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RESUME

INTRODUCTION

Contexte

Les différentes plantes aromatiques, médicinales et d'épices sont une immense source de ressources naturelles et durables de composés avec des propriétés bénéfiques. Elles sont utilisées, depuis la préhistoire, comme aromatisants ou préservent des aliments ou comme traitement médicaux. Actuellement, certains d'entre eux sont déjà cultivés commercialement pour utiliser ses propriétés. Néanmoins, il existe encore certaines plantes médicinales et aromatiques qui ne sont pas encore valorisées et donc elles sont dénommées comme « plantes médiévales oubliées ». Par exemple, *Calamintha grandiflora, Myrrhis odorata* et *Tussilago farfara* correspondent à ce type de plantes et lesquelles seront considérées dans ce manuscrit.

Afin de revaloriser ces plantes oubliées, la bioprospection est mise en place et laquelle permet la recherche, la collection de nouveaux composés bioactifs naturels et son utilisation dans les produits pharmaceutiques et cosmétiques, par exemple. De plus, la bioprospection permet d'analyser les profils chimiques de différentes plantes et définir une liste des composés phytochimiques associés à chaque plante. Ainsi, par exemple, au travers de l'analyse d'une plante spécifique, il est possible définir les différents types antioxydants existant ainsi que sa quantité. Donc, cette plante peut utiliser comme une plante médicinale ou aromatique pour la consommation humaine afin d'absorber et neutraliser les radicaux libres ou la décomposition des peroxydes dangereux.

L'apparition des plantes médicinales et aromatiques et leur composition dépendent de nombreux facteurs, mais particulièrement, sur les conditions climatiques et le type de sol. La biogenèse des composés biologiquement actifs dépend de plusieurs facteurs: l'origine de la plante, la zone géographique, son génotype, phénotype et chémotype, ainsi que les fluctuations climatiques annuelles. La plupart des composés naturels possèdent plusieurs activités biologiques: antioxydants, antimicrobiens, anti-inflammatoires et autres. En conséquence, la comparaison des espèces végétales cultivées dans des sites géographiques différents afin d'évaluer leurs propriétés biologiques, il est une tâche intéressante et importante, en particulier, pour la valorisation de ces plantes pour leur culture commerciale.

Par ailleurs, une autre tâche importante pour la valorisation est l'utilisation rationnelle des plantes dans toute sa totalité, c'est-à-dire, la valorisation de tout le produit avec le moins de déchets. Les déchets ou sous-produits obtenus lorsque la transformation ou modification de transformer des matières premières des plantes peuvent représenter une ressource potentielle des antioxydants et d'autres substances importantes. Par exemple, les déchets produits pendant la distillation des plantes aromatiques pour la récupération de l'huile essentielle peuvent constituer jusqu'à 99,5 % des matières premières lesquels sont utilisés inefficacement ou ne sont pas utilisés. En effet, il y a des preuves croissantes que les composés non volatils présents dans ces résidus peuvent posséder diverses activités, qui pourraient être appliquées dans les aliments comme antioxydants et des additifs antimicrobiens pour la prévention de diverses maladies telles que le cancer, l'athérosclérose et des difficultés neurologiques. De plus, en considérant les études récentes [1-3] sur la fraction volatile de plantes, traditionnellement appelées huiles essentielles, qui considèrent leurs propriétés antioxydantes comme des substituts potentiels pour les antioxydants synthétiques [4]. Il est possible d'utiliser (ou revaloriser) les sous-produits, qui sont obtenus après l'élimination de l'huile volatile par diverses techniques de distillation, afin d'améliorer la faisabilité commerciale de la production de composés bioactifs de différentes plantes.

Dans notre recherche nous sommes intéressés par des espèces qui sont considérées comme de « plantes oubliées médiévales » et dans lesquelles les sous-produits ne sont pas encore analysés dans les études récentes. Ainsi, *Calamintha grandiflora, Myrrhis odorata* et *Tussilago farfara*, qui sont des plantes de type et qui poussent en Lituanie et au sud de la France, sont considérées comme candidat à réaliser une étude afin d'identifier les composant leurs propriétés et ses activités (antioxydants ou odorants) et définir la variation des composés bioactifs entre les différentes origines, une tâche considérée importante tant du point de vue chimique et pour l'optimisation de la production d'ingrédients fonctionnels bioactifs.

Objectif et les tâches du travail

Le but de ces travaux de thèse était d'évaluer les possibilités de la transformation des plantes *Calamintha grandiflora, Myrrhis odorata* et *Tussilago farfara* en arômes et ingrédients antioxydants en utilisant le concept d'agro-raffinerie.

