	-	**Precursor [M-H]-**		

No fragmentation data potentially due to rt shift

IDENTIFICATION

PC-group	N/A		
Category	Oligoester	**Substance**	**lin(2TCDDM+2PA)**
Chemical formula	$C_{40}H_{46}O_9$	**Monoisotopic mass**	670.3142
Apex t_R (min)	11.56	**Δppm**	+0
Deduced neutral mass	670.3142	**ΔmDa**	+0
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 669.31

Annotated adduct ion $[M-H]^-$

Most intense coating

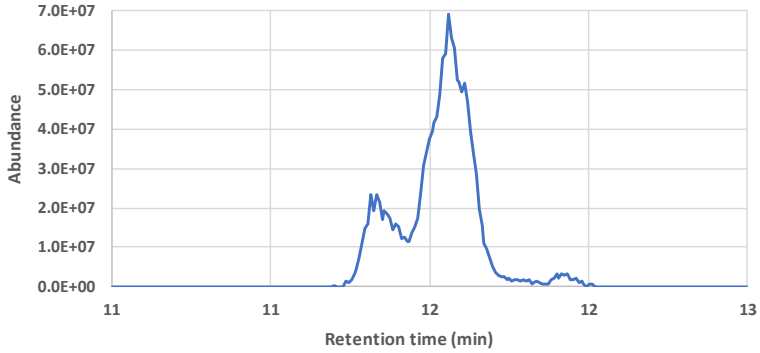


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
669.3069	100%	$[M-H]^-$

ONE-DIMENTIONAL DATA

negative mode	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
669.3078	100	71.64	-	**Precursor [M-H]-**		
447.1820	-	15	1	$[C_{27}H_{27}O_6]^-$	TCDDM+2PA fragment	
403.1921	-	3	5	$[C_{26}H_{27}O_4]^-$	TCDDM+2PA fragment	
343.1555	-	9	-	$[C_{20}H_{23}O_5]^-$	TCDDM+PA fragment	
225.0560	-	2	4	$[C_{14}H_9O_3]^-$		
207.0453	-	1	8	$[C_{14}H_7O_2]^-$		
197.0610	-	0	7	$[C_{13}H_9O_2]^-$		
193.1235	-	2	4	$[C_{12}H_{17}O_2]^-$	TCDDM fragment	
181.0661	-	-	7	$[C_{13}H_9O]^-$		
149.0245	-	2	6	$[C_8H_5O_3]^-$	PA fragment	
121.0296	0	100	100	$[C_7H_5O_2]^-$	PA fragment	

IDENTIFICATION

PC-group	N/A		
Category	Oligoester	**Substance**	**lin(TCDDM+PA)**
Chemical formula	$C_{20}H_{24}O_5$	**Monoisotopic mass**	344.1624
Apex t_R (min)	4.93	**Δppm**	+0
Deduced neutral mass	344.1624	**ΔmDa**	+0
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z*	343.1600
Annotated adduct ion	$[M-H]^-$
Most intense coating	

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
343.1551	100%	$[M-H]^-$

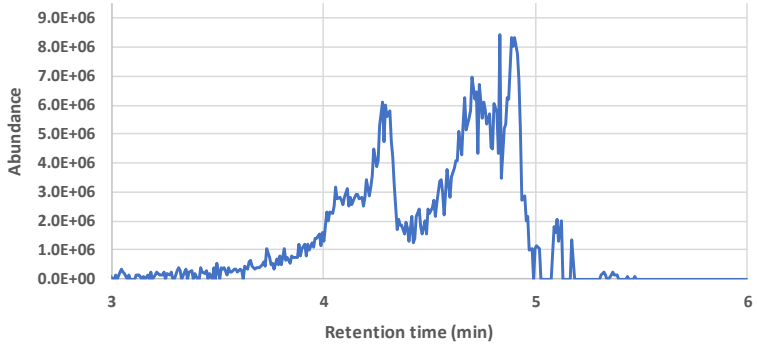


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

ONE-DIMENTIONAL DATA

negative mode	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
343.1555	100	63	0	**Precursor $[M-H]^-$**		
299.1657	0	5	0	$[C_{19}H_{23}O_3]^-$		
283.2646	1	2	0	$[C_{18}H_{35}O_2]^-$	TCDDM+PA fragment	
193.1235	0	9	3	$[C_{12}H_{17}O_2]^-$	TCDDM fragment	
191.1081	0	2	3	$[C_{12}H_{15}O_2]^-$	TCDDM fragment	
175.1130	-	2	3	$[C_{12}H_{15}O]^-$	TCDDM fragment	
134.0375	0	2	1	$[C_8H_6O_2]^-$	PA fragment	
121.0296	4	100	100	$[C_7H_5O_2]^-$		

IDENTIFICATION

PC-group	N/A		
Category	Oligoester	**Substance**	**lin(2BD+TCDDM+3PA)**
Chemical formula	$C_{44}H_{48}O_{13}$	**Monoisotopic mass**	784.3095
Apex t_R (min)	11.10	**Δppm**	+0
Deduced neutral mass	784.3095	**ΔmDa**	+0
Confidence level	4		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z*	802.34	783.3022
Annotated adduct ion	$[M+NH_4]^+$	$[M-H]^-$?
Most intense coating		

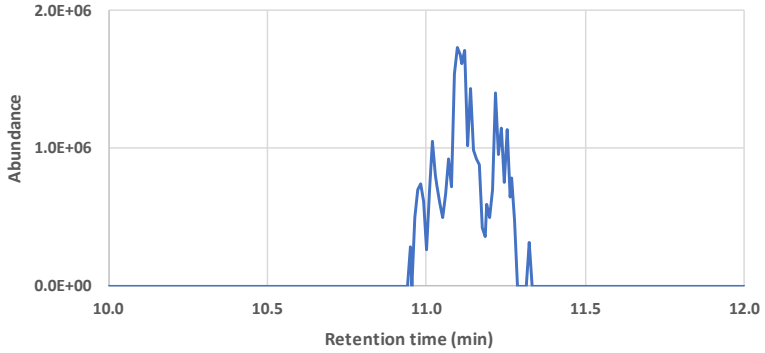


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
783.3022	100%	$[M-H]^-$?

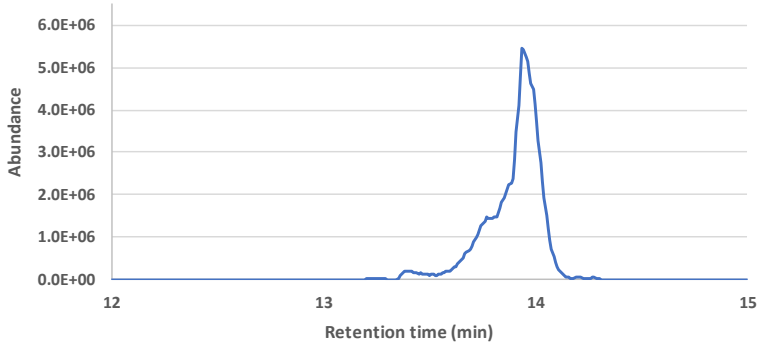
ONE-DIMENTIONAL DATA

positive mode	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
802.3400	-	-	-	**Precursor $[M+NH_4]^+$**		
Signal too low for fragmentation						

negative mode	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
783.5057	8	-	-	**Precursor [M-H]-**		
Signal too low for fragmentation						

IDENTIFICATION			
PC-group	N/A		
Category	Oligoester	**Substance**	**lin(BD+2TCDDM+3PA)**
Chemical formula	$C_{52}H_{58}O_{13}$	**Monoisotopic mass**	890.3877
Apex t_R (min)	13.94	**Δppm**	0.000
Deduced neutral mass	890.3877	**ΔmDa**	0
Confidence level	2b		

FRAGMENTATION OF SELECTED ION(S)		
Selected ion *m/z*	908.42	889.38
Annotated adduct ion	$[M+NH_4]^+$	$[M-H]^-$
Most intense coating		



Observed mass spectrum

m/z	Relative area (%)	Annotated ion
891.3950	8%	[M + H]+
908.4216	100%	[M + NH4]+
913.3770	12%	[M + Na]+
-	-	$[M+K]^+$
-	-	$[M+NH_4+ACN]$

889.3805	100%	[M - H]-

Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

ONE-DIMENTIONAL DATA

positive mode	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
908.4200	-	-	-	**Precursor $[M+NH_4]^+$**		

Signal too low for fragmentation

negative mode	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
889.3811	100	-	-	**Precursor [M-H]-**		
343.1555	-	2	-	$[C_{20}H_{23}O_5]^-$	BD+2PA fragment	
237.0772	-	100	1	$[C_{12}H_{13}O_5]^-$	TCDDM fragment	
181.0661	-	-	2	$[C_{13}H_9O]^-$		
165.0194	-	18	-	$[C_8H_5O_4]^-$	PA fragment	
121.0296	-	56	100	$[C_7H_5O_2]^-$	PA fragment	

IDENTIFICATION			
PC-group	N/A		
Category	Oligoester	**Substance**	**lin(BD+TCDDM+2PA)**
Chemical formula	$C_{32}H_{36}O_9$	**Monoisotopic mass**	564.2359
Apex t_R (min)	8.89	**Δppm**	0.000
Deduced neutral mass	564.2359	**ΔmDa**	0
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)		
Selected ion *m/z*	565.24	563.23
Annotated adduct ion	$[M+H]^+$	$[M-H]^-$
Most intense coating		

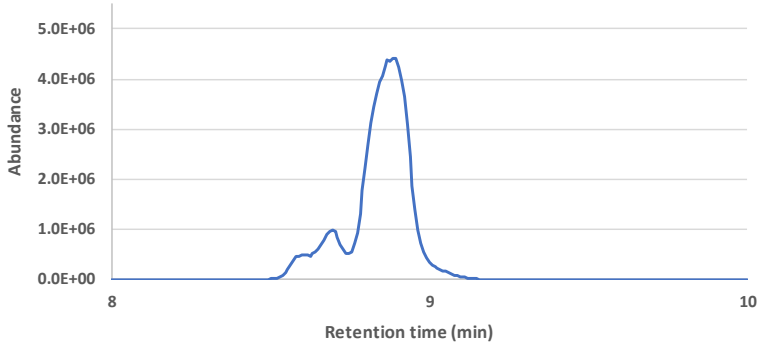


Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating,

Observed mass spectrum

m/z	Relative area (%)	Annotated ion
565.2432	100%	[M + H]+
582.2698	98%	[M + NH4]+
587.2252	36%	[M + Na]+
-	-	$[M+K]^+$
-	-	$[M+NH_4+ACN]$
563.2287	100%	[M - H]-

ONE-DIMENTIONAL DATA

positive mode

	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
565.2432	7	-	-	**Precursor $[M+H]^+$**		
547.2328	6	-	-	$[C_{32}H_{35}O_8]^+$	H_2O loss	
493.1857	68	-	-	$[C_{28}H_{29}O_8]^+$	TCDDM+2PA fragment	
327.1592	7	-	-	$[C_{20}H_{23}O_4]^+$	TCDDM+PA fragment	
161.1325	100	100	86	$[C_{12}H_{17}]^+$	TCDDM fragment	
149.0234	-	3	41	$[C_8H_5O_3]^+$	PA fragment	
133.1013	1	30	81	$[C_{10}H_{13}]^+$	TCDDM fragment	
119.0858	1	16	75	$[C_9H_{11}]^+$	TCDDM fragment	
105.0703	0	17	81	$[C_8H_9]^+$	TCDDM fragment	
95.0861	-	4	50	$[C_7H_{11}]^+$		
93.0704	-	3	26	$[C_7H_9]^+$		
91.0548	-	8	86	$[C_7H_7]^+$	Tropilium cation	
81.0706	-	18	100	$[C_6H_9]^+$		
79.0549	-	7	60	$[C_6H_7]^+$		
73.0656	1	5	14	$[C_4H_9O]^+$	BD fragment	
67.0550	-	3	37	$[C_5H_7]^+$		

negative mode

	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
563.2294	100	24	-	**Precursor [M-H]-**		
491.1719	2	70	-	$[C_{28}H_{27}O_8]^-$	TCDDM+2PA fragment	
447.1819	0	100	-	$[C_{27}H_{27}O_6]^-$	TCDDM+2PA fragment	
403.1920	-	28	15	$[C_{26}H_{27}O_4]^-$		
225.0560	-	28	23	$[C_{14}H_9O_3]^-$		
224.0481	-	-	6	$[C_{14}H_8O_3]^-$		
223.0403	-	1	11	$[C_{14}H_7O_3]^-$		
207.0453	-	15	37	$[C_{14}H_7O_2]^-$		
197.0610	-	4	28	$[C_{13}H_9O_2]^-$		
193.1235	-	2	2	$[C_{12}H_{17}O_2]^-$	TCDDM fragment	
181.0661	-	4	51	$[C_{13}H_9O]^-$		
121.0296	0	46	100	$[C_7H_5O_2]^-$	PA fragment	

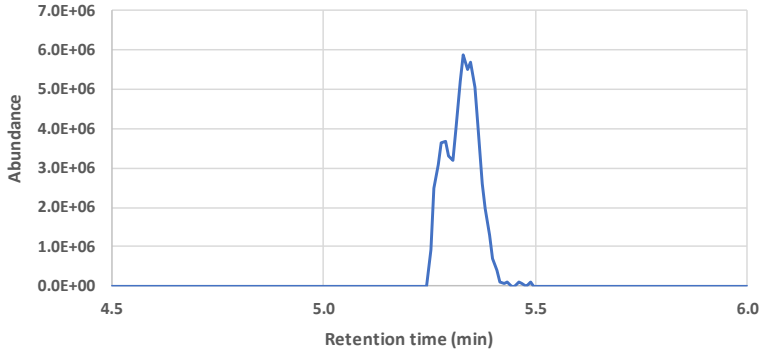
IDENTIFICATION			
PC-group	N/A		
Category	Oligoester	**Substance**	**lin(2BD+2PA)**
Chemical formula	$C_{24}H_{26}O_9$	**Monoisotopic mass**	458.1577
Apex t_R (min)	5.33	**Δppm**	+0
Deduced neutral mass	458.1577	**ΔmDa**	+0
Confidence level	2a		

FRAGMENTATION OF SELECTED ION(S)

Selected ion *m/z* 457.15

Annotated adduct ion $[M-H]^-$

Most intense coating



Observed mass spectrum

m/z	Relative area (%)	Annotated ion
457.1504	100%	$[M-H]^-$

Figure Extracted ion chromatogram (±5 ppm) obtained for the base peak ion in the most intense coating.

ONE-DIMENTIONAL DATA

negative mode	Relative area (%)					
m/z	**NCE 10%**	**NCE 30%**	**NCE 50%**	**Ion formula**	**Annotation**	***Comment***
457.1512	100	8	-	**Precursor [M-H]-**		
385.0935	4	25	-	$[C_{20}H_{17}O_8]^-$	BD+2PA fragment	
341.1034	1	100	0	$[C_{19}H_{17}O_6]^-$	BD+2PA fragment	
297.1136	-	2	-	$[C_{18}H_{17}O_4]^-$		
225.0560	0	98	18	$[C_{14}H_9O_3]^-$		
199.0767	-	0	6	$[C_{13}H_{11}O_2]^-$		
181.0661	-	17	65	$[C_{13}H_9O]^-$		
121.0297	0	60	100	$[C_7H_5O_2]^-$	PA fragment	

Annex 4

a. Lowest effective concentration (ng/well) of negative and positive references tested in the absence and in the presence of metabolic activation on *S. typhimurium* TA98 strain (each cell represents an individual assay)

b. Lowest effective concentration (ng/well) of negative and positive references tested in the absence and in the presence of metabolic activation on *S. typhimurium* TA100 strain (each cell represents an individual assay)

a.

References	Test article	GenEvolution						PackTox										
		Replicates						Replicates										
Positive References	2NF (-S9)	2,5	2,5	1,25	1,25	2,5	1,25	1,25	0,5	2,5	2,5	1,25	2,5	1,25	2,5	0,5	0,5	4
	SA (-S9)																	
	4NQO (-S9)	1		0,5	2,5	0,5	1	0,5	0,1	10	10	10						
	MMS (-S9)	<2		0,5				C1	C1	C1	C1	C1	n,6000	n,6000	n,6000	n,6000		
	2AA (+S9)	2	2	2	0,5	5	2,5	2	2	5	n	5	5	2	2	2		
	B[a]P (+S9)	1	10	2,5	1	40	20	10	25	2,5	2,5	25	25	10				
	CPA (+S9)	n		220	C1		C1	C1	C1	C1	C1	C1	n, 6000	n, 6000				
	ENU (-S9)	n		6000		1000		n,5032	n,5032	n,5032	n,5032	3500	n,4500	3500				
	ENU (+S9)	2000		1000		n		n,5032	5320	3000	3000	2000	4000					
	DMBA (-S9)	n		200				200	1400	2400	800	600	2000	100	200	100		
	DMBA (+S9)	50		n		50		1200	500	50	50	100	100					
	AFB1 (-S9)	Tox		n				100	50	50	50	50	100	10				
	AFB1 (+S9)	0,1	0,2	0,1	1	0,4	6	0,1	0,1	1	10							
	ICR (-S9)	Tox		0,25				0,5	1	1	0,5	0,5						
	ICR (+S9)	1		2		2		1	2	0,5	10							
Negative References	D-Mannitol (-S9)	C1		C1		C1		C1	C1	C1								
	D-Mannitol (+S9)	C1		C1		C1		C1	C1	C1								
	Melamine (-S9)	C1		C1		C1		C1	C1	C1								
	Melamine (+S9)	C1		C1		C1		C1	C1	C1								
	4AP (-S9)	C1		C1		C1		C1	C1	C1								
	4AP(+S9)	C1		C1		C1		C1	C1	C1								

b.