Les tâches suivantes ont été définies pour atteindre l'objectif:

1. Déterminer la composition d'huiles essentielles à partir de plantes aromatiques collectées en Lituanie et sud de la France et les comparer avec les données publiées antérieurement.
2. Développer de nouvelles techniques d'extraction rapide des composés volatils et les comparer avec les méthodes traditionnelles. Pour ce faire, les composés volatils des huiles essentielles de *Calamintha grandiflora* obtenues par la méthode d'hydrodistillation sont comparés avec ceux obtenus avec les nouvelles méthodes Artificial Crushing Finger device (CFD) et Design of Flash Aroma Dispenser (FAD).
3. Évaluer les propriétés antioxydantes des sous-produits d'hydrodistillation au travers des différents essais *in vitro* avec différents solvants.
4. Évaluer les propriétés antioxydantes des sous-produits d'hydrodistillation *in situ* (huile de tournesol) en le comparant avec l'antioxydant synthétique commercial.
5. Déterminer les composés phytochimiques des sous-produits d'hydrodistillation et évaluer les propriétés antioxydantes de composants individuels en utilisant des méthodes chromatographiques et spectrométriques.

Originalité de la thèse

La nouveauté scientifique suite à ces travaux de thèse a été réalisée par l'accomplissement des tâches énumérées ci-dessus:

1. Les profils chimiques des composés volatils dans les plantes sélectionnées qui poussent en Lituanie et au sud de la France ont été compares par première fois. Les différences entre les plantes de Lituanie et de France ont été observées, ainsi qu'avec des travaux précédents rapportés dans la littérature.
2. Deux nouvelles méthodes analytiques (Artificial Crushing Finger device et Design of Flash Aroma Dispenser) pour l'évaluation rapide de native huile essentielle de plantes aromatiques ont été développées et testées.
3. L'évaluation systématique et complète des composés phytochimiques dans les sous-produits d'hydrodistillation, particulièrement les composés phénoliques, dans *Calamintha grandiflora, Myrrhis odorata* et *Tussilago farfara* a été réalisée pour la première fois.
4. Résidus désodorisés de *Calamintha grandiflora, Myrrhis odorata* et *Tussilago farfara* ont été valorisés à partir des bioressources d'un antioxydant pour la première fois.

5. La comparaison de profil chimique et des propriétés antioxydants de *Myrrhis odorata* et *Tussilago farfara de* Lituanie et la France a été réalisée pour la première fois et certains composés n'ont pas été identifiés dans les études précédemment publiées.

Apports de la thèse

Les sous-produits après hydrodistillation sont des ressources les plus prometteuses d'antioxydants naturels et qui permettent une utilisation rationnelle des matières premières. L'information obtenue dans cette étude peut être utilisée pour la sélection des plantes avec le plus grand contenue des composants actifs en fonction de leur origine géographique. L'information obtenue sur la composition des plantes sélectionnées et leurs propriétés biologiques élargir nos connaissances scientifiques sur la biodiversité des produits naturels. En effet, cette information peut être utile pour les scientifiques dans le domaine concerné et aux spécialistes de l'alimentation, des produits pharmaceutiques et cosmétiques, en termes d'applications pratiques. Enfin, les méthodes développées pour l'évaluation rapide des composés volatils pourraient trouver application dans la reproduction de diverses espèces de plantes aromatiques et d'épices.

Les points clés de la thèse

1. Des arômes et ingrédients avec des antioxydants peuvent être obtenus des sous-produits après hydrodistillation de *Calamintha grandiflora, Myrrhis odorata* et *Tussilago farfara* en utilisant le processus de l'agro-raffinerie.
2. Les méthodes de rapid arôme profilage, à savoir 'Artificial Crushing Finger device (CFD)' et 'Design of Flash Aroma Dispenser (FAD)' peuvent être utilisées pour l'évaluation des composés volatils des plantes comme des méthodes alternatives aux techniques d'extraction traditionnelle.

Les résultats de cette recherche ont été publiés dans trois articles scientifiques dans les revues indexées par 'ISI Web of Science' et dans deux d'actes de colloque internationaux. De plus, les résultats de cette recherche ont été présentés également dans les huit conférences scientifiques internationales.

Résultats

Analyse chimique des plantes aromatiques de deux différentes zones géographiques

La composition chimique des plantes aromatiques et médicinales varie selon la région de culture, les conditions climatiques, la phase végétative, la modification génétique pour n'en citer que quelques-uns. Par conséquent, la caractérisation de la diversité de la flore dans les différentes zones géographiques et pays est une tâche importante. Cette caractérisation est possible au travers de l'analyse des huiles essentielles des plantes aromatiques, car ils sont une grande source des composés chimiques. Les facteurs qui déterminent la composition chimique et sa quantité dans les huiles essentielles sont nombreuses et, dans certains cas, il est difficile de différencier entre eux, car ils peuvent être interdépendants et d'influencer réciproquement [37]. Par exemple, dans ces facteurs où variables, on peut considérer les différentes variations: saisonnière, l'origine géographique, génétique, les stades de croissance, manipulation post-récolte et le stockage [15, 37]. Ainsi, l'objectif de ce manuscrit a été d'examiner les compositions chimiques des huiles essentielles de trois plantes aromatiques (*Myrrhis odorata, Tussilago farfara* et *Calamintha grandiflora)* de deux différentes origines afin de déterminer les facteurs plus importants qui influencent la composition et la quantité chimique de ces plantes et les comparer avec des résultats publiés antérieurement à ce manuscrit. Pour ce faire, les huiles essentielles de ces plantes ont été obtenues par hydrodistillation. Puis, pour l'identification des composées volatiles, les huiles sont analysées avec la méthode de chromatographie en phase gazeuse (GC) et détecteur à ionisation de flamme et avec la méthode chromatographie en phase gazeuse et de spectrométrie de masse (GC-MS). Les résultats de ces analyses sont détaillés ci-après.

Myrrhis odorata

La quantité d'huile essentielle extrait a été de 0.40±0.02% (Lituanie) et 0.50±0.01% (France) (w/w). vingt et vingt-deux composants volatiles ont été détectés dans les huiles essentielles de Lituanie et de France, respectivement. De plus, six composants volatils qui ont été détectés dans l'huile d'origine française ne sont pas trouvés dans l'huile d'origine lituanienne: camphre, *iso*-menthone, *cis*-thujone, pinocarveol, carvacrol et *p*-anisaldéhyde. Par ailleurs, les deux huiles essentielles ont montré une plus grande quantité des hydrocarbures sesquiterpènes que d'hydrocarbures monoterpènes (tableau R.1). Les composants de major quantité ont été *E*-anéthol, methyleugenol et *E*-nerolidol, dans lequel aucune différence significative n'a pas été constatée entre les deux origines. Par rapport à ses arômes, ce trois composants ont été

analysés et décrits comme sucrée, de l'anis étoilé et de réglisse (E-anéthole); clou de girofle épicé (methyleugenol), et floraux, fruités, cireux boisé (E-nérolidol). La comparaison entre les pourcentages des principales composantes des huiles analysées dans notre étude et précédemment rapporté dans la littérature sont résumées dans le tableau R.2

Tableau R.1. Composition (%) chimique de l'huile essentielle de *Myrrhis odorata* (L.) Scop. De la Lituanie et la France.