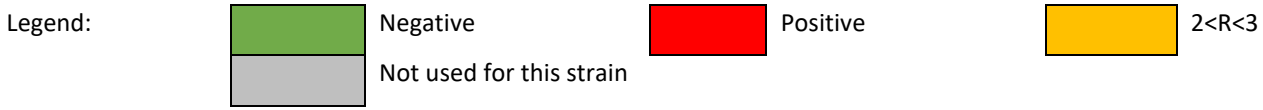
References	Test article	GenEvolution						PackTox										
		Replicates						Replicates										
Positive References	2NF (-S9)																	
	SA (-S9)	2,5	5	50	30			480	480	200	200	120	60	50				
	4NQO (-S9)	1	0,5	1	0,5	0,5		0,5	1	1	5	1						
	MMS (-S9)	n	5	Tox				C1	C1	C1	C1	5000	5000	4000	4000	3000	2500	
	2AA (+S9)	10	10	20	20	5	5	6	10	C1	5	5	C1	50	50	10	5	10
	B[a]P (+S9)	2,5	2,5	n	30	40		C1	15	10	10	30	10					
	CPA (+S9)	22	n	n	220	n		C1	C1	C1	100	C1	10	C1	n, 6000	220		
	ENU (-S9)	2000		6000	2000			4000	n, 5320	4000	3500	2000	1000	1000				
	ENU (+S9)	2000		2000	2000			4000	5320	3500	2000	1000	2000	2000				
	DMBA (-S9)	200	400	600	400			C1	C1	C1	C1	C1	600	600	C1			
	DMBA (+S9)	100		n	n			2500	C1	500	100	C1	50	200	500	50		
	AFB1 (-S9)	n		100				n	n	n	n	75	75	n	5	n		
	AFB1 (+S9)	0,4	n	0,1	60			1	n	n	1	n	5	0,5	2,5	1	2,5	
	ICR (-S9)	5		5	2,5			n	2,5	2,5	n	2,5	0,5	1				
	ICR (+S9)	n		10	15			1	n	n	n	10	2,5	25	n	10		
Negative References	D-Mannitol (-S9)	C1		C1	C1			C1	C1	C1								
	D-Mannitol (+S9)	C1		C1	C1			C1	C1	C1								
	Melamine (-S9)	C1		C1	C1			C1	C1	C1								
	Melamine (+S9)	C1		C1	C1			C1	C1	C1								
	4AP (-S9)	C1		C1	C1			C1	C1	C1								
	4AP(+S9)	C1		C1	C1			C1	C1	C1								

C1= up to high dose 2500 ng/well (maximum recommended),

Positive: number of revertant colonies divided into number of revertant colonies in negative control (ratio (R)) is higher than 3, R>3,

Negative: R<3,

2NF: 2-nitrofluorene, SA: Sodium azide, 4NQO:4-nitroquinoline, MMS: Methyl methanesulfonate, 2AA: 2-aminoanthracene, B[a]P: Benzo[a]pyrene, CPA: cyclophosphamide, 4AP: p-aminophenol, ENU: N-ethyl-N-nitrosourea, DMBA: 7,12 Dimethylbenzanthracene, AFB1: Aflatoxin B1 crystalline, ICR: ICR191acridine mutagen hydrochloride



Annex 5

Article: The cell transformation assay to assess potential carcinogenic properties of nanoparticles

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The cell transformation assay to assess potential carcinogenic properties of nanoparticles

Ruzanna Hayrapetyan [a], Théo Lacour [b], Annette Luce [a], Francis Finot [b], Marie-Christine Chagnon [a], Isabelle Séverin [a,*]

[a] Nutrition Physiology and Toxicology Laboratory (NUTOX), INSERM U1231, Univ. Bourgogne Franche-Comté (UBFC) University of Burgundy, L'Institut Agro Dijon, 1 Esplanade Erasme, F-21000 Dijon, France
[b] GenEvolutioN - SEQENS' Lab Porcheville - Bâtiment 1, 2-8 rue de Rouen-ZI de Limay-Porcheville, F-78440 Porcheville, France

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ABSTRACT

Nanoparticles (NPs) are present in many daily life products with particular physical-chemical properties (size, density, porosity, geometry …) giving very interesting technological properties. Their use is continuously growing and NPs represent a new challenge in terms of risk assessment, consumers being multi-exposed. Toxic effects have already been identified such as oxidative stress, genotoxicity, inflammatory effects, and immune reactions, some of which are leading to carcinogenesis. Cancer is a complex phenomenon implying multiple modes of action and key events, and prevention strategies in cancer include a proper assessment of the properties of NPs. Therefore, introduction of new agents like NPs into the market creates fresh regulatory challenges for an adequate safety evaluation and requires new tools. The Cell Transformation Assay (CTA) is an *in vitro* test able of highlighting key events of characteristic phases in the cancer process, initiation and promotion. This review presents the development of this test and its use with NPs. The article underlines also the critical issues to address for assessing NPs carcinogenic properties and approaches for improving its relevance.

1. Introduction

In Europe, **cancer** is the most frequently occurring form of non-communicable disease and the second most common cause of death, after cardiovascular diseases. Estimates for 2018 indicate 4.2 million new cancer cases and 1.94 million cancer deaths, as reported by the European Cancer Information System, in spite of major improvements in diagnosis and therapy and a slight decrease in deaths linked to certain cancers [1]. In 2017, the 70th World Health Assembly of the World Health Organization (WHO) adopted a resolution on cancer prevention and control, with a broad consensus that "Cancer is a growing public health concern which requires increased attention, prioritization and funding" [2].

Nanomaterials (NMs) are any type of material of nanosized thickness, where at least one dimension is less than approximately 100 nanometers [3]. They are widely used in different technologies and across almost all industrial sectors and product categories, whether cosmetics, industrial chemicals, food contact materials, or medicines because at this scale, unique optical, magnetic, electrical, and other properties emerge.

The term **nanoparticles** (NPs) is used for constituent particles of NMs or for nanoscale particles generated by different sources (natural or engineered, *e.g.* biotechnology, in healthcare technology, medicinal imaging applications, in food, gas sensing, electronic computer, energy harvesting (Khan et al., 2019)[4].

Due to the advances in nanoscience and the NPs widespread use increasing in the last two decades, consumers, workers, and the environment are exposed to them in many different ways.

This exposure makes it urgent to study more deeply the hazardous effects of NPs to assess the risk for human and animal health, and the environment.

Nanotoxicology represents a complex and growing research area in toxicology. Inherent properties of NPs as well as environmental factors collectively influence NPs behavior, fate and transport, available fraction for uptake into the cells (bioavailability) and ultimately toxicity. Then, nanotoxicology needs multidisciplinary knowledge such as biology, toxicology, chemistry and physic to be able to get a proper characterization of NPs [5],6-8.

* Corresponding author.
E-mail address: isabelle.severin@agrosupdijon.fr (I. Séverin).

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Introduction of NPs into the market creates fresh regulatory challenges for an adequate, low cost and rapid safety evaluation using alternative methods such as *in vitro* testing strategies, which requires new tools to better understand the NP mode of actions. Moreover, the 3 R rule (Replace, Reduce and Refine) preconized by the European commission to avoid animal testing involves alternative strategies such as *in vitro* testing usually used as first intention tier to study NPs, with mechanistic information to support a weight of evidence for further use and application of NMs [9].

Alternative methods have been tentatively used to predict the toxicity of a new NP. Among them, QSAR techniques are extensively applied in the area of toxicology and drug design to predict physicochemical properties of chemicals and their effects on the human organism and in the environment. But the development of QSAR models dedicated to NMs and NPs requires both the development of nano-specific descriptors and curated experimental datasets [10]. Read-across could also be used to assess NP toxicity as a technique for predicting endpoint information for one or more substances (target substance(s)) by using data from the same endpoint from (an) other substance(s) provided that these substances are similar, *i.e.* have similar physicochemical, toxicological and ecotoxicological properties, or follow a regular pattern as a result of structural similarity that allows them to be considered as a group (REACH Annex XI, [11]). However, the identification of structurally similar substances is more challenging for NMs and/or NPs than regular chemicals because NMs do not have a uniquely defined structure [12]. The European Chemicals Agency (ECHA) has recently released guidance on how to justify grouping for read-across between nanoforms of the same substance [13] as differences in the toxicological behavior of NPs at different sizes have been reported (Li et al., 2011 [14], Sahu et al., 2016 [15]). However, to date, the read-across approach has only been used with a few NPs, among them the nanoTiO_2 NPs [16,17,12].

1.1. Nanoparticles and toxicological endpoints

NPs can have toxicological impacts in many ways. Toxicity of NPs can be tested using, *in vivo* and/or *in vitro* studies with the particularity that it will be a case-by-case approach. In contact with organisms, they can trigger inflammatory process, immune reactions, oxidative stress, DNA damage, cell death… (for general review, [18]).

NMs are used in every field of manufacturing with different regulations, and requests for carcinogenicity assessment could vary from one to another. For the food and feed area, EFSA has published in 2018 a guidance on nanotechnologies and has proposed a stepwise framework for nano-related hazard identification and characterization. In this decision tree, carcinogenicity studies are asked only if there are indications for slow elimination or accumulation and distribution to specific tissues (EFSA, 2018 [19]). In this case, targeted in-depth investigations are required *e.g.* additional toxicokinetic study (optionally in human studies), reproductive and developmental toxicity, immunotoxicity, neurotoxicity, carcinogenicity/mutagenicity, endocrine properties, and effects on gut microbiome.

In the cosmetic field, a guidance on the Safety Assessment of Nanomaterials in Cosmetics has been published in 2019 (SCCS/1611/19) [20]. A carcinogenicity study is in general only submitted when already available; *e.g.*, when carried out before the animal testing ban or when generated for compliance under a different (non-cosmetic) legislative framework. The decision on the carcinogenic potential of mutagenic or genotoxic substances may thus be made on the outcome of *in vitro* mutagenicity tests. A positive *in vitro* result in mutagenicity tests is then seen as indicative of the carcinogenic potential of the substance (SCCS/1602/18) [21].

In regulatory toxicology, *in vitro* evaluation methods need to be faster cost-effective alternative methods, robust, reliable, and standardized [22]. In addition, in nanotoxicology, compared to standard chemicals, a proper characterization of NPs is essential, as it will determine the testing strategy due to potential interferences between NPs and the selected assay [23]. Furthermore, NMs are engineered to have various unique material properties. These properties will also affect their possible direct or indirect interaction with the DNA. Depending on the structure, size: surface (μg/cm2) and volume (μm3/cell), hydroscopic properties of the NP may have an impact on the genotoxicity test results. Catalytic activity and inflammation potential (including but not limited to ROS generation) may have an impact on general toxicity [24].

Moreover, it is also recommended for testing NPs *in vitro*, to perform a battery of tests on cells of human origin, either cell lines or primary cells, in conditions as close as possible to an *in vivo* situation [22].

Damage to DNA leading to gene mutations, larger-scale chromosomal aberration, and aneuploidy (numerical chromosome changes) are generally considered as the first step in the multi-stage process of malignancy [25]. NP genotoxicity can be a consequence of three mechanisms: a direct interaction of NPs with genetic materials, an indirect action *via* ROS damage, due to toxic lesions (*e.g.* transition of metal's ions, *via* Fenton type reaction, due to binding with DNA bases, released from soluble NP) or a result of oxidative DNA damage by ROS during NP elicited inflammation (for review, see [26]). Indeed, the induction of **oxidative stress** induced by NPs due to an overproduction of reactive oxygen species (ROS) is also often searched as it has been shown to be an underlying mechanism of possible toxicity of NPs leading to immunotoxicity, genotoxicity [22] and carcinogenicity [27].

However, **carcinogenesis process** involves sequential genetic alterations in a single target cell corresponding to several steps known as initiation, promotion, and progression events that are not necessarily dependent on mutations in the genomic material. Smith et al. [28] identified 10 key events for pertinent mechanistic studies characteristics for human carcinogenesis. For example, events can trigger carcinogenesis, such as involvement of growth factors-like mechanisms in this process leading to an uncontrollable proliferation of cells corresponding to a promotion process. Those alterations cause a growth disturbance leading the cells to form malignant tumors [29].

Moreover, epigenetic phenomena, including changes to the DNA methylome and chromatin compaction states, along with histone modification can also impact the carcinogenic process by affecting gene expression and DNA repair dynamics (Herceg et al. [30]), epigenetic process being relevant for both genotoxic and non-genotoxic agent [31].

1.2. Genotoxic and non-genotoxic carcinogens

Carcinogenic agents (natural, chemicals, drugs, contaminants) are considered to fall into two broad categories, depending on their mode of actions.

The first one is called **initiators**, they are DNA reactive carcinogens being able to initiate cells to carcinogenesis through direct interaction with DNA. These interactions result in DNA damage and/or structural/numerical chromosomal aberrations, which can be detected by genotoxicity tests. However, not all genotoxic agents are mutagenic; apoptosis and DNA repair can occur. To date, the standard approach to identify this class of carcinogens is an evaluation of mutagenic potential focused on the assessment of gene mutations and structural/numerical chromosomal aberrations and is generally carried out using a battery of *in vitro* bioassays with high specificity and sensitivity according to OECD guidelines. Recommended test battery strategy for genotoxicity evaluation has been issued by regulatory agencies. The bacterial reverse mutation assay (Ames test, OECD 471), which is the keystone test of the evaluation of chemicals, is in contrast not considered suitable for NPs owing to the inability of bacterial cells (unlike mammalian cells) to internalize particles. The use of mammalian cell models, especially human with a functional p53 is then recommended as a suitable battery of tests dedicated to NP assessment proposed by EFSA [16,17] to address the critical genotoxicity endpoints. Both the *in vitro* mammalian cell gene mutation tests using the HPRT and XPRT genes (OECD 476) [32] and the thymidine kinase gene (OECD 490)[33] are appropriately

completed with the use of a test for structural and numerical chromosome damage, *i.e.* the *in vitro* mammalian cell micronucleus test (OECD 487)[34]. To note, regarding to NPs, the most performed *in vitro* genotoxicity assays are micronucleus test and comet assay (Magdolenova et al. [26]).

A second category of carcinogens is non-DNA reactive agents called **promotors**. It has been estimated that 10–20 % of recognized human cancer carcinogens are classified as class I by the IARC act by non-genotoxic mechanisms [35]. These carcinogenic agents are, at least initially, devoid of direct interaction with DNA. Promotion, the second step of carcinogenesis process is reversible, and induced by different events. Indeed, the indirect modifications of DNA structures or function may induce altered gene expression and/or signal transduction. Then, non-genotoxic carcinogens refer to carcinogens negative in genotoxicity assays.

To date, there are no specific requests (and no OECD *in vitro* guidelines) to identify non-genotoxic mechanisms of carcinogenicity. In several chemical regulatory frameworks, then, for now, the risk posed by non-genotoxic carcinogenic agents for the consumer cannot be managed.

The only way to detect compounds with non-genotoxic action is to perform long-term *in vivo* study but animal testing can be waived when results from genotoxicity are clearly negative and when no structural alerts are identified for most of regulations. Limited assessment of carcinogenicity increases the probability of consumers to be exposed to agents able to promote tumors and alter hormone responsiveness; this is also true for NPs as demonstrated by Fontana et al. [36] with silica NPs.

1.3. Carcinogenicity studies and their drawbacks

I*n vivo* studies are essential for a complete hazard evaluation of carcinogens; they are used in the risk assessment process, with internationally agreed testing methods, which rely on rodents assays. The standard approach is to conduct a 2-year study on two different rodent species according to the OECD guidelines (OECD 451 [37]or OECD 453 [38]). But, these assays have some limitations; using a large number of animals (600–800 per chemical), they have a prolonged duration (2 years), are very expensive (at least one million euros), raised ethical concerns, and gave scarce mechanistic information making difficult to completely understand the human relevance ([39], Madia et al., 2019 [40]).

Lifetime rodent cancer bioassays have to be performed if any *in vivo* genotoxicity tests are positive but under most chemical regulations except pharmaceutical, plant protection, and biocide safety this is rare [41]. Moreover, retrospective analysis of carcinogenicity data revealed that mouse studies have not a substantial benefit in regulatory decision-making [42].

In accordance with the 3 R rules which support efforts to find alternative methods for testing on animals, the development of an *in vitro* strategy based on mode of action to evaluate carcinogen potential is becoming increasingly an appreciated aspect of hazard assessment urgently required, especially for the promotion step, as the best strategy to drive new developments [43].

To identify promotion property, cell transformation assays, as an *in vitro* alternative approach, were developed, using Balb/c 3T3 derived cells or SHE cells.

Sasaki et al. [44] developed a cell transformation assay (CTA), based on the ability of normal cells to transform into malignant cells detecting both genotoxic and non-genotoxic carcinogens, mimicking, by the way, some key stages of the *in vivo* multistep carcinogenesis.

Although being validated by the European Center for Validation of Alternatives Methods (ECVAM), the CTA is not currently used for regulatory purposes. CTA is often performed to clarify *in vitro* genotoxicity results, evaluate classes of chemicals that have a low predictive capacity in traditional *in vitro* genotoxicity assays (*e.g.* aromatic amines), and investigate tumor initiation/promotion activity (mechanisms of action of specific carcinogens) [45]. It is used by academia, and by chemical, agro-chemical, cosmetic, tobacco and pharmaceutical industries and as part of weight of evidence assessment [46]. Furthermore, CTA is recognized as a useful assay for substances with structural alerts for carcinogenicity to demonstrate similarities (or not) across a chemical category [45]. The scientific committee on consumer safety (2018) mentioned the use of CTA in its guidance for testing cosmetic ingredients, as a possible alternative to animal models to detect genotoxic and non-genotoxic carcinogens. A tiered approach based on Ames test and the SHE assay demonstrated detection of up to 90 % of carcinogens [47]. EFSA [46] in its scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment, recommended also CTA as a promising new test method.

However, CTA by measuring induction of phenotypic alterations does not provide any information on molecular key events, nor mechanism of action making this bioassay not fully contemplated in the regulatory context. Three main concerns were raised for final OECD acceptance: the reproducibility of the data between laboratories, the subjective nature for assessing transformation (morphological characteristics), and the lack of knowledge on the molecular mechanisms involved in the transformation.

This article reviews the use of CTA with NPs and highlights important key points to perform and improve this bioassay for a relevant assessment of the NP carcinogenic potential. To our knowledge, no study using SHE was performed with NPs, then this review will focus only on the Balb/c 3T3 cells-based assays.

2. Cell transformation assay methods

CTA measures the change in phenotype between normal and malignant cells in mammalian cells after exposure to genotoxic or non-genotoxic carcinogens. The cell transformation assay was demonstrated to involve a multistage process that closely models key stages of *in vivo* carcinogenesis [48]. Formation of transformed foci is the consequence of the complex process of transforming cells into a malignant state. Transformed cells can lead to tumor formation *in vivo* when injected into a suitable host, underlining their biological relevance [39]. This assay was developed on different cell lines [45].

2.1. Cell transformation assay on immortalized Balb/c 3T3 cells

The history of the CTA development is presented in Fig. 1 and the development on Balb/c 3T3 cells has been reviewed by Schechtman [49].

The chemical transformation of 3T3 cells resulting in the induction of morphologically aberrant foci was reported by Kakunaga and Kamahora [50] from immortalized cells of the BALB/c 3T3 A31 subclone issued from whole embryo cultures of BALB/c mice, established by Aaronson and Todaro [51]. These cells grow as a monolayer, they stop dividing when the culture surface is covered. However, upon treatment with carcinogens at the beginning of growth, some cells do not stop proliferation and grow as morphologically aberrant foci over the monolayer of normal cells. They grow as multilayers, are spindle-shaped, dense with basophilic staining, and are able to induce tumor if injected in nude mice [52].

Several sub-clones of the original clone A31 were generated and some were analyzed for their suitability for cell transformation. The subclone, A31–714 isolated by Aaronson and Todaro [51] got also good properties such as contact-inhibition after reaching confluence, strong cell-to-substrate adhesion, high cloning efficiency, cell doubling time < 20 h, high sensitivity to transformation induced by chemical, and low background of spontaneous transformation [53].

Later, Kakunaga and Crow [54] established an A31–1–1 clone, stable for transformation after chemical treatment and UV sensitive, and they recommended its use for the cell transformation assay. This clone has erroneously been considered to be derived from Balb/c 3T3 A31. A

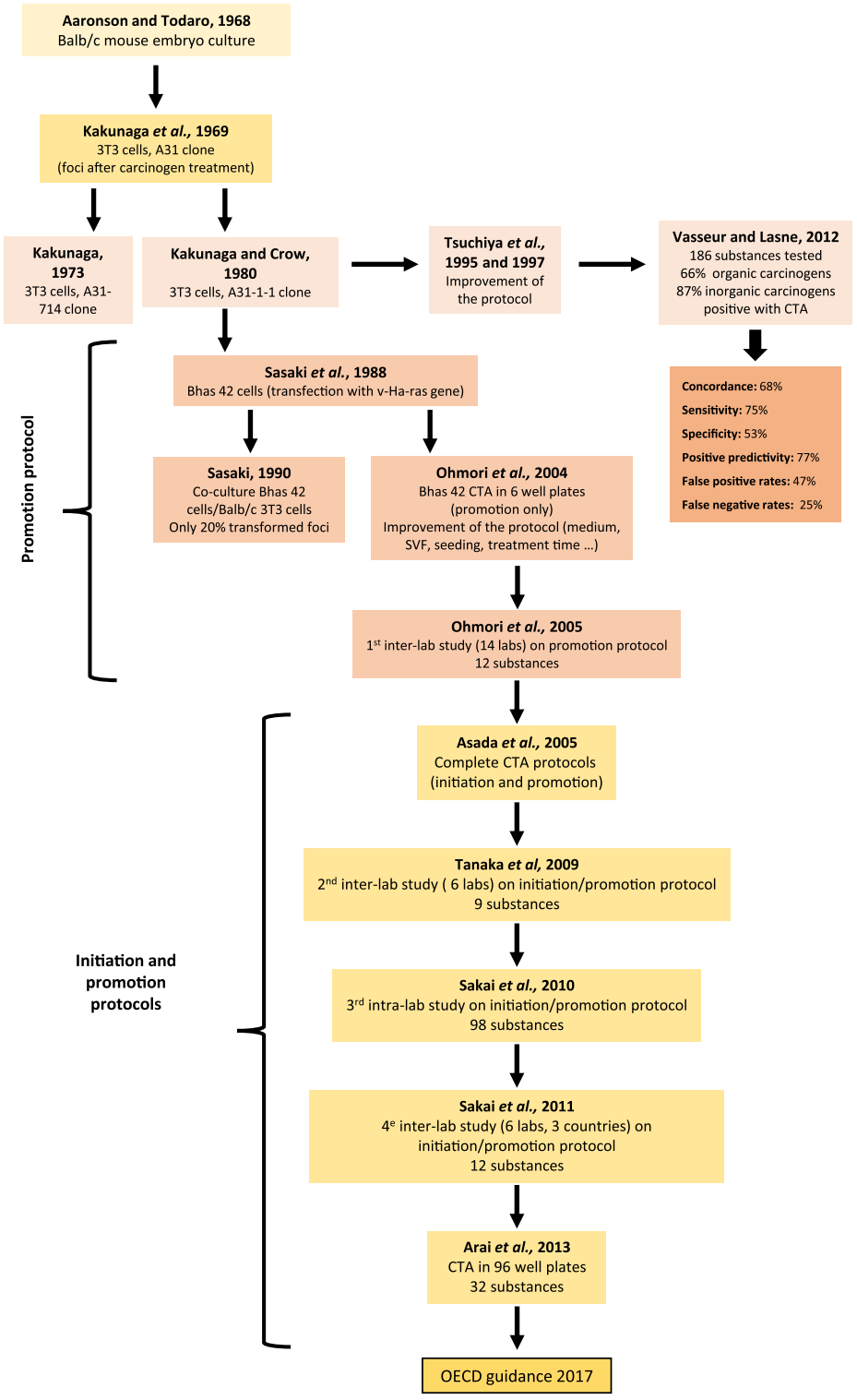

Fig. 1. Overview of the development of the cell transformation assay from derived Balb/c 3T3 cells.

subsequent characterization performed by Uchio-Yamada et al. (2017) [55] confirmed that this clone is from Swiss mouse.

Among all the clones studied, those exhibiting low frequencies of cell transformation appeared to have a reduced sensitivity to certain chemical carcinogens. They differ also substantially both in their capacities to endogenously metabolize test compounds, and in their spontaneous transformation frequencies [56].

Indeed, *in vitro*, different parameters could influence the cell proliferation and the transformation frequencies such as cell culture method (pH, medium, serum, seeding density), cell passage number [57,58], treatment period and time, cell division time required for fixation and expression of the foci.

Some authors demonstrated that treatment with well-known tumor agents after exposure to chemicals enhanced the transformation potential of cells (two-step method) [59]. Cell trypsinization at confluence increased the number of transformed foci [58] and increasing the seeding rate and processing the cells one day after seeding gave better cell transformation [60]. Lastly, decreasing the FBS content in the

culture medium improved the sensitivity of the test [61].

A new protocol was developed to address some of these items, especially to shorten the duration of the assay (Tsuchiya and Umeda [62]). This protocol involves clone A31–1–1 cells, recommended by Kakunaga and Crow [54] and a modified medium, in conjunction with a new treatment schedule. This resulted in a substantial improvement in the transformation frequency, the formation of clearly transformed foci at an earlier stage of incubation, an enhanced responsiveness of the assay to several carcinogens, and a new protocol suitable for investigating promoting activity (Tsuchiya and Umeda [63]).

In parallel to the development of the protocol on the clone, a set of 186 chemicals tested in the Balb/c 3T3 CTA were reviewed by Vasseur and Lasne [64]. It includes 165 organic chemicals (112 classified as rodent carcinogens, 53 as rodent non-carcinogens) and 21 inorganic chemicals (15 rodent carcinogens, 6 rodent non-carcinogens). Sixty-six percent of the rodent organic carcinogens and 87 % of the inorganic carcinogens were positive in the assay giving a concordance of 68 %, a sensitivity, specificity and positive predictivity of 75 %, 53 % and 77 %, respectively. False-positive and negative rates were 47 % and 25 % respectively.

However, there are still some drawbacks with this assay, notably the time (4–6 weeks of cell culture) required for the development of unequivocally transformed foci; the relatively low frequency of transformation making this assay quite labor-intensive and relatively time-consuming.

2.2. Cell transformation assay on Bhas 42 cells

2.2.1. Origins and characteristics of Bhas 42 cells

From the subclone A31–1–1 of Balb/c 3T3 cells, Sasaki et al. [44] established a new cell line by stable transfection using v-Ha-*ras* gene, with a few copies of v-Ha-*ras* genes integrated into the chromosomal DNA (chromosomes 17 and 19), (Sasaki et al., 2015)[65].

Bhas 42 (clone n°42 of v-Ha-*ras* transfected Balb/c 3T3 cells) are morphologically non-transformed cells sensitive to contact inhibition [44]. They do not form foci but they can be converted to transformed foci after treatment with a well-known promoter, e. g. 2-*O*-tetradecanoylphorbol-13-acetate (TPA). To check the tumor-inducing capacity of transformed cells, they were inoculated subcutaneously into nude mice and form tumors (latent period: 20–60 days) in contrast to non-transformed cells by 90 days. Thus, Bhas 42 cells are regarded as being initiated cells [66].

This assay is a surrogate system to observe the transition of cells from premalignant to malignant stages.

The new method on Bhas 42, markedly scaled down from the Balb/c 3T3 one, has many advantages, *e.g.* a high sensitivity, a shorter experimental period, and the use of smaller amounts of materials with a simplified procedure and a more efficient cost ([67]; Sakai et al., 2007 [68]).

2.2.2. History of the test improvement

The first study of transiently transfected Bhas 42 cells showed their ability to transform after 2 weeks of treatment with TPA, followed by 3 weeks of growth to allow the developing of foci, instead of 4–6 weeks for the Balb/c 3T3 CTA. This transformation is characterized by significant morphological changes not so easy to quantify by the scorer under microscope after Giemsa staining (spindle shape, loss of contact inhibition, criss-cross of cells and appearance of multilayers) [44].

The second experiment used the previously stably transfected cells established by Sasaki et al. [44] in co-culture with Balb/c 3T3 cells. Inoculated at 100 cells/dish for 10^4 Balb/c 3T3 cells, they were treated for 2 weeks with TPA and foci were counted 6 weeks after seeding (Sasaki, 1990). However, only 20 % of the Bhas 42 cells formed foci suggesting that normal cells prevent division of the transformed cells.

Thus, Bhas 42 cells have been used alone to develop a specific CTA [69], with only a protocol for the detection of chemical promoting activity. Many parameters were studied to determine the conditions allowing the maximum number of well-identifiable foci and a cell growth assay was developed in parallel to choose the concentration range to test in the CTA. For this, media with different percentages of fetal calf serum, cell seeding rates, promoter treatment and post-treatment culture times were compared. Finally, the percentages in the culture medium of solvents (DMSO, ethanol, acetone) used to dissolve compounds were also studied on the foci appearance. The main improvements were: omission of Bhas42 cell treatment with an initiating agent and with a subsequent cultivation for an expression period, a shortened period to 3 weeks, with a half of the time needed for the Balb/c 3T3 assay. The assay was also miniaturized: the size of the plates and the volume of medium used were then decreased to 6-well dishes and 2 mL, respectively. Only 3–6 wells were mandatory to test one dose instead of 10–20 dishes and the percentage of fetal bovine serum in the medium decreased from 10 % to 5 %.

Ohmori et al. [70] published the first inter-laboratory study with this optimized protocol. Fourteen laboratories tested 12 compounds in the project. The first step was to implant the cell growth test and the Bhas 42 promotion assay in the different laboratories using two tumor promoters, TPA and lithocholic acid (LCA) treatment. The latter was to test positive controls for the ability of the laboratories to perform the test. Finally, 12 compounds were tested and analyzed by 4 laboratories. Same results were obtained by the 4 labs with eight substances (4α-phorbol, 17β-estradiol, mezerein, phorbol 12,13-didecanoate, progesterone, diethylstilbestrol, insulin, sodium saccharin) giving a quite high consistency rate. For two substances (okadaic acid, catechol), only one of the four labs presented discordant results. Inconsistent results for two chemicals (dexamethasone, anthralin) required further improvements and technical refinement of the protocol.

In 2005, Asada et al.[67] published the first study using a complete CTA test (detecting both the initiating and promoting activities of chemicals), using two protocols performed in parallel and based on a difference in the cell seeding number and in the chemical treatment time. In the promotion assay, Bhas 42 cells were exposed to chemicals when cells were near confluence. Since initiators need several cell divisions for gene mutation fixation, a new treatment protocol was designed: cells were seeded at a lower rate to divide several times in culture dishes after chemical treatment. After checking the feasibility of this new protocol using positive controls (MMNG, MCA for initiation and TPA, LCA, and okadaic acid for promotion), they tested 16 PAHs. Then, PAHs were divided into 4 groups according to the results. Benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene induced only foci formation in the initiation assay (group 1) in contrast to benzo[*e*]pyrene, benzo[gly]perylene, 1-nitropyrene and pyrene inducing foci only in the promotion assay (group 2). Positive answers were observed with benz[*a*]anthracene, benz[*b*]anthracene, chrysene and perylene in both initiation and promotion (group 3). For acenaphthylene, anthracene, coronene, 9,10-diphenylanthracene, naphthalene and phenanthrene, results were negative or equivocal (group 4). These findings suggested different modes of action of PAHs and an increased risk when people are exposed to PAH mixtures.

Further, 3 large-scale, intra- or inter-laboratory studies were undertaken to validate the complete test. Tanaka et al. [71] tested 9 substances in 6 laboratories, and then Sakai et al. [72] tested 98 compounds in his own lab. Performance indices were calculated from the results of 52 carcinogens and 37 non-carcinogens. The concordance was 78 %, sensitivity 73 %, specificity 84 %, positive predictivity 86 %, negative predictivity 69 %, false-negative 27 % and false-positive 16 %. The concordance, specificity, negative and false-positive predictivity were superior while the other performance indices were equivalent to conventional genotoxicity test indices, identifying genotoxic carcinogens making the assay promising. In 2011, Sakai et al. published the results of an international validation study, carried out in 6 laboratories of 3 different countries (Japan, USA, Germany) with 12 compounds, positive or negative in the *in vivo* carcinogenesis assay. The Bhas 42 CTA

correctly distinguished all six carcinogens and two tumor promoters from four non-carcinogens, demonstrating the transferability, reproducibility between laboratories, and its applicability to predict a mode of action in the carcinogenicity.

Finally, in 2013, Arai et al. (2013) [73] proposed a miniaturized version with the use of 96-well plates (instead of 6-well plates), for high-throughput automated applications. This protocol was validated using 32 compounds already tested in the 6-well method. The results were concordant between the two techniques, except for one compound, sodium arsenite.

Then, these data using 6-well and 96-well studies have led to an OECD guidance on the in vitro Bhas 42 cell transformation assay [74].

2.2.3. Protocols of Bhas 42 CTA according to the OECD guidance (2017)

In parallel to the CTA, a proliferation assay (Fig. 2) is necessary to establish the concentrations affecting the cell growth. The OECD guidance recommends testing chemicals on a large concentration range: from weak or no toxicity to the highest acceptable level corresponding to less than 20 % survival compared to the negative control. At least 5 concentrations should be tested: (a) one concentration below the non-toxic level (around 80–120 % of cell growth), (b) two concentrations between the non-toxic level and the 50 % inhibitory concentration (IC_{50}) and (c) two concentrations between the IC_{50} and the IC_{90} [74].

In the initiation assay, the cells are inoculated at a low density (4 000 cells/well in 6-well protocol) and exposed at the beginning of growth phase to a test chemical for 3 days, 24 h after seeding to detect initiating activity of the chemical.

For the promotion assay, the seeding is higher (14,000 cells/well in 6-well protocol) with cells being sub-confluent on day 4. Cells are treated continuously when they are sub-confluent for 10 days. This condition allows cell-to-cell communication and particularly favors the promoting activity of chemicals. The total culture duration is then, 21 days in each assay with a fixing and staining step at the end to observe the wells under light microscope. Representative protocols issued from the OECD guidance (2017) are shown in Fig. 2.

Regards the application of this guidance for NPs testing, some adjustments are needed. It is discussed in the #4 "Keypoint to perform CTA for NPs testing".

2.2.4. Controls (MCA/TPA)

Negative and positive controls must be included each time an experiment is performed. For negative controls, culture medium alone should not induce more than 10 foci/well (initiation test) and 12 foci/well (promotion test) in the 6-well protocol. Solvent used to dilute chemicals is always included for the positive controls (diluted in DMSO). The positive controls are depending on the protocol. The typical carcinogen 3-methylcholanthrene (MCA, 1 μg/mL) is used for initiation assay. It induces many transformed foci (52) in the initiation assay, but none in the promotion assay (less than 10). For the promotion test, the well-known tumor promoter 2-*O*-tetradecanoylphorbol-13-acetate (TPA, 50 ng/mL) produces a great number of foci in the promotion assay (55), but none in the initiation assay (less than 5) (Sakai, 2007).

These positive controls were used in all the published studies but variabilities in the number of foci/well with the same chemical concentration were observed between laboratories [70,72,75]. This specific point is discussed later (see #4.3.).

3. Literature review on the use of CTA with NPs

Few articles were published regards to NP toxicity performing the CTA. NPs tested on Balb/c 3T3 and Bhas 42 CTAs are compiled thereafter (Table 1). In these articles, the NPs studied with CTA were all properly characterized before testing as *in vitro* assessment may vary according to physical-chemical parameters of NPs.

3.1. Studies with Balb/c 3T3 protocol

All the articles published with the Balb/c 3T3 protocol only focused on the initiation step, except one (Sighinolfi, 2014[76]), where the two-steps were performed. The Joint Research Center of the European Commission published the 5 following articles using the Balb/c 3T3 assay to assess NPs to support the European strategy to develop *in vitro* systems and to understand the manufactured NP mechanisms of action:

Ponti et al. [77] were the first authors to use the CTA for studying NPs. They assessed the morphological transforming activity of **cobalt NPs** (Co-nano) and **cobalt ions** (Co^{2+}) in Balb/c 3T3 cells after 72 h treatment (initiation assay) using sodium chromate (50 μM) as a positive control [78]. A total of 1.5×10^3 cells/mL in 10 mL of complete culture medium were seeded in each 100 mm dish (10 replicates for each

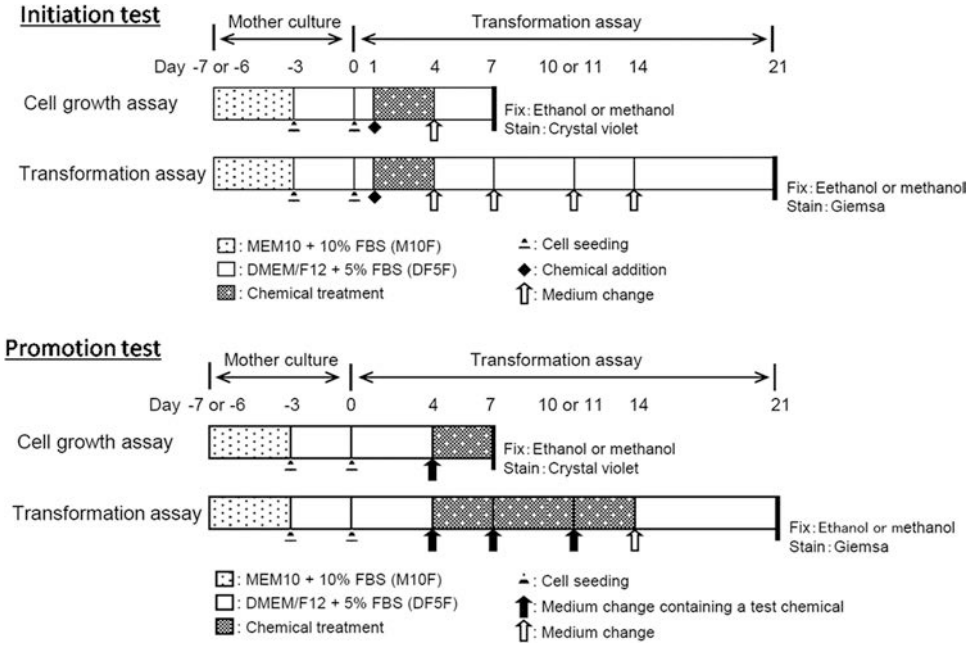

Fig. 2. Time line for the initiation and promotion test component of the transformation assay (6-well format) (OECD guidance, 2017).

Table 1
Overview of the studies performing cell transformation assay for testing NPs. Blue: positive in initiation protocol. Red: positive in promotion protocol.

Year	First author	Co-authors	Cell Model	Nanoparticles	Solvent	Concentrations	Treatment time	Results	Positive control
2009	Ponti	JRC	CTA Balb 3T3	cobalt NP $cobalt^{2+}$	Ultrapure sterile water	1, 7, 10, 30 µM 1, 7, 30, 70 µM	72h (initiation)	positive except 1 µM negative	$Na_2CrO_4,4H_2O$ (50 µM)
2012	Uboldi	JRC	CTA Balb 3T3	amorphous $SI0_2$ NP	Ultrapure sterile water	100 µg/mL	72h (initiation)	negative	MCA (4 µg/mL)
2013	Ponti	JRC	CTA Balb 3T3	multiwall carbon nanotubes nude and functionalized	Dimethylsulfoxide	1, 10, 100 µg/mL	72h (initiation)	positive without dose effect at least at one concentration	MCA (4 µg/mL)
2014	Sighinolfi		CTA Balb 3T3	Fe_30_4, CeO_2, Au NP	Phosphate Buffer Saline	10 µM	2 stage treatment (initiation: NP + TPA promotion: MCA + NP)	negative as initiators positive as promoter (Fe_3O_4 and Au)	Co NP (10 mM)
2016	Stoccoro	JRC	CTA Balb 3T3	pristine,remediated citrate/silica ZrO_2, TiO_2 NP	Ultrapure sterile water	10, 20, 40 µg/cm²	72h (initiation)	positive for pristine, low and high citrate coated ZrO_2 NP positive for low and high citrate coated TiO_2 NP	MCA (4 µg/mL)
2016	Uboldi	JRC	CTA Balb 3T3	titanium dioxide nanoparticles	Deionized water	1, 5, 10 µg/mL	72h (initiation)	positive for nanosized and bulk rutile TiO_2 negative for nanosized and bulk anatase TiO_2	MCA (4 µg/mL)
2017	Choo		CTA Balb 3T3	AgNP	MEM medium	0.17, 0.66, 2.65, 5.30, 10.60 µg/mL	72h (inititiation)	positive with dose effect	B[a]P 1µg/mL
2011	Hasegawa		CTA Bhas 42	micro-sized In203, Dy 203 nano-sized In 203, Dy 203 WO_3 et Mo	Ultrapure sterile water	0.01 to 5 µg/mL	10 days (promotion)	positive for nano-sized Ln_2O_3 and Dy_2O_3, negative for WO_3 and Mo	not mentionned
2016	Gabelova		CTA Bhas 42	nanosilver (AgNM300), AgNM302) MNP differing in surface modifications	MilliQ water	0.1-10 µg/cm² for nanosilver 0.25-50 mM for MNPs	3 days (initiation)	negative for spherical AgNM300, SO-MNP, SO-PEG-MNP positive for nanorods/wires Ag NM302 and SO-PEG-PGLA-MNP	MCA (3 µg/mL)
2017	Fontana		CTA Bhas 42	SiO_2 (NM 200, NM 201, NM 202, NM 203), Min-U-Sil, diatomaceous earth	Sterile deionized water	2 to 80 µg/cm²	3 days (initiation) 10 days (promotion)	negative as initiators positive as promoters	MCA (1 µg/mL) TPA (0.05 µg/mL)
2020	Jalili	Fontana	CTA Bhas 42	Al^0, Al_2O_3 and $AlCl_3$	Distilled water	0.3 to 28 µg/cm²	3 days (initiation) 10 days (promotion)	negative as initiators and promoters	MCA (1 µg/mL) TPA (0.05 µg/mL)

treatment). After 24 h, the medium was changed and replaced with one containing selected concentrations of Co-nano or Co^{2+}. Co-nano was tested at 1, 7, 10, 30 µM and Co^{2+} at 1, 7, 30, 70 µM corresponding respectively to IC20, 30, 50, 80, determined by Colony-Forming Efficiency (CFE). After 72 h treatment, cultures were maintained for 5 weeks, with the culture medium renewed twice a week. At the end of the experiment, the cells were fixed and stained. Co-nano were characterized in water and in complete culture medium for their morphology by Scanning Electron Microscope/energy dispersive using X-ray (SEM-EDX) technique and size distribution by NP tracking analysis.

An increase in morphological transformation was observed for all concentrations of Co-nano (except for Co-nano at 1 µM); while no statistically significant morphological transformation was observed for Co^{2+}. These results agree with Lison et al. [79], who demonstrated a genotoxic potential for cobalt *in vitro* without testing the morphological transforming potential. Concerning the morphological transformation by cobalt and its compounds, the IARC [80] concluded that they are possibly carcinogenic to humans (Group 2B) since there was sufficient evidence in experimental animal studies but inadequate evidence for carcinogenicity in humans (lung cancer) [81].

In 2012, Uboldi et al. [82] analyzed the initiation property of **amorphous silica NPs** ($aSiO_2$NPs) with the Balb/c 3T3 CTA. Compared to the previous article, the protocol was used with minor modifications such as the seeding number (3.3×10^3 cells/mL instead of 1.5×10^3) and the positive control (MCA at 4 µg/mL instead of sodium chromate at 50 µM). NM-200 and NM-203 belong to the list of NMs of interest to the OECD and were delivered in powder form, while the synthesis of NRT-808, NRT-817, NRT-820 and NRT-944 was performed in the laboratory. Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM) were used to study the size distribution and morphology of the particles, respectively. NPs were not cytotoxic after 72 h as determined by CFE.$aSiO_2$NPs (100 µg/mL) did not induce any morphological transformation in the Balb/3T3 cells despite the internalization (uptake and intracellular distribution) of the NPs confirmed by fluorescence microscopy following exposure to 85 nm fluorescently labeled $aSiO_2$NPs. They were located exclusively in the cytoplasmic region. Neither small (16–80 nm) water dispersed aSiO2NPs, nor larger (NM-200; 200 nm size diameter) silica particles, nor pyrogenic $aSiO_2$NPs (NM-203; 300 nm size diameter) showed any carcinogenic potential in CTA, suggesting that size or synthesis method of $aSiO_2$NPs had no impact.

Few years later, Ponti et al. [83] studied the morphological transformation of Balb/c 3T3 induced by **multiwall carbon nanotubes** (mw CNTs) mainly used for the production of smart textile. They investigated the toxicity of nude and chemically functionalized (-NH2, -OH and -COOH groups) mw CNTs. Minor modifications of the protocol were applied compared to Uboldi et al. [82]: after 72 h of exposure, the suspensions were changed with fresh M10F instead of directly adding the DMEM/F12 medium and only from day 7, DMEM/F12 medium was used till day 24 and the medium was not changed during the last culture week.

The size distribution of the mwCNTs suspended in complete culture medium at the highest concentration tested (100 mg/mL) was measured by DLS. The sedimentation of different types of mw CNTs (nude, -OH, -COOH and -NH2), dispersed as described above, was characterized by visible and near-infrared light absorption (VIS-NIR). Mw CNTs interaction with cells was investigated, using a Scanning Electron Microscopy (SEM) both in reflection and transmission configurations, and using a Transmission Electron Microscope (TEM). In this case, some mw CNTs were internalized by the cells, but they strongly interacted with cells and cell membrane with persistence.

The authors observed morphological transformation of mw CNTs in four independent experiments and the average data showed statistically a significant increase at least at one concentration for all the nude forms or functionalized mw CNTs but without any dose-response.

Three non-cytotoxic concentrations were tested (1, 10 and 100 µg/mL). Transformed foci increased at 1 mg/mL ($p < 0.001$) and 100 µg/mL ($p < 0.01$) for –NH2; at 1 µg/mL ($p < 0.05$) for -COOH; at 10 µg/mL ($p < 0.01$) for –OH and at 100 mg/mL ($p < 0.001$) for nude mw CNTs. However, as recommended by the OECD guidance, a second experiment is necessary to confirm the positivity.

Stoccoro et al. [84] studied the morphological neoplastic transformation potential on Balb/c 3T3 cells of **titania** (TiO_2) and **zirconia** (ZrO_2) NPs, either in their **pristine** (uncoated) form or **modified with citrate and/or silica** on their surface. As benchmark material, commercial TiO_2 preparation (Aeroxide®P25) was used. The chemical-physical characterization of the pristine and modified ZrO_2 and TiO_2 samples was performed by TEM, DLS and Zeta-potential. No or low cytotoxicity (<30 %) was noted by the authors, whatever the concentrations and treatment time tested.

CTA was carried out as reported by Ponti et al. [83] on Balb/3T3 cells treated with ZrO_2 and TiO_2 NPs at concentrations of 10, 20 and 40 µg/cm², corresponding to 91.6, 183.3 and 366.6 µg/mL, respectively.

The pristine and low/high citrate coated **ZrO_2** NPs induced statistically significant morphological transformations with dose-effect suggesting initiation properties. In contrast, the silica-coated form did not induce any cell transformation suggesting the silica coating able to preserve cells from the foci formation and hence from morphological transformation.

For **TiO_2** NPs, only citrate-coated and P25 induced the formation of foci while pristine and silica-coated NPs did not induce any cell transformation. In 2010, TiO_2 was recognized as a possible carcinogen for humans (group 2B) [85]. After France in 2019, since January 2022, TiO_2 is now forbidden in Europe as a food additive (Regulation 2022/63).

Uboldi et al. [86] examined the effects of **nanosized and bulk titanium dioxide** (TiO_2) **in two different crystalline forms** (anatase and rutile) with CTA, performed according to Ponti et al. [83]. Inductively Coupled Plasma-Mass Spectrometry (ICPMS) was used to study the uptake of titania in Balb/3T3 cells as it allowed to relate the amount (expressed as mass and number of particles) of TiO_2 NPs internalized and their toxic potential. DLS and TEM were applied to characterize stock suspensions and Zeta potential was also measured at pH 7 for all NPs. Nanosized rutile TiO_2 (Ru10), bulk rutile (BRU), nanosized anatase (An-10) and bulk anatase (BAN) were tested at 1, 5, 10 μg/mL, as low cytotoxic concentrations (<30 %).

Crystalline forms had no impact on the internalization; in contrast, nano-titania NPs were more internalized than their respective bulk materials suggesting that the uptake was size-dependent.

Ru-10 induced a statistically significant dose-dependent increase in cell transformation. Similarly, BRU resulted in transforming Balb/c 3T3 cells at all concentrations. In contrast, although An-10 and BAN slightly enhanced foci formation whatever the concentrations, their effect was not statistically significant. In fact, while a significantly higher internalization of anatase TiO_2 NPs was measured, only the rutile crystalline form induced morphological transformation in Balb/c 3T3 fibroblasts. These results might be due to different reactivity of the two investigated crystalline forms. Actually, rutile TiO_2 NPs are more photocatalytic than anatase and consequently, are able to produce greater numbers of oxygenated free radicals on their surface [87,88] leading to indirect genotoxicity and then mutations. Recently, EFSA [16,17] published a scientific opinion on the safety assessment of TiO_2 with TiO_2 particles having the potential to induce DNA strand breaks and chromosomal damages but not gene mutations and by the way, EFSA concluded that genotoxicity cannot be ruled out due to uncertainty concerning the mode of action involved (threshold or not).

Independently from the JRC, two teams published results on the Balb/c 3T3 CTA performed with NPs:

Choo et al. [89] carried out the CTA to assess carcinogenic potential of **silver NPs (AgNPs)**, after a 72 h treatment. The Ag nanopowder (<100 nm), was completely dispersed in an experimental medium by sonication and was characterized by energy dispersion X-ray spectrum (EDS) analysis, TEM, SEM, DLS, and Zeta potential. Protocol was the same as described previously with minor modifications compared to Uboldi et al. [82]. 2×10^3 cells/mL were seeded in M10F complete medium, after 24 h of incubation, medium was changed with media containing benzo[*a*]pyrene (B[*a*]P) as a positive control (instead of MCA in [82]), anthracene as a negative control, or AgNPs at five concentrations (0.17, 0.66, 2.65, 5.30, and 10.60 μg/mL). Two cytotoxicity tests were performed, Cristal Violet and CFE with different determinations of t IC_{50} (0.17 μg/mL and 5.91 μg/mL respectively). The chosen concentrations were as requested by the OECD guidance (from no observable adverse effective concentration to IC_{90}) corresponding to CV cytotoxic results.

The positive control (B[*a*]P) (1 μg/mL) showed a 21.33-fold increase of foci formation per plate in contrast to negative control (Anthracene, 20 μg/mL). Cells exposed to AgNPs for 72 h showed significantly induced morphological transformation at all concentrations with a concentration dependency up to IC_{90}.

Sighinolfi et al. [76] **is the only** study with the two-stage cell-transformation protocol using BALB/c 3T3 cells. They assessed the carcinogenic potential of **metal (Fe_3O_4, CeO_2,** and **Au) NPs**, used for medical applications as contrast agents in NMR, or as catalysts and UV adsorbent and tumor hyperthermia agents.

Treatments with a tumor initiator 3-methylcholanthrene (MCA, 0.5 μg/mL) at the beginning of cultivation and then with TPA (300 ng/mL), as tumor promoter to induce preneoplastic foci were used. This two-stage transformation assay was based on the improved method proposed by Kajiwara et al. [90]. In particular, it was used a special medium supplemented with a mixture of insulin, transferrin, ethanolamine, and sodium selenite (ITES) and a low concentration of fetal bovine serum (FBS). Then, it was demonstrated that this medium together with MCA+TPA treatment caused high-frequency transformation with foci appearing earlier (after 45 days instead of the 90 days). Cytotoxicity was studied on a range of concentrations from 1 to 100 μM with CFE and XTT assays, after 24 h or 72 h of treatment. Mild cytotoxic effect (<30 %) was noted for all NPs at 50 and 100 μM after 24 or 72 h of treatment, except for Co, which was concentration dependent cytotoxic, with high cytotoxic effect (IC_{90}) at the highest concentrations (50 and 100 μM).

As NPs were tested in various combinations as potential initiators, promoters or both, NPs were used at the concentration of 10 μM with/without MCA or TPA, following the two-stage protocol. Actively growing cells were seeded at a density of 5×10^4 cells/dish in 5 mL of DMEM culture medium. Twenty-four hours after seeding, the cells were treated with/without the tumor initiator MCA at the concentration of 0.5 mg/mL with/without NPs. After three days, the medium was changed and ITES medium was added for another three days. Cells were then exposed (or not) to the tumor promoter TPA, at the concentration of 300 ng/mL with/without NPs. After two weeks, cells were normally seeded with ITES medium for another four weeks, fixed and stained.

Cobalt NPs at a 10 mM concentration was selected as reference carcinogenic compound to perform the two-stage transformational assay [77]. The NP suspensions were characterized using the Zeta Potential (Z-Potential) and DLS to define the hydrodynamic diameter of the NP dispersion.

Data with NPs were depending on their respective chemical-physical properties. Indeed, while Fe_3O_4, Au, and CeO_2 NPs, when used as tumor initiators, did not give rise to neoplastic transformation. In contrast, Co NPs were able to induce foci formation, confirming the data of Ponti et al. [77].

Fe_3O_4 and Au NPs, when tested as a promoter, showed a dramatic increase of neoplastic foci, as compared to MCA alone, reaching values similar to the well-known promoter TPA. The fact that Co NPs were not able to generate foci as a promoter could be attributable to its intrinsic cytotoxic activity.

Findings regarding the activity of CeO_2 NPs were quite interesting. In fact, CeO_2 NPs treatment after MCA treatment did not change the number of foci after treatment by MCA alone (17 and 19 respectively) but decreased significantly the foci formation when compared to the number of foci induced by treatments with positive controls (MCA+TPA) (38), suggesting a potential antagonism in the promotion of cell transformation at non-cytotoxic concentrations.

3.2. Studies with the Bhas 42 protocol

To date, only four articles related to NPs and using the Bhas 42 protocol are available in the literature (Table 1).

Hasegawa et al. (2012)[91] were the first to publish in 2012 a study on the tumor promotion activity of rare metals; (**molybdenum (Mo)** and metal oxide (**indium oxide (In_2O_3), dysprosium oxide (Dy_2O_3) and tungsten oxide (WO_3)** NPs according to the protocol of Ohmori et al. [69].

Each metal was characterized. Purity, diameter, primary crystallite size, surface area and impurities were provided by the manufacturer, mean diameter in solution was measured by DLS and Zeta potential.

Growth inhibition of Bhas 42 cells was observed when more than 10 mg/mL of rare metals (microsized) were added in the medium, while a lower transformed colony number was observed in the group treated with the nano-sized In_2O_3 and Dy_2O_3 at the concentration of 5 mg/mL, which could be attributed to metal oxides cytotoxicity.

Both nano- and micro-sized In_2O_3 increased significantly transformed colonies at concentrations above 1 mg/mL and 2 mg/mL, respectively. For both **nano- and micro-sized Dy_2O_3** also the number of transformed colonies increased significantly at concentrations above 2 mg/mL. At the top concentration (5 mg/mL), the number of transformed colonies induced by the nano-sized In_2O_3 and Dy_2O_3 was lower than in the group treated with micro-sized NPs suggesting that size may play a role in the cytotoxicity. In contrast, **WO_3** or **Mo** did not increase the number of transformed colonies in the concentration range of 0.01–5 mg/mL whatever the size (nano- or micro-particles). These results showed that particle sizes of these metals did not affect the promotion activity. However, in this study, six independent experiments were performed for each and in duplicate with a high standard deviation suggesting a poor reproducibility of the assay, perhaps due to the subjectivity of scoring and difficulties to detect unequivocally foci. Furthermore, no positive control for the CTA was used in this article.

Gabelova et al., [92] investigated the capacity of two frequently used metallic engineered nanomaterials (ENMs), nanosilver and magnetite NPs (MNPs), to induce transformed foci in Bhas 42 cells using the tumor initiation protocol. Two types of nanosilver, **spherical NPs (AgNM300) and fibrous (AgNM302) nanorods/wires, and MNPs differing in surface modifications [MNPs coated with sodium oleate (SO-MNPs), MNPs coated with SO + polyethylene glycol (SO-PEG-MNPs) and MNPs coated with SO + PEG + poly(lactide-*co*-glycolic acid) SO-PEG-PLGA-MNPs]** were included in this study. Particles were characterized in terms of particle size, magnetism and Zeta potential in stock and in culture medium. The silver NPs were tested at 0.1, 0.3, 1, 3 and 10 μg/cm^2 and the MNPs at 0.25, 0.5, 1, 2.5, 10, 25, 50 mM.

Two negative controls were used in these experiments: (i) Control 1, cells cultivated in medium supplemented with 5 % serum during treatment with nanosilver and (ii) Control 2, cells cultivated in medium supplemented with only 2 % serum during exposure to surface-modified MNPs. The average number of transformed foci per well determined in control 1 was 0.5 ± 0.5, and in control 2 was 3.0 ± 2.1. The average number of transformed foci per well determined in MCA-treated cells was 23.2 ± 2.4.

Neither spherical AgNM300, nor SO-MNPs or SO-PEG-MNPs increased substantially the number of transformed foci. In contrast, a significant increase in the transformed foci number was observed when Bhas 42 cells were exposed to silver nanorods/wires (AgNM302) or SO-PEG-PLGA-MNPs, mainly at lower concentrations (1–10 mM) with the highest level of transformed foci with SO-PEG-PLGA-MNPs. These results showed that the carcinogenic potential of nanosilver is underlying by fibrous shape.

Fontana et al. [36] analyzed the transformation initiating and promoting activities of four **synthetic amorphous silica (SAS)** NPs, two precipitated, NM-200 and NM-201, and two pyrogenic, NM-202 and NM-203. These NPs were characterized previously [93]. The Bhas 42 CTA, with the initiation and the promotion assay, was performed in accordance with the OECD recommendations [74] and validated in the laboratory using known carcinogenic substances. The concentrations tested ranged from 2 μg/cm^2 to 80 μg/cm^2 for SAS. MCA (1 μg/mL) and TPA (0.05 μg/mL) were used as positive controls in the CTA performed with silica micro-and nano-particles. In this study, the number of transformed foci per well induced by MCA in initiation assays was 13.2 ± 3.1 *versus* 3.2 ± 1.6 transformed foci in the control (0.1 % DMSO), and the number of transformed foci per well induced by TPA in promotion assays was 14.9 ± 3.0 *versus* 5.7 ± 2.0 transformed foci in the control (0.1 % DMSO).

Transformed foci were observed with all SAS but only in the promotion assay, suggesting a tumor promotor mode of action of the silica NPs. NM-203 was active at lower concentrations compared to other SAS, due to high production of oxygen species in an aqueous solution [93].

Jalili et al. [94] focused on initiation and promotion properties of **aluminium NPs (Al^0 and Al_2O_3)** by using CTA and Bhas 42 cells, as performed by Fontana et al. [36]. In this study, the supplier (IoLiTec (Heilbronn, Germany) provided the NP characteristics. The results for the positive controls were in agreement with the study of Fontana et al. [36]. The average number of transformed foci per well induced by MCA in initiation assays was 14.2 = 7 ± 2.9 *versus* 5.8 ± 1.9 transformed foci in the control (0.1 % DMSO), and the average number of transformed foci per well induced by TPA in promotion assays was 20.3 ± 3.0 *versus* 8.8 ± 2.1 transformed foci in the control (0.1 % DMSO).

Concerning cell proliferation, in the initiation assay, Al^0 induced a decrease on Day 7 at the two highest concentrations (1 (38 %) and 3 μg/cm^2 (84 %) for Al^0 NP). In contrast, Al_2O_3 in the initiation assay and the two forms of Al (Al^0 and Al_2O_3) in the promotion assay induced a moderate decrease in cell proliferation for all concentrations tested (<25 % at the highest concentration, respectively, 3 μg/cm^2 for Al^0 and 9 μg/cm^2 for Al_2O_3), suggesting a weak cytotoxicity. No transforming activity was then observed with Al^0 and Al_2O_3, in the initiation or promotion assays.

Briefly, to date, very few studies on NPs and CTA are published, seven articles using the Balb/c 3T3 and four with the Bhas 42 cells. Five of these articles were performed by the JRC, using only the initiation protocol and only three were carried out with the two-stage protocols (initiation and promotion). In all studies, as recommended, characterization of tested NPs was always performed in parallel using different techniques such as TEM, SEM, DLS, Zeta potential for a proper interpretation of the results.

Different NPs (cobalt, silice, carbon, silver, aluminium…) were tested giving various results (positive or negative with initiation and/or promotion protocol and even some antagonism activities). No rules about NP mode of action can be established regards to these studies. For example, for Balb/c 3T3 initiation, 57 % of the NPs were negative (8/14) and 43 % positive (6/14). Taking into account the promotion studies, 44 % of the NPs were negative (4/9) and 56 % positive (5/9) leading to new information on NPs mode of action, compared to the genotoxicity test battery recommended. Regards to Bhas 42 protocols, 40 % positive effects (6/15) were found for promotion step.

4. Key points to perform CTA for NPs testing

Some particular points could be specified for a CTA OECD guidance dedicated to NPs assessment.

4.1. Preparation of NPs before testing

NPs are prone to change properties in different media and treatments and many characteristics have to be taken into consideration to assess toxicity.

For any *in vitro* assessment regards to NPs, **a prerequisite step is to characterize first the NP suspension in the culture medium** and not just as supplied in powder form or water suspension. Properties of NPs change depending on the surrounding environment; a lot of reactions can occur when NPs are into biological media. Proteins can be adsorbed to the surface of NPs and Zeta potential can be modified when NPs are at physiological pH. NPs can precipitate/agglomerate/aggregate, changing then their toxic potential. Therefore, it is necessary to perform an accurate characterization of NPs before, during and after testing [95]. Particle size, state of agglomeration, surface properties and stability of the dispersion stock solution as well as how the NPs dispersed in the final treatment medium should be measured and monitored.

The size and the shape must be determined as two crucial parameters for the internalization mechanism. Studies have shown that there is a size- and shape-dependent toxicology associated with NPs. For the

size, smaller is the NP, higher is the toxicological effect and for the shape, nanotubes have a greater ability to translocate inside cells than nanospheres or nanocubes [96]. The **surface properties** have to be assessed, as this is the most critical parameter regarding interactions with the biological medium. **Surface area** has an important impact; larger is the surface, higher is the probability for biomolecules present in the medium to interact with NPs. The **surface chemistry** (nanoparticle functionalization) impacts also the toxic effect of NPs. Lastly, the **surface charge** of the NPs in the medium is required as it determines the NP stability. Indeed, if they are all negatively or positively charged, they are stable in suspension thanks to electrostatic repulsion, but when NPs have both positives and negatives charges on their surface, it conducts to their agglomeration/aggregation. Agglomeration or aggregation increases the size and changes the shape of NPs, influences NP interactions in biological medium and most importantly, modify the stability and homogeneity. It is crucial then, to determine surface charge of NPs at culture medium pH (7.4) before *in vitro* assessment (Caballero-Díaz & Valcarcel Cases, 2016)[6]. The presence of mutagens and transitions metals affiliated with the particles must also be identified [97,98].

Homogeneity of NP suspension is required to correctly investigate toxicological effects, but many parameters can affect it. NPs are simply nano-objects in suspension, a sedimentation phenomenon can occur in addition to agglomeration and aggregation. To avoid this phenomenon, an ultrasound step allows to homogenate NP suspensions. Ultrasounds are currently used with a bath or a probe depending on NP properties. Ultrasound probes break stronger aggregates or agglomerates, but this method has the drawback to heat the sample. Ultrasound bath has a weaker impact avoiding the increase of NP suspension temperature [99]. Another drawback of the ultrasound step is the foam formation due to the presence of proteins and salts in the culture medium. Then, it is recommended to prepare NP suspension into water or phosphate buffer saline (PBS) and to homogenate with ultrasound before incorporating NPs into the culture medium [100].

Properties of NPs and their toxic effects can also be influenced by the different physical and chemical characteristics of **solvents** used for dispersing or dissolving them. Factors such as pH, salinity, water hardness, temperature and presence of dissolved or natural organic particles in the medium can influence the biological reactivity of NPs. Thus, they may behave differently in water, culture medium, PBS and other solvents with pronounced effects on their uptake, cellular localization and hence the observed toxic response [101].

Furthermore, in the culture medium, a selective set of proteins with a high affinity for the NP surface will adhere and remain more or less bound, forming **a corona** (Gonzalez et al., 2010; [102,103]). Protein corona could be schematically represented as a double-layer coating wrapping the NPs, consisting of a first tightly bound monolayer, usually defined as "hard" corona, composed of proteins with high affinity for the NP surface (Rocker et al., 2009 [104]), whereas the outer layer, termed "soft" corona, consists of a loose protein layer reflecting serum proteins abundance, predominantly comprising albumin and derivatives (Walczyk et al., 2010 [105]; Casals et al., 2010 [106]) easy to be eluted [107]. This protein corona will greatly modify NP parameters, their hydrodynamic diameter, surface charge and especially their surface chemistry. In addition, the proteins making this corona differ according to the type of NPs and the medium composition. These are just as many parameters that affect the ability of NPs to penetrate inside cells and therefore their toxicity.

Two effects of the protein corona can be distinguished. On one hand, the surface of the NPs coated with proteins allows better **stability** by steric hindrance as well as by the surface charges modification. It also enhances biocompatibility of NP by ligand-receptor recognition [108]. On the other hand, these bound proteins with many antibodies at their surface can activate the mechanisms of cellular internalization [109] and increase NP uptake. Proteins (FBS, BSA…) in NP dispersions and cell cultivation may vary from 0 % to 10 %, Toyooka et al. [110] demonstrated that the genotoxic effect was less extensive with BSA coated than with uncoated TiO2NP.

With regards to **internalization**, the most cells concerned are immune cells. Among the proteins identified of the protein corona, many are proteins of the complement system [107]. These proteins are then recognized by the different cells of the immunity system, which internalize the NPs. As the immune cells can move into the body through the lymphatic system, they carry with them NPs that can be internalized, degraded, or excreted [111]. Indeed, it has been demonstrated that a lot of NPs have immunotoxic effects.

As shape, size, surface charge and surface chemistry can affect cellular uptake of NPs and not all *in vitro* models possess all the endocytic mechanisms, it is important to check the ability of the tested NPs to be internalized in the chosen cell model. Each type of NPs is internalized by the cell *via* preferentially uptake pathway [112]. *In vitro*, the principal route of entry of NPs into the cells is *via* endocytosis. This uptake could be investigated by diverse analytical techniques based on transmission electron microscopy (TEM) and scanning electron microscopy (SEM), induced coupled-plasma (ICP)-based techniques and fluorescent-based methods such as microplate reader, flow cytometry and confocal imaging [113].

Then, to conclude and for all these reasons, to adapt the CTA OECD guidance to NPs, a paragraph should be added to specify, using recognized methods, the characterization of the tested NPs as a pre-requisite step.

4.2. Selection of NP concentration range using CTA

In the studies on NPs, dose metrics can be expressed in mass (μg/mL), surface area (m^2/mL) or particle number (n/mL) [114]. In the majority of studies published with CTA, concentrations are expressed in mass units, *i.e.* as NP dispersion concentration (μg/mL) or μM, or as NP mass per surface of exposure cells (μg/cm^2). Generally, regards to *in vitro* toxicity, a correlation with surface area has been proposed as a recommended dose expression [8,115] as it can be compared to *in vivo* exposure results (even if attention to differences in the exposure method should be considered). The use of mass, number or concentration per cell appears to be particularly appropriate for NP testing; however, it is recommended to express concentrations in the three different dose metrics (mass, surface area and number), which is not mentioned in the published articles. This allows conversion from one dose to another when needed for determination of the best fitting dose metric for a toxic effect. If the particles are thoroughly characterized, this should be anyhow feasible [116].

In the CTA guidance for standard chemicals (2017), the highest concentration of the test chemical in the medium should be 2 mg/mL or 10 mM or 2 μL/mL if no precipitate or limiting cytotoxicity is observed. Regards to the highest concentrations (Table 1), for most of the studies, NPs were tested up to 100 μg/mL or to 70 μM, which is 20 or 142 fold respectively below the limits recommended in the guidance, suggesting that for NP testing, these limits could not be attained.

The cell growth assay (as a marker of cytotoxicity) associated with CTA enables to fix the concentration tested in the initiation and promotion test. Unlike conventional genotoxicity assays where concentrations to be tested are up to IC_{50}, in the CTA, the range of concentrations can be wider (up to IC_{90}). Nevertheless, this top dose does not appear easily achievable as in the previous mentioned published articles, NPs showed weak cytotoxic effects with a decrease of viability, only up to 30 %, except for Co [76] and Ag [89] which were demonstrated strongly cytotoxic, allowing a IC_{90} determination. Regards to the guidance, if the IC_{90} could not be reached, then, the highest concentration to be tested should be determined by agglomeration of NPs.

In the international validation study [75], five or more concentrations were selected based on cell growth assay results, respectively performed for the initiation or promotion protocol.

The chosen concentration range will not be the same depending on the protocol studied, initiation or promotion. Indeed, in the initiation

protocol, the cells are treated for only 3 days with NP while in the promotion protocol, the cells are exposed for 7 days to NP, resulting in a greater cytotoxic effect.

4.3. CTA reproducibility between laboratories (negative and positive controls)

A trend is observed with experiments: when negative controls are weak, positive controls induce also a lower number of transformed foci and conversely.

In 2005, Ohmori et al. published the first inter-laboratory study on the Bhas 42 CTA for tumor promoters. They observed a dose-dependent increase of transformed foci in cells treated with the positive control TPA in the 12 laboratories, compared to the control cells. However, there were variations in the results among them; the response was highest in two laboratories with many foci also observed in negative controls. In contrast, in two other laboratories, few foci appeared in control cultures [70].

The first inter-laboratory study for the complete (initiation and promotion) assay in 2009 [71] reveals again some variability in the inducibility of transformed foci either in negative controls or after treatment with the same concentrations of the positive controls (MCA and TPA). Incidentally, laboratories with no experience in CTA reading, give lower results with positive controls than laboratories performing CTA routinely, suggesting an impact of the reading skills and experience. In 2011, in another inter-laboratory study with 6 laboratories, this finding was again noticed. There were always significant differences between negative and positive controls in both the initiation and promotion assays, but the number of foci induced by each positive/negative controls varied between laboratories and even sometimes within laboratories [75].

To minimize variability between experiments, it is of first importance to control the cell maintenance conditions [70], especially serum batch and cell culture period. For example, the use of the same cell passage is recommended to obtain reproducible results of CTAs [117, 118].

In the OCED guidance dedicated to NP assessment, nanoform positive controls could also be used instead of standard chemicals (MCA and TPA). Considering the results published in the previous mentioned articles, TiO_2 and SiO_2 could be potential candidate as initiator and promoter positive controls, respectively.

4.4. Subjective nature of using morphological characteristics for assessing transformation

The transformed foci of Bhas 42 cells are judged based on their morphological characteristics. The foci must have more than 100 cells, with spindle-shaped cells different from the contact-inhibited monolayer cells, a deep basophilic staining, a random orientation of cells at the edge of foci, a dense multilayering of cells and an invasive growth into the monolayer of surrounding contact-inhibited cells. As all transformed foci do not have all of these characteristics, a certain level of subjective judgments cannot be avoided (Sasaki et al., 2014)[119].

Photo catalogue were published with different cell models [120-122]. It is a practical tool to help with the foci identification and cell colonies classification after the staining step. Several conditions are necessary for a reliable transposition of the test in a new laboratory: staff training, detailed protocol and use of the photo catalogue.

4.5. Metabolic status of cells used in CTA

The cell model used for the CTA possesses limited metabolic status, Cyp isoforms, (except Cyp1A1) are not expressed [123] and various approaches have been employed to enhance their endogenous metabolic activity [124,125]. Later, Schechtman and co-workers developed methods for supplementing endogenous metabolizing activity of Balb/c 3T3 A31 cells, with a rat liver S9 metabolizing enzymes [56,67].

Regards to NP assessment, most poorly soluble NMs are not metabolised and the metabolic activation system (S9) may interfere with the assay reducing the NM bioavailability. Organic NMs or some inorganic NMs coated with organic functional groups may however exert their genotoxic effects in the presence of the metabolic activation system (Sharifi et al., 2012[126]). To date, S9 has never been used with CTA, however, it could be evaluated case by case,

5. Future improvement of the CTA

5.1. Improvement of foci scoring

The major drawback of the CTA is the visual detection of tumor foci, which impact inter-reproducibility data as discussed previously. They are manually scored for morphological transformation under a stereomicroscope as described by the International Agency for Cancer Research (IARC) Working Group (IARC/NCI/EPA [118]).

Alterations in adhesion protein profiles or enzyme activities modulating cellular adhesion and migration are involved in the process of focus formation. These may be promising candidates for the identification of molecular markers leading to a more objective scoring of foci and a greater acceptance of cell transformation tests in general. In 2007, Poth et al. (2007) [127] proposed to measure the butyrylcholinesterase (BChE) activity in the type III foci induced by chemical treatment, as a biomarker. Indeed, Bhas42 cells growing as a monolayer of non-transformed cells showed no increased activity of BChE. Morphologically transformed cells issued from positive foci gave a marked response in the activity of BChE.

Sasaki et al. [128] found that transformed Bhas 42 cells but not the non-transformed parental cells can survive in the presence of H_2O_2. They determined that, after treatment with H_2O_2, transformed foci are selectively stained with dyes used for monitoring cell viability, such as WST-8 or resazurin. The stained foci could then be quantified by absorbance with a microplate reader.

Using this analytical technique, they demonstrated that sodium arsenite in the 96-well method could be judged positive as transformed foci on the sidewall are stained and became measurable. Wells with transformed foci showed high OD values, whereas OD values of wells without transformed foci showed a blank level. The total OD values and the number of wells containing transformed foci in each plate increased dose-dependently with MCA concentration. Then, the Bhas 42 transformation assay in 96-well plates and subsequent treatment with H_2O_2 followed by the referenced dye staining may be a novel approach to the scoring of transformed foci and, as such, may be readily applicable as a rapid and objective high throughput screening system for the detection of chemical carcinogens.

Since 2013, an Italian team [129-131] works on another solution to improve the last step of the Balb/c 3T3 CTA, namely the visual scoring of foci. This reading is based on morphology of foci under a light microscope by trained experts, which implies a fair degree of subjectivity. They proposed to use digital image analysis to support the scoring of malignant foci and they develop statistical descriptors, by translation of morphological features (foci size, multilayering and invasive cell growth into the background monolayer, spindle-shape of transformed cells and texture heterogeneity of foci). The aim is to use these descriptors to mimic the classification performances of the visual scorer to discriminate between transformed and non-transformed foci.

5.2. Use of CTA in the battery for potential carcinogenic properties of NPs

CTA offers a good phenotypic anchoring, which links the cause (exposure) to the event (oncotransformation) [39,43].

The toxicogenomic approach applied to this transformation assay is an interesting way, for the identification of the transcriptional pathways, significantly modulated as the consequence of the agent exposure

and which are strictly related to the formation of the transformed phenotype. Colacci [132] developed an experimental protocol, which combined the Balb/c 3T3 CTA and a global gene expression analysis to highlight the cross-talk between genotoxic and non-genotoxic carcinogenic mechanisms in the pathway leading to malignant cell transformation. The toxicogenomics approach applied to the *in vitro* CTA allowed the identification of the transcriptionally activated pathways [39]. This integrated approach has the potential to be considered part of an Integrated Approach for Testing and Assessment for non-genotoxic carcinogenesis [43]. Transcriptomics may highlight the key events along the pathway to the adverse outcome. CTA can be considered to provide endpoints related to morphological transformation, if NPs induce also oxidative stress we can wonder if it is linked to morphological transformation, if both the transformation and oxidative stress is observed, level of concentration may inform as a possible link or not [29]. Then, CTA used as part of a testing strategy and/or in a weight of evidence approach, could contribute to assessing carcinogenic potential not only of chemicals [45] but also of NPs. CTA combined with toxicogenomic profiling will be a way to better highlight underlying mechanism with information on molecular mechanisms of NPs as already demonstrated for chemicals [39]. Indeed, some authors propose a path for implementation of innovative cancer risk assessment including genomic signatures for mechanistic relevance of carcinogenicity. Transcriptomic signatures have been demonstrated to be powerful prediction of toxicological changes that can be integrated in risk assessment [133]. More recently it was reported in a first article on gene expression over time during the process of cell transformation with only a tumor-promoting compound. Pathways that were activated or inactivated during the process of cell transformation in the Bhas 42 cells treated with TPA were related not only directly to *RAS* but also to various pathways in the hallmarks of cancer [134].

6. Conclusion

Nanotechnology is regarded as the technology of the future, but a better understanding of NP mode of actions for their safe use is needed. Alternative *in vitro* testing has to be considered with NPs, carcinogenesis being even more challenging for the risk assessment. In the respect of the 3 R rules, cell transformation assay is recognized to be able to detect various types of carcinogens, including those that are thought to act *via* genotoxic and non-genotoxic mechanisms. To date, this bioassay has been performed with a dozen of NPs either with the protocol of initiation or promotion alone or both. Concerning the promotion assay, SiO_2, LnO_2, Dy_2O_3, Fe_3O_4 and Au showed non-promotor/active properties.

The accuracy of prediction of NP carcinogenicity would be improved by using the CTA in the battery of *in vitro* assays for a weight of evidence approach and in an Integrated Approach for Testing and Assessment, CTA could be one of the possible building blocks. As discussed earlier a further guidance regards CTA would b need to be adapted for testing specifically NMs.

This assay with future developments especially coupled to genomic approaches, accompanied regarding to the specific NP properties a complete characterization, internalization and stability studies in the experimental conditions, is a promising tool, especially for NPs, which will probably never be tested in an *in vivo* carcinogenic study according to OECD guidelines.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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_____ INTRODUCTION_____

Il est difficile d'imaginer un monde moderne sans emballage à contact alimentaire, tant leur importance est cruciale à l'échelle mondiale. La principale fonction de l'emballage alimentaire est de protéger le produit contre les chocs, les variations de température et les facteurs environnementaux. Il agit comme barrière à l'oxygène et à l'humidité, prévient la contamination, facilite le transport, conserve la qualité et l'arôme, informe le consommateur, soutient le marketing et la sécurité, tout en améliorant la praticité et le contrôle des portions (Marsh and Bugusu, 2007; Bix *et al*., 2004).

Les matériaux au contact des denrées alimentaires (MCDA) désignent tous les articles et matériaux destinés à entrer en contact avec des denrées alimentaires (ILSI, 2015). Les MCDA sont rarement inertes : des interactions entre les aliments et les MCDA peuvent entraîner la migration (transfert) de substances chimiques de l'emballage vers les aliments.

Le règlement (CE) n° 1935/2004 de l'Union européenne concerne 17 groupes de matériaux et garantit que ces matériaux soient suffisamment inertes afin de ne pas libérer leurs constituants dans les aliments à des niveaux dangereux pour la santé humaine, ni altérer la composition, le goût ou l'odeur des aliments au-delà de limites acceptables (EC, 1935).

Les substances chimiques qui migrent des emballages vers les aliments sont classées en deux catégories : les substances intentionnellement ajoutées (IAS en anglais) et les substances non intentionnellement ajoutées (NIAS en anglais). Les IAS sont délibérément incorporées dans les formulations d'emballages, comme les monomères ou les additifs. Les NIAS, souvent présentes à l'état de traces, peuvent provenir des procédés de fabrication ou de l'utilisation de matériaux recyclés. Selon le règlement (UE) n° 10/2011 sur les plastiques (article 3), les NIAS sont des « substances ajoutées involontairement » : il peut s'agir d'une impureté dans les substances utilisées, d'un intermédiaire de réaction formé au cours du processus de production, ou encore d'un produit de décomposition ou de réaction (EU, 2011)

De plus, l'article 19 précise que les substances non inscrites à l'annexe I doivent faire l'objet d'une évaluation des risques, conformément à l'article 3 du règlement (CE) n°1935/2004 et selon des principes scientifiques reconnus à l'échelle internationale (EU, 2011).

L'évaluation des risques des NIAS reste complexe, car leur origine et leur formation sont souvent inconnues, leur identification et leur quantification sont difficiles en raison du manque de standards analytiques, et très souvent, leurs effets toxiques ne sont pas

documentés (Kato and Conte-Junior, 2021). Les NIAS peuvent migrer à partir de divers matériaux d'emballage, y compris les revêtements. Appliqués sur la surface intérieure des boîtes métalliques, les revêtements empêchent les interactions métal–aliment susceptibles de provoquer de la corrosion et la migration d'ions métalliques. Fabriqués à partir de vernis complexes variant selon les fabricants, ces revêtements ne sont actuellement pas régis par une réglementation harmonisée au niveau européen en matière de matériaux en contact avec les aliments.

Le Conseil de l'Europe a publié une résolution sur ces revêtements (ResAP(2004) 1, 2009). Les fabricants sont invités à l'utiliser comme ligne directrice, mais son application n'est pas obligatoire. De plus, certains pays ont élaboré leur propre réglementation nationale applicable aux revêtements. Les revêtements à base d'époxy étaient largement utilisés en raison de leurs performances techniques et de leur faible coût de production, jusqu'à ce que les préoccupations croissantes concernant les risques sanitaires du bisphénol A incitent à rechercher des alternatives plus sûres. Les revêtements en polyester ont depuis gagné en popularité à l'échelle mondiale, mais peu d'études ont été menées sur les NIAS qu'ils libèrent, leur potentiel de migration et les risques associés. Certaines études ont signalé la migration de divers NIAS, notamment de nombreux oligoesters, à partir de revêtements en polyester destinés au contact alimentaire (Hayrapetyan *et al*., 2024; Cariou *et al*., 2022; Lestido-Cardama *et al*., 2022a; Omer *et al*., 2019). Il est donc nécessaire de mener des recherches supplémentaires pour combler ces lacunes et statuer sur leur innocuité.

Les objectifs de cette thèse étaient :

- d'identifier et de caractériser les NIAS provenant des extraits et des migrats de revêtements en polyester destinés au contact alimentaire ;
- d'évaluer le danger et le risque présentés par les NIAS susceptibles de migrer dans les aliments ;
- d'étudier leur devenir dans le tractus gastro-intestinal,

afin de garantir la sécurité des consommateurs exposés par le biais de la consommation d' aliments en boite de conserve.

_____ STRATÉGIE DE RECHERCHE _____

Cette thèse est composée de six chapitres, chacun abordant des aspects essentiels de l'identification, de la caractérisation et de l'évaluation de la toxicité des NIAS provenant des revêtements en polyester. La stratégie de recherche s'est concentrée sur l'identification des NIAS volatiles, semi-volatiles et non volatiles à travers des essais d'extraction et de migration utilisant un simulant alimentaire ou un solvant, suivis d'une identification analytique. L'évaluation des dangers a été réalisée à l'aide de modèles *in silico* et, lorsque cela était possible, confirmée par des biotests *in vitro* utilisant des standards commerciaux.

En complément de l'analyse chimique, la génotoxicité et un mode d'action non génotoxique d'une potentielle cancérogénicité des extraits et des migrats ont été évaluées à l'aide d'essais *in vitro*.

Le devenir des NIAS non volatiles dans le tractus gastro-intestinal a aussi été étudié à l'aide d'une nouvelle méthode d'extraction à l'acétonitrile (ACN) et d'un modèle de digestion intestinale simulée, afin de suivre les composés parents et leurs produits de transformation.

Le dernier chapitre présente une étude de pré-validation du test NanoAmes — une version miniaturisée du test d'Ames — pour détecter des génotoxiques à de faibles concentrations. Des substances de référence ont été testées avec et sans activation métabolique (mélange S9), en utilisant les souches de *Salmonella* TA98 et TA100 dans deux laboratoires. Les résultats ont été comparés pour évaluer la fiabilité et la pertinence de ce test.

_____ RÉSULTATS _____

Chapitre 1- Évaluation *in silico* des dangers des NIAS identifiés (fenozan et quatre oligoesters cycliques)

Introduction

Dans cette étude, le fenozan (voir Chapitre 3) ainsi que quatre oligoesters cycliques (voir Chapitre 2) composés de monomères 1,4-butanédiol (BD), acide téréphtalique (tPA) et tricyclodécane-diméthanol (TCDDM) —c(2TCDDM+2tPA), c(2BD+TCDDM+3tPA), c(BD+2TCDDM+3tPA), c(3TCDDM+3tPA) ont été identifiés.

Ces composés ont été détectés dans les extraits d'acétonitrile (ACN) et dans les migrats d'éthanol à 95 % (EtOH 95 %) réalisés à partir de plaques métalliques revêtues de polyester. Une hydrolyse est nécessaire pour confirmer la présence d'isomères monomériques

spécifiques, car la chromatographie liquide-spectrométrie de masse à haute résolution (LC-HRMS) ne permet pas de les distinguer.

À l'aide du logiciel Freelance Chamespider, tous les isomères structuraux possibles des oligoesters identifiés ont été générés pour l'évaluation de leur toxicité.

L'objectif de cette étude était d'évaluer la toxicité potentielle des NIAS identifiés — fenozan et quatre oligoesters cycliques — à l'aide d'outils QSAR *in silico*, cette évaluation servant de dépistage de premier niveau pour prioriser les composés préoccupants et orienter les futurs biotests visant à confirmer certains risques toxicologiques.

Divers outils QSAR ont été utilisés : Derek Nexus v6.1, Sarah Nexus v6.1, VEGAHUB v1.1.5, Danish (Q)SAR Database, et ADMETlab 3.0, afin de prédire la génotoxicité, la cancérogénicité, les perturbations endocriniennes, la toxicité développementale, la toxicité d'organe et les valeurs de Log $k_{o/w}$ pour ces NIAS.

Résultats

Évaluation prédictive *in silico* des dangers du fenozan

- ***Mutagénicité*** *:* VEGAHUB a signalé un possible effet sur les chromosomes *in vitro* avec une fiabilité modérée. La base de données Danish (Q)SAR a identifié des alertes pour la liaison à l'ADN (phénols alkylés), des mutations par décalage du cadre de lecture, des mutations sur le locus de la thymidine kinase dans les cellules de lymphome murin *in vitro*, et *in vivo* des micronoyaux chez la souris des mutations létales dominantes chez les rongeurs, et des cassures d'ADN dans le test des comètes chez la souris.
- ***Cancérogénicité*** *:* la base de données Danish (Q)SAR a prédit une cancérogénicité chez la souris et une similitude structurelle avec des composés de type phénol.
- ***Toxicité d'organe*** *:* VEGAHUB a classé le fenozan comme hépatotoxique avec une faible fiabilité. Derek Nexus a également indiqué une hépatotoxicité plausible et une prédiction équivoque pour un effet toxique sur la thyroide.
- ***Perturbation endocrinienne*** *:* VEGAHUB et la base danoise ont prédit une faible affinité de liaison aux récepteurs des œstrogènes alpha (ERα). Cette dernière a également signalé une inhibition possible de la thyroperoxydase, une interférence avec le symporteur sodium/iodure (NIS 1), et une interaction avec les récepteurs X des pregnanes et les récepteurs constitutifs aux androstanes.

- ***Toxicité développementale*** *:* ADMETlab et VEGAHUB ont prédit une toxicité développementale avec une fiabilité modérée.

Évaluation *in silico* des dangers des oligoesters

- ***Nombre d'isomères*** *:* 147 isomères au total ont été générés : 7 isomères pour c(2TCDDM+2PA), 12 isomères pour c(3TCDDM+3PA), 56 isomères pour c(BD+2TCDDM+3PA), 72 isomères pour c(2BD+TCDDM+3PA).
- ***Valeur de*** *Log* $k_{o/w}$*:* Supérieure à 10 pour tous les oligoesters.
- ***Mutagénicité*** *:* Tous les outils QSAR ont prédit une absence de potentiel mutagène pour tous les isomères. Toutefois, ADMETlab a détecté 3 alertes de génotoxicité pour certains isomères de quatre oligoesters.
- ***Classification Cramer*** *:* Tous les isomères ont été classés en Classe III par VEGAHUB.
- ***Cancérogénicité*** *:* VEGAHUB a prédit une cancérogénicité pour tous les oligoesters. ADMETlab n'a donné des prédictions positives que pour les isomères de deux oligoesters contenant le monomère BD.
- ***Perturbation endocrinienne*** *:* Derek Nexus n'a prédit aucune activité endocrinienne. VEGAHUB a indiqué une liaison aux récepteurs œstrogènes (ER) de faible fiabilité pour 27 isomères de c(2BD+TCDDM+3PA). Plusieurs isomères ont également montré une activité potentielle envers l'aromatase et les domaines de liaison aux ER.
- ***Toxicité développementale*** *:* VEGAHUB et Derek Nexus ont tous deux prédit une toxicité développementale potentielle. VEGAHUB a classé plusieurs isomères comme actifs avec une fiabilité faible, tandis que Derek a suggéré une tératogénicité plausible pour de multiples isomères.

Chapitre 2 – Article 1 – Identification de substances non volatiles non intentionnellement ajoutées provenant de revêtements alimentaires en polyester et évaluation de la génotoxicité des migrats de ces revêtements

Introduction

Cette étude vise à identifier les NIAS non volatiles et à évaluer leur génotoxicité potentielle *in vitro*, en utilisant des échantillons issus de cinq lots d'une même boîte en fer blanc revêtue de polyester, afin de garantir la sécurité des consommateurs exposés à des aliments conditionnés dans des boites de conserve.

Matériels et méthodes

Cinq lots de plaques en fer blanc revêtues de polyester, ainsi qu'une plaque témoin non revêtue, ont été soumis à des tests de migration et d'extraction. Deux échantillons circulaires (1,02 dm^2 chacun) de chaque matériau ont été hermétiquement scellés dans des cellules de migration Calipack (norme CEN - EN 1186). Chaque cellule a été remplie avec 430 mL soit d'ACN, soit d'EtOH 95%, et incubée à 40 °C pendant 1 jour pour les échantillons ACN, ou à 60 °C pendant 10 jours pour ceux à EtOH 95 %, au contact à la fois des plaques revêtues et non revêtues.

L'ACN, un solvant de polarité intermédiaire, a été utilisé pour simuler un scénario d'extraction de type « pire des cas », tandis que l'EtOH 95 %, a été utilisé comme substitut de simulants alimentaires lipophiles comme l'huile végétale, conformément au Règlement UE 10/2011 (EU, 2011). Après incubation, les extraits et les migrats ont été regroupés par type de solvant, concentrés 54 fois par évaporation rotative suivie d'un séchage à l'azote à 40 °C. Des blancs de solvant ont été préparés selon les mêmes procédures.

Après migration ou extraction, les échantillons ont été concentrés et analysés par LC-HRMS pour une identification non ciblée. Les oligoesters identifiés ont été semi-quantifiés à l'aide de standards internes marqués au deutérium. Le logiciel Toxtree (v3.1.0, Ideaconsult Ltd., Sofia, Bulgarie) a été utilisé pour attribuer les classes de Cramer aux composés identifiés non génotoxiques, et les valeurs prédictives de Log $k_{o/w}$ ont été estimées à l'aide du modèle Meylan/Kowwin dans le logiciel VEGA HUB (v1.1.5).

Le potentiel génotoxique des extraits et des migrats a été évalué à l'aide de deux essais *in vitro* : Mini Mutagenicity test (MMT), une microméthode avec plusieurs souches de *Salmonella typhimurium* par incorporation directe (TA100, TA98, TA102, TA1537) et pré-incubation (TA100, TA98) (Flamand *et al*., 2001), et le test DNA Damage Response (DDR) sur cellules lymphoblastoïdes humaines TK6 (Bryce *et al*., 2016). Pour exclure les faux négatifs dus à des effets de matrice, les échantillons ont été enrichis avec des témoins positifs correspondants et testés en parallèle dans les essais MMT et DDR.

Résultats

L'analyse non ciblée par LC-HRMS a révélé la présence de quatre oligoesters cycliques composés de monomères BD, tPA et TCDDM — c(2TCDDM+2tPA), c(2BD+TCDDM+3tPA), c(BD+2TCDDM+3tPA) et c(3TCDDM+3tPA) — dans les extraits ACN et les migrats EtOH 95 %

des plaques métalliques revêtues de polyester. Ces oligoesters ont été identifiés avec un niveau de confiance 2, leurs masses moléculaires étaient inférieures à 1000 Da, et ils présentaient des valeurs élevées de Log $k_{o/w}$ (>11).

L'analyse semi-quantitative a montré que les concentrations de c(BD+2TCDDM+3tPA), c(3TCDDM+3tPA) et c(2TCDDM+2tPA) allaient de 45,4 à 85,0 µg/dm² (équivalent à 272,4–510 µg/kg d'aliment), tandis que le c(2BD+TCDDM+3tPA) était présent à des niveaux plus faibles, entre 3,1 et 6,1 µg/dm² (18,6–36,6 µg/kg d'aliment).

Les extraits ACN comme les migrats EtOH 95 % ont donné des résultats négatifs dans les tests de génotoxicité ; dans le MMT quelles que soient les souches bactériennes et les conditions expérimentales. De même, le test DDR n'a révélé aucun effet clastogène, aneugène dans aucun des échantillons testés. Les essais avec ajout de témoins positifs ont confirmé l'absence d'effets de matrice dans les conditions utilisées.

Chapitre 3 – Article 2 – Évaluation du danger du fenozan, une substance non intentionnellement ajoutée libérée à partir d'un revêtement en polyester

Introduction

Cette étude porte sur l'identification des NIAS volatiles et semi-volatiles issues de revêtements à base de polyester, afin d'évaluer leurs dangers potentiels et de garantir la sécurité des consommateurs exposés *via* les aliments conditionnés dans des boites de conserve.

Matériels et méthodes

Cinq échantillons de fer-blanc revêtus de polyester (les mêmes que ceux utilisés au Chapitre 2) ont été testés, ainsi qu'un échantillon témoin non revêtu. Les extractions et les tests de migration ont été réalisés selon les mêmes procédures que celles décrites au Chapitre 2. Les échantillons ont ensuite été concentrés et analysés par chromatographie en phase gazeuse couplée à la spectrométrie de masse à haute résolution (GC-HRMS). La présence d'un NIAS identifié (fenozan) a été confirmée à l'aide d'un standard analytique.

La base de données Danish (Q)SAR a été utilisée pour prédire l'activité hormonale thyroïdienne des composés identifiés.

Afin d'évaluer la toxicité du composé identifié, plusieurs biotests *in vitro* ont été réalisés, incluant le test d'Ames (OECD, 2020), le test du micronoyau avec lecture en cytométrie en

flux (Bryce *et al*., 2007; Avlasevich *et al*., 2006) et le test d'incorporation de l'iodure (de l'iode sous forme I^-) (Waltz *et al*., 2010).

***Test d'Ames** :* Réalisé avec trois souches de *Salmonella typhimurium* (TA98, TA100, TA1535) et une souche *Escherichia coli* (WP2), avec et sans activation métabolique (mélange S9), en utilisant les méthodes d'incorporation directe et de pré-incubation.

***Test du micronoyau** :* Effectué sur des cellules humaines d'origine hépatique HepG2, avec et sans activation métabolique (S9) : 48 heures de traitement en absence d'activation métabolique, ou 4 heures avec activation métabolique S9 suivies d'une période de récupération de 44 heures. La cytotoxicité et la génotoxicité ont été évaluées par cytométrie en flux.

***Test d'absorption de l'iodure** :* Fait sur des cellules thyroïdiennes de rat FRTL-5 afin d'évaluer l'impact du fenozan sur l'absorption de l'iodure nécessaire à la synthèse des hormones thyroidiennes, indicateur d'une perturbation potentielle d'une activité thyroïdienne.

Résultats

L'analyse GC-HRMS a identifié la présence de l'acide 3-(3,5-di-tert-butyl-4-hydroxyphényl)propionique, communément appelé fenozan (CAS RN : 20170-32-5), un produit de dégradation (par hydrolyse) d'un antioxydant, à une concentration estimée à environ 0,2 µg/dm², selon l'analyse semi-quantitative. Le fenozan a été détecté dans les extraits ACN et les migrats EtOH à 95 %. Son identité a été confirmée à l'aide d'un standard de référence analytique.

Dans le test d'Ames, le fenozan a donné des résultats négatifs pour toutes les souches testées, avec et sans mélange S9, que ce soit par incorporation directe ou pré-incubation. La cytotoxicité a été observée à 2,5 mg/plaquette sur la souche TA98, avec et sans mélange S9, en utilisant les méthodes d'incorporation directe et de pré-incubation, ainsi que sur la souche TA100 sans mélange S9, en utilisant la méthode de pré-incubation.

Le test du micronoyau *in vitro*, réalisé sur des cellules HepG2, a également donné un résultat négatif après un traitement de 4 heures avec S9 et un traitement de 48 heures sans S9. La cytotoxicité a été observée à une solution de fénosan à 0,5 mg/mL, la concentration la plus élevée ayant été déterminée en divisant la dose cytotoxique par deux.

Dans le test d'absorption de l'iodure, le fenozan a montré un effet inhibiteur faible mais statistiquement significatif, à partir de 800 µM, avec une inhibition de 47 % à 1000 µM et une valeur IC_{50} de 1152 µM. Aucune cytotoxicité significative n'a été observée dans ce test.

Chapitre 4 – Évaluation du potentiel cancérogène des extraits et migrats de revêtements en polyester

Introduction

L'analyse *in silico* a mis en évidence des alertes de promotion tumorale pour quatre oligoesters cycliques identifiés dans les extraits ACN et les migrats EtOH 95 % des revêtements polyester (voir Chapitres 1 et 2). Ces substances n'étant pas disponibles dans le commerce, leur potentiel cancérogène a été évalué, globalement, dans les extraits, à l'aide du test de transformation cellulaire (CTA en anglais) sur cellules Bhas 42. Il s'agit d'un biotest *in vitro* permettant de détecter des initiateurs et des promoteurs tumoraux en comptant les foyers de transformation après traitement des cellules avec le produit testé. Cette approche vise également à évaluer les effets « cocktail » et le potentiel cancérogène de composés non identifiés présents dans les mélanges.

Matériels et méthodes

Deux mécanismes d'action cancérogène ont été investigués : l'initiation et la promotion. Avant de réaliser le test de transformation cellulaire, des essais de croissance cellulaire ont été effectués afin d'évaluer la cytotoxicité des extraits et migrats issus des vernis polyester, et de déterminer les plages de dilution adéquates pour les tests ultérieurs.

Ensuite, les extraits et migrats ont été testés à divers niveaux de dilution dans le CTA afin d'évaluer de potentiels effets dose-réponse. Les protocoles d'extraction et de migration utilisés dans cette étude ont suivi les procédures décrites au Chapitre 2, avec une modification clé : les échantillons ont été concentrés 108 fois (au lieu de 54 fois) à l'aide d'un flux doux d'azote. Une étape supplémentaire a été nécessaire pour les extraits ACN, car ce solvant n'est pas compatible avec la culture cellulaire. Ces extraits (3 mL) ont été concentrés presque à sec (environ 100 µL) sous azote, puis reconstitués dans 3 mL d'EtOH à 95 %, pour atteindre une concentration finale d'ACN de 3,33 %.

Résultats

Les essais de croissance cellulaire ont montré que ni les extraits ni les migrats non dilués n'étaient cytotoxiques. La validité expérimentale a été confirmée : les valeurs obtenues pour les témoins négatifs et positifs étaient dans les plages historiques du laboratoire.

Tous les extraits et migrats testés ont donné des résultats négatifs, aussi bien dans les essais d'initiation que de promotion, pour toutes les concentrations testées (non dilué, ½ dilution, ¼ dilution) et pour les deux séries expérimentales.

Chapitre 5 – Identification des substances non volatiles non intentionnellement ajoutées par LC/ESI-HRMS dans les extraits ACN du vernis polyester et étude de leur devenir gastro-intestinal

Introduction

Dans les chapitres précédents, quatre oligoesters cycliques ont été identifiés dans les extraits ACN et les migrats EtOH 95 % des revêtements en polyester à l'aide de la LC-HRMS (voir Chapitre 2). L'étude du devenir gastro-intestinal des NIAS identifiées est essentielle, car le métabolisme par les enzymes du tractus gastro-intestinal peut modifier leur propriétés (toxiques ou non). Cette étude vise à:

1) identifier les NIAS non volatiles issues des revêtements en polyester

2) évaluer leur devenir dans le système digestif.

Matériels et méthodes

Afin d'examiner le devenir gastro-intestinal des oligoesters précédemment identifiés, une extraction supplémentaire à l'ACN (par râpage) a été réalisée sur le revêtement polyester (voir Chapitre 2) pour obtenir des extraits plus concentrés, permettant de détecter d'autres composés parents non identifiés précédemment. Chaque plaque métallique revêtue (4 cm^2) a été râpée, placée dans un flacon de 15 mL avec 4 mL d'ACN, puis incubée pendant 24 h à 40 °C. Les flacons ont été centrifugés, les surnageants transférés dans un nouveau flacon de 20 mL et concentrés sous flux d'azote doux jusqu'à 4 mL. Cet extrait est désigné dans le manuscrit sous le nom de « CA_{mix} ». Le CA_{mix} a ensuite été soumis à une digestion intestinale simulée, suivant un protocole modifié d'après Eckardt *et al.* (2019), et les composés parents ainsi que leurs produits de transformation attendus ont été suivis par LC/Electrospray ionization (ESI)-HRMS. Une portion du CA_{mix} a été concentrée 50 fois afin de permettre la détection de substances à très faibles concentrations, désignées comme composés parents.

Résultats

En mode non ciblé, la LC/ESI-HRMS en polarité positive et négative a permis d'identifier 18 composés parents dans l'extrait ACN concentré 50 fois, incluant 9 oligoesters cycliques et 5 linéaires.

Après digestion, l'analyse a révélé que les oligoesters possédant des liaisons TCDDM–PA étaient résistants à l'hydrolyse, tandis que ceux contenant des esters BD–PA et TCDDM– acide adipique (AA) étaient facilement dégradés.

Huit produits de dégradation linéaires ont été identifiés, leur concentration ayant augmenté, indiquant une dégradation continue. Il est probable que d'autres produits plus petits aient été formés, bien qu'ils n'aient pas été détectés avec les méthodes actuelles. La quantité de TCDDM sont restés inchangés, suggérant qu'il ne s'agit pas de produits de dégradation et que le TCDDM est stable.

Chapitre 6 – Étude interlaboratoire du test NanoAmes, une version miniaturisée proposée du test d'Ames – Données préliminaires

Introduction

Dans les chapitres précédents, les résultats des tests MMT réalisés sur les extraits ACN et les migrats EtOH 95 % des revêtements en polyester ont été négatifs. Cette absence de réponse pourrait s'expliquer par la sensibilité biologique limitée de ces essais, étant donné que les NIAS présentes dans les extraits sont souvent en très faibles concentrations, et la quantité nécessaire pour les tester n'est pas toujours disponible pour identifier un danger. Le test d'Ames standard (OCDE TG 471) manque de sensibilité biologique pour détecter des substances génotoxiques à de tels concentrations. Pour pallier cette limitation, différentes versions miniaturisées du test d'Ames (ex. : Ames MPF, MMT) ont été développées pour améliorer la sensibilité et évaluer le potentiel génotoxique des extraits de MCDA, notamment pour les NIAS imprévisibles. Toutefois, aucune de ces variantes n'est encore validée comme alternative à la norme de l'organisation pour la coopération et le développement économiques (OECD en anglais) (OECD, 2022).

Le test **NanoAmes est** une version fortement miniaturisée et développée par GenEvolution, qui vise à abaisser le seuil de sensibilité biologique pour détecter les génotoxiques à de faibles concentrations dans des matrices complexes. Ce test a été mis en place dans le laboratoire

dans un premier temps. Ce chapitre présente une pré-validation inter-laboratoire du test NanoAmes menée par le CTM INSERM-UMR1231 (« plateforme PackTox ») et GenEvolution.

Matériels et méthodes

Quatorze substances de référence (génotoxiques et non génotoxiques), ainsi que des témoins solvant, ont été testés avec les souches *Salmonella typhimurium* TA98 et TA100, avec et sans activation métabolique (S9). L'objectif était d'évaluer la répétabilité et la reproductibilité du test NanoAmes. Les concentrations effectives minimales (CEM) et les relations dose-réponse ont été déterminées, et les résultats entre les deux laboratoires comparés.

Résultats

Témoins positifs et négatifs

Les valeurs des témoins négatifs étaient cohérentes entre les deux laboratoires pour les deux souches, avec ou sans S9. Les témoins positifs sans S9 (nitrofluorène (2NF) pour TA98, 1-oxyde de 4-nitroquinoline (4NQO) pour TA100) ont également montré des réponses similaires.

Cependant, avec le S9, les réponses au 2-aminoanthracène (2AA) étaient plus faibles au laboratoire PackTox que chez GenEvolution, avec des écarts de concentration notables, en particulier pour TA100.

Détermination des concentrations effectives minimales

- **Souche TA98** : Les substances 4-aminophénol (4AP), D-mannitol et mélamine ont donné des résultats négatifs cohérents dans les deux laboratoires. Les témoins positifs (2NF, 4NQO, 2AA, benzo-α-pyrène (BaP), N-Nitroso-N-Néthylurée (ENU), ICR 191 chlorhydrate d'acridine mutagène (ICR) ont donné les réponses attendues dans les deux laboratoires. En revanche, le méthanesulfonate de méthyle (MMS)/-S9, le cyclophosphamide monohydraté (CPA)/+S9, le 7,12-dimethylbenzanthracene (DMBA)/-S9 et l'aflatoxine B1 (AFB1)/-S9 n'ont pas montré les réponses attendues au laboratoire PackTox.
- **Souche TA100** : Des résultats négatifs similaires ont été obtenus pour le 4AP, D-mannitol et mélamine. Les résultats positifs pour 4NQO, 2AA, BaP, CPA, ENU et ICR étaient cohérents entre les deux laboratoires. Toutefois, au sein du laboratoire, il n'a pas été détecté le MMS/-S9 comme positif seulement à de très fortes concentrations (2500–5000 ng/puits), tandis que GenEvolution l'a détecté à 5 ng/puits. Le DMBA/-S9

et l'AFB1/-S9 n'ont pas donné les réponses attendues comme prévu lors des expérimentations dans la plateforme PackTox.

Relation dose-réponse

Des relations dose-réponse claires ont été observées pour certaines substances (par exemple 2NF, 4NQO, BaP) dans nos experimentations, mais d'autres composés n'ont pas montré les réponses attendues.

Répétabilité

Des problèmes de répétabilité ont été observés dans les deux laboratoires, en particulier dans notre structure. Avec la souche TA98, des variabilités ont été notées pour ENU, DMBA et AFB1. Avec la TA100, les substances SA, MMS, ENU, DMBA et AFB1 n'ont pas donné la réponse attendue.

_____ DISCUSSION _____

Cette étude a évalué la migration (avec de l'EtOH 95 %) et l'extraction (avec de l'ACN) de revêtements en polyester, avec une analyse des échantillons par LC-HRMS pour les NIAS non volatiles, et par GC-HRMS pour les NIAS semi-volatiles et volatiles. Le **fenozan** (acide 3-(3,5-di-tert-butyl-4-hydroxyphényl)propionique), un produit connu de dégradation d'antioxydants comme l'Irganox 1010 et 1024, a été identifié dans tous les échantillons par GC-HRMS à l'aide d'un standard de référence (Bertoldo and Ciardelli, 2004; Haider and Karlsson, 2002; Ji *et al.,* 2024; Liu and Mabury, 2021). L'analyse LC-HRMS des NIAS non volatiles a révélé un tétramère cyclique et trois hexamères cycliques (c(BD+2TCDDM+3tPA), c(3TCDDM+3tPA), c(2BD+TCDDM+3tPA), c(2TCDDM+2tPA)) correspondant à des oligoesters attendus selon la composition des revêtements.

L'étude s'est concentrée sur l'évaluation des dangers des NIAS de masse moléculaire inférieure à 1000 Da, car ces substances peuvent être absorbées par l'organisme humain (EFSA, 2008). La toxicité (cancérogène, mutagène, reproductive/développementale, toxicité d'organe et perturbation endocrinienne) du fenozan et des oligoesters a été prédite par des outils QSAR *in silico*, avec des biotests prévus pour les substances disposant de standards commerciaux (fenozan). Les valeurs de Log $K_{o/w}$ ont été utilisées pour évaluer le potentiel de

bioaccumulation, et la règle des 5 de Lipinski (Chapitre 2) a permis d'estimer l'absorption et la perméabilité en l'absence de données bibliographiques.

À ce jour, la toxicité du fenozan reste peu étudiée, les données existantes étant souvent confidentielles et non publiées. Des études rapportent un Log $K_{o/w}$ de 4,7 pour le fenozan, et une biodisponibilité comprise entre 46,8 % et 57,8 % (issus d'études sur les rats), ce qui suggère une absorption rapide et une élimination lente par l'organisme humain (Prokopov *et al*., 2006a, Prokopov *et al*., 2006b).

L'évaluation prédictive du danger du fenozan a révélé des alertes positives pour la mutagénicité, la cancérogénicité, la toxicité développementale, la perturbation endocrinienne (liaison ERα, inhibition de la thyroperoxydase, interférence avec le symporteur sodium/iodure (NIS1), liaison aux récepteurs pregnane X et androstane constitutif) et la toxicité d'organe (foie et thyroïde). Pour valider ces prédictions QSAR, des biotests *in vitro* — le test d'Ames et le test du micronoyau par cytométrie en flux — ont été réalisés conformément aux recommandations de l'autorité européenne de sécurité des aliments (EFSA en anglais) pour les substances mutagènes (EFSA, 2019a). Les deux tests ont donné des résultats négatifs, indiquant l'absence de génotoxicité pour le fenozan.

Le test d'absorption de l'iodure réalisé pour évaluer une possible perturbation thyroïdienne montré un effet inhibiteur faible du fenozan mais mesurable (IC_{50} 3840 fois plus élevé que le perchlorate, inhibiteur de référence) sur des cellules thyroïdiennes de rat FRTL-5. Ces résultats suggèrent un mode d'action potentiellement antithyroïdien du fénosan.

L'ANSES rapporte que le 2,6-di-tert-butyl-4-methylphenol (BHT), un analogue du fenozan, a induit des modifications de la physiologie thyroïdienne chez le rat dans plusieurs études (ANSES, 2016a). Pour mieux comprendre les effets possibles de l'inhibition du NIS1 par le fenozan, un essai *in vivo* Larval Amphibian Growth and Development Assay (LAGDA) pourrait être envisagé (OECD, 2015). De plus, un essai visant à verifier l'activité de peroxydase impliquée dans la synthèse des hormones thyroidiennes (Thyroid PerOxidase ou TPO) pourrait être réalisé pour vérifier l'alerte *in silico* concernant l'inhibition de la thyroperoxydase.

Plusieurs outils QSAR ont prédit une toxicité développementale pour le fenozan, potentiellement liée à la perturbation thyroïdienne — un lien à explorer. La perturbation de la synthèse des hormones thyroïdiennes par le fénosan pourrait représenter une voie

potentielle d'effets neurodéveloppementaux chez le fœtus à la suite d'une exposition maternelle, ce qui justifie des investigations supplémentaires afin d'élucider le lien entre ces deux effets toxicologiques (Mughal *et al*., 2018).

D'autres biotests ciblés pourraient être utilisés pour confirmer les alertes : le CTA pour la cancérogénicité, des essais de liaison aux récepteurs prégnane X humains et murins, et un test CAR-3 humain pour l'activation du récepteur androstane constitutif en utilisant des tests d'activation transcriptionnelle ou des études « omiques ».

En ce qui concerne l'évaluation des risques, l'approche TTC n'est pas applicable car le fénosan présente une activité de perturbation endocrinienne (EFSA, 2019b).

Concernant les quatre oligoesters identifiés, tous les isomères structurels possibles ont été conçus en tenant compte des monomères spécifiés par le fabricant (et non listés avec des limites de migration spécifique dans ResAP(2004)). Les quatre oligoesters ont été testés dans divers outils QSAR.

Tous les oligoesters ont été prédits avec des Log $k_{o/w} > 10$, ce qui suggère un fort potentiel d'accumulation, alors qu'un Log $k_{o/w} > 3$ nécessite déjà un examen approfondi selon l'EFSA (EFSA, 2008). Selon la règle des 5 de Lipinski, seul c(2TCDDM+2tPA) pourrait présenter une faible absorption et perméabilité, bien que des données expérimentales soient nécessaires pour confirmation (Lipinski *et al.,* 2001).

Ces oligoesters ne sont pas disponibles dans le commerce et doivent être synthétisés — une étape coûteuse et longue — avant que des biotests individuels puissent être réalisés. Pour l'instant, seules des prédictions *in silico* ont été effectuées, montrant une absence de mutagénicité, mais une toxicité potentielle sur les plans cancérogène, développemental et endocrinien (ex. : liaison à l'aromatase et aux ER). Les quatre oligoesters cycliques ont été classés Cramer III, avec des expositions estimées (0,05–0,84 µg/kg pc/jour) (calculée pour la population française uniquement, en considérant un poids corporel moyen de 60 kg) inférieures au seuil de préoccupation toxicologique (TTC en anglais) de 1,5 µg/kg pc/jour, ne suggérant pas de risque pour la santé.

Les bioessais restent essentiels pour évaluer la toxicité globale des extraits et migrats des MCDA, car ils peuvent détecter les NIAS non identifiées et tenir compte des effets "mélange" ce qui n'est pas le cas des méthodes analytiques ou *in silico*.

Ainsi, la génotoxicité et la cancérogénicité potentielles des extraits ACN et migrats EtOH 95% ont été évaluées par des biotests *in vitro*. Le MMT et le DDR ont été utilisés : le MMT miniaturisé est adapté aux faibles volumes d'échantillons ; le test DDR détecte plusieurs mécanismes mutagènes et biomarqueurs de clastogénicité, d'aneuploïdie et de génotoxicité, avec une sensibilité de 92,1 % et une spécificité de 96,2 % (Bryce *et al*., 2017). À notre connaissance, c'est la première fois qu'il a été utilisé pour tester des extraits ou migrats de MCDA.

Tous les extraits ACN et migrats EtOH 95 % ont été négatifs avec les tests MMT et DDR. Des tests de dopage avec des références positives ont confirmé la validité des tests et donc permis d'exclure un effet de matrice.

Pour vérifier les alertes de cancérogénicité *in silico* des oligomères, le CTA a été mis en œuvre au laboratoire. Les données des témoins dans les bornes des contrôles historiques ont confirmé le bon fonctionnement du test dans ces conditions expérimentales. Tous les échantillons ont été négatifs que ce soit en phase d'initiation ou de promotion, suggérant une absence d'activité de mutagénicité et de promotion (absence d'une quantité significative de foyers). C'est la première fois que le test CTA est utilisé avec des extraits/migrats de MCDA. Les résultats négatifs pourraient indiquer une absence ou une concentration très faible de composés génotoxiques, soulignant l'importance d'une sensibilité analytique adaptée et d'une concentration d'échantillons réaliste pour l'exposition. Il est important de souligner que, bien que le CTA demeure un outil de criblage précieux pour identifier des substances chimiques potentiellement cancérogènes, il ne permet pas de couvrir l'ensemble des modes de promotion tumorale. Cette limitation s'explique par le fait que l'essai ne reproduit pas la complexité de l'environnement *in vivo*, et que la promotion tumorale implique de multiples processus clés qu'aucun test *in vitro* unique ne peut détecter de manière exhaustive (Smith *et al*., 2016).

Une approche potentielle pour pallier cette limitation consiste à combiner le CTA avec des analyses toxicogénomiques et des biomarqueurs épigénétiques, susceptibles de mieux révéler les mécanismes sous-jacents des substances cancérogènes (Coppedè, 2023; Guichard *et al.*, 2023; Kirsch *et al.*, 2020; Mascolo et *al*., 2018; Pillo *et al*., 2024).

Ces résultats sont cohérents avec des études antérieures sur les revêtements polyester ou similaires, qui n'ont pas révélé d'effets génotoxiques (tests d'Ames, micronoyaux, SOS-Umu-C) (Meyer, 2020 ; Rainer *et al*., 2019 ; Séverin *et al*., 2016; Mittag *et al*., 2006).

Évaluer uniquement les NIAS identifiées ou la toxicité globale des MCDA est insuffisant, car de nouveaux métabolites peuvent apparaître lors de la digestion gastro-intestinale. Pour investiguer cela, la stabilité de quatre oligoesters cycliques a été étudiée par extraction ACN ciblée. Dix-huit composés parents ont été identifiés, dont le TCDDM, l'acide maléique et le trimère isocyanurate de diisocyanate d'isophorone (IPDI). L'extrait a été soumis à une digestion intestinale *in vitro* et analysé par LC/ESI-HRMS. Les oligoesters TCDDM–PA sont restés stables, alors que ceux contenant BD–PA et TCDDM–AA se sont partiellement dégradés en huit oligoesters linéaires. La dégradation a augmenté avec la pancréatine, bien que les produits plus petits n'aient pu être détectés.

Ces produits de dégradation peuvent présenter des profils ADMET (absorption, distribution, métabolisme, excrétion, toxicité) différents de ceux des composés parents. Par exemple, les oligoesters cycliques peuvent s'hydrolyser en formes linéaires moins toxiques, certaines classées Cramer I, alors que les formes cycliques sont classées Cramer III (Lestido-Cardama *et al.*, 2022b; Eckardt *et al.*, 2020, 2019).

Des investigations supplémentaires avec davantage de réplicats sont nécessaires pour bien comprendre le devenir digestif de ces oligoesters. Si ces résultats se confirment, des études de toxicité sur les produits de dégradation seront nécessaires, bien qu'entravées par l'absence de standards commerciaux.

Dans cette étude, tous les extraits et migrats de revêtements en polyester ont été négatifs dans les tests MMT, DDR et CTA, dans les conditions expérimentales. Pour améliorer le seuil de détection biologique et la sensibilité, un test Ames miniaturisé — le NanoAmes — a été développé par GenEvolution. Cette thèse présente une pré-validation inter-laboratoire, où GenEvolution et PackTox ont testé 14 substances de référence (avec et sans S9) sur *S. typhimurium* TA98 et TA100.

Le test a montré des témoins négatifs cohérents, indiquant une bonne répétabilité et reproductibilité. Pour la majorité des substances, les deux laboratoires ont donné des résultats similaires. Cependant, des réponses inattendues ont été observées pour l'AFB1 et le

DMBA sans S9, peut-être dues à l'instabilité des composés ou à une activation métabolique involontaire lors de la pré-incubation.

Les résultats préliminaires sont prometteurs : le NanoAmes a montré des CEM inférieurs à ceux des tests Ames classiques et MPF pour toutes les substances sauf AFB1/+S9, indiquant une meilleure sensibilité. Toutefois, des problèmes de répétabilité ont été constatés, avec des incohérences pour ENU, DMBA et AFB1 (TA98), et pour SA, MMS, ENU, DMBA et AFB1 (TA100). Cela pourrait résulter de différences dans la qualité des cultures bactériennes, des lots S9, des équipements ou du maniement par les opérateurs.

_____ CONCLUSION _____

Les tests de migration et extractions effectués avec de l'EtOH 95 % et de l'ACN sur les revêtements en polyester ont permis d'identifier quatre oligoesters cycliques (niveau de confiance 2) ainsi que le **fenozan**, un produit de dégradation connu d'antioxydants, confirmé à l'aide d'un standard de référence.

Aucune différence significative liée au solvant utilisé n'a été observée dans les quantités de ces composés. Les oligoesters **c(BD+2TCDDM+3tPA)**, **c(3TCDDM+3tPA)** et **c(2TCDDM+2tPA)** ont été quantifiés à des concentrations comprises entre **45,4 et 85,0 µg/dm²** (soit **272,4 à 510 µg/kg d'aliment**), le **c(2BD+TCDDM+3tPA)** était présent à des niveaux plus faibles, entre **3,1 et 6,1 µg/dm²** (18,6–36,6 µg/kg d'aliment). Le **fenozan** a été détecté autour de **0,2 µg/dm²** (1,2 µg/kg d'aliment) (concentrations de NIAS calculées en considérant qu'1 kg d'aliments est conditionné dans un emballage de 6 dm²).

Les prédictions QSAR ont indiqué que les isomères des oligoesters ne sont pas mutagènes, mais pourraient être **cancérogènes**, **tératogènes** et **perturbateurs endocriniens**. Le **fenozan**, quant à lui, a été prédit comme étant potentiellement toxique sur plusieurs cibles, non genotoxiques **dans les tests d'Ames et de micronoyaux**, il a toutefois **inhibé l'incorporation de l'iodure dans des cellules thyroïdiennes** (IC_{50} = 1152 µM), suggérant une activité antithyroïdienne.

Les extraits et migrats (n=10) ont donné des résultats **négatifs aux essais de génotoxicité** (MMT, DDR) et au **CTA**. L'implémentation du CTA au laboratoire PackTox a permis d'établir des valeurs de référence, confirmant l'absence de foyers dans les conditions testées.

Pour étudier le devenir gastro-intestinal, des extraits ACN ont été préparés à partir de revêtements polyester râpés. L'analyse LC/ESI-HRMS a identifié **18 composés parents** (oligoesters cycliques et linéaires, monomères). La digestion *in vitro* a montré que les oligoesters **TCDDM–PA résistaient à la dégradation**, tandis que les composés **BD–PA** et **TCDDM–AA** se **dégradaient partiellement** en oligoesters linéaires. Les plus petits produits de dégradation n'ont pas été détectés en raison des limites chromatographiques.

Une pré-validation interlaboratoire du test NanoAmes, utilisant 14 substances chimiques, a montré des résultats cohérents pour la majorité des substances positives et pour l'ensemble des substances négatives dans les deux laboratoires (GenEvolution et PackTox). Des relations dose-réponse claires n'ont pu etre déterminées que pour quelques composés (par ex., 2NF, 4NQO, BaP).

Le test NanoAmes a présenté des CEM inférieurs à ceux du test Ames classique et du test Ames MPF (à l'exception de l'AFB1/+S9), ce qui suggère une **sensibilité accrue**. Des problèmes de **répétabilité** et de **reproductibilité** subsistent et nécessitent des améliorations.

_____ PERSPECTIVES _____

Cette thèse a permis d'investiguer des effets indésirables essentiels dans la sécurité sanitaire des revêtements en polyester, en se concentrant sur l'identification et la caractérisation des NIAS. Des recherches supplémentaires sont nécessaires pour évaluer la sécurité de ces revêtements.

Voici les principales recommandations :

- **Vérifier les modes d'action (notamment oestrogéniques,** thyroïdiens) pour **les effets perturbateurs endocriniens** des extraits et migrats. En cas de résultat positif, procéder à un **fractionnement des mélanges** et à de nouveaux tests pour **identifier les substances actives**.
- **Optimiser les paramètres analytiques** pour améliorer l'identification des NIAS.
- **Appliquer des approches de modélisation QSAR pour prédire la toxicité des mélanges** chimiques.
- **Identifier la composition en monomères** des quatre oligoesters identifiés.

- Tester le **fenozan** dans des biotests ciblés sur les effets toxiques prédits (par ex. : **test TPO** pour l'inhibition de la thyroperoxydase, **CTA** pour la cancérogénicité). Un **essai *in vivo* LAGDA** (OECD, 2015) pourrait être utilisé pour évaluer l'inhibition du symporteur sodium/iodure (NIS1) et les effets thyroïdiens associés.
- Inclure **au moins trois réplicats** pour les essais de digestion des extraits ACN. Utiliser les **modèles QSAR** pour prédire la toxicité des **composés parents** et de leurs **produits de dégradation**. **Une évaluation de l'exposition et des risques** liés aux produits de dégradation pourrait être réalisée, en testant avec des enzymes intestinales, gastriques et salivaires, ainsi qu'en évaluant leur absorption.
- **Étendre la sélection des souches bactériennes** pour répondre aux normes OCDE dans les tests NanoAmes. Examiner les facteurs de variabilité, appliquer une **analyse statistique rigoureuse**, et **impliquer d'autres laboratoires** pour renforcer la robustesse de la validation interlaboratoire.