No .	Composés	RI[th]	RI[♦]	(%)		Perception de l'odeur [c]
				Lituanie	France	
1	α- Pinène	939	936	0.1±0.0[a]	0.1±0.0[a]	frais, camphre, de pin doux, terreux, boisé
2	β-Myrcène[*]	992	986	1.7±0.1[a]	1.8±0.1[a]	poivré, terpène, épicé, balsamique, plastique
3	*p*-Cymène	1027	1022	0.2±0.0[a]	0.5±0.1[a]	frais, agrumes, terpène, boisé, épices
4	Limonène	1029	1030	1.7±0.0[a]	0.4±0.0[b]	citrus, frais, poivré
5	*E*-β-Ocimène	1038	1032	0.3±0.1[b]	0.8±0.0[a]	doux, vert, céleri épicé, floral
6	γ-Terpinène	1074	1058	0.3±0.0[b]	0.6±0.1[a]	huileux, boisé, terpène, citron, tropical, de fines herbes
7	*cis*-Thujone	1102	1092	-	0.2±0.0	feuille de cèdre
8	Nonanal	1104	1103	0.2±0.0[b]	0.4±0.0[a]	aldéhyde rose, frais, d'orange
9	Camphre	1139	1131	-	0.3±0.0	médicinales, camphrée
10	*iso*-Menthone	1162	1168	-	0.2±0.1	puissant, rafraîchissant menthe propre, frais, menthe poivrée
11	Pinocarveol	1169	1169	-	0.5±0.0	camphre, boisé, le pin, le sapin
12	Carvacrol	1192	1192	-	0.4±0.0	épice, boisé, le camphre, le thymol
13	Estragole[*]	1200	1197	0.9±0.1[b]	4.1±0.4[a]	douce, de réglisse, d'épices d'anis, de fenouil herbes vertes
14	*p*-Anisaldéhyde	1263	1271	-	0.3±0.0	doux, mimosa, floral, aubépine en poudre, le sapin baumier
15	*E*-Anethole[*]	1285	1290	48.1±1.4[a]	50.7±1.1[a]	sucrée, de l'anis étoilé et de réglisse
16	Methyleugenol[*]	1407	1401	14.3±0.5[a]	13.1±0.4[a]	clou de girofle épicé, cannelle
17	β-Caryophyllène	1419	1409	9.7±0.3[a]	6.1±0.3[b]	sucré, boisé, d'épices
18	α-Bergamotene	1431	1430	0.9±0.1[a]	0.5±0.0[b]	boisé, chaud, thé
19	α-Humulene	1467	1459	0.9±0.2[a]	0.5±0.0[a]	Boisé, herbes
20	Germacrene-D	1487	1485	5.0±0.1[a]	4.3±0.3[a]	boisé, d'épices
21	β-Bisabolène	1498	1496	0.1±0.0[a]	0.1±0.0[a]	balsamique, d'agrumes, de la myrrhe, épicé
22	α-Farnesène	1500	1504	0.3±0.0[a]	0.2±0.0[b]	herbes, lavande, bergamote, myrrhe, vert
23	*E*-Nerolidol[*]	1563	1559	10.2±0.8[a]	12±0.3[a]	floraux, fruités, cireux boisé
24	Spathulenol	1619	1618	2.1±0.5[a]	0.7±0.0[a]	terreux, herbes, fruité
25	Hexadécanol	1870	1864	1.2±0.2[a]	0.7±0.0[a]	cireux, floral
26	Phytol	2128	2125	1.4±0.1[a]	0.4±0.0[b]	délicat, floral, le sapin baumier, poudreuse, cireux
	Total			99.6±0.2	99.9±0.1	
Phénylpropanoïdes (%)				63.3±0.7[a]	68.2±0.6[a]	
Hydrocarbures monoterpènes (%)				4.1±0.1[a]	3.7±0.1[a]	
Monoterpènes oxygénés (%)				-	1.2±0.0	
Hydrocarbures sesquiterpéniques (%)				16.9±0.1[a]	11.7±0.1[b]	
Sesquiterpènes oxygénés (%)				12.3±0.4[a]	12.7±0.3[a]	
Autres (%)				3.0±0.1[a]	2.4±0.1[b]	

RI[♦]**:** Indices de rétention relatifs à C8-C22 alcanes sur la colonne AnHP-5MS
RI[th] : Indice présenté à partir de la base de données http://www.flavornet.org
[*] standard authentiques ont été co-injectées pour l'identification des composés
[c]Description Odeur adapté de www.thegoodscentscompany.com
Valeurs avec des lettres différentes (a-b) sont significativement différentes à P <0,05

La comparaison entre les pourcentages des principales composantes des huiles analysées dans notre étude et précédemment rapporté dans la littérature sont résumées dans le tableau R.2. Ainsi, dans la littérature, *E*-anéthole a été le composant la plus significative (82–85%) dans les huiles d'origine Finlande [137], de la Russie [139] et des États-Unis [140] et on peut considérer que ces plantes ont été que de chémotype *E*-anéthole. En autre, les plantes des États-Unis ont eu une raisonnable quantité de méthyleugénol qui n'a pas été trouvé dans les plantes d'origine de la Russie ou de Finlande. Par ailleurs, dans les plantes d'origine de la Russie [139] et de la Serbie [141], le chémotype *E*-nerolidol n'est pas identifié. De plus, un chémotype complément différent a été identifié dans l'huile d'origine de la Serbie (région Prokletije) avec p- cymène comme le composant le plus représentatif constituant le 62.0% du total d'huile [141].

Tableau R.2. Comparaison des principaux constituants d'huiles essentielles de *Myrrhis odorata* (L.) Scop.

Composés	**Lituanie[a] (%)**	**France[a] (%)**	**Finlande[b] (%)**	**Russie[c] (%)**	**Serbie[d] (%)**	**Etats-Unis[e] (%)**
β- Myrcène	1.7	1.8	1.1	-	-	-
p-Cymène	0.2	0.5	-	-	**62.0**	-
Limonène	1.7	0.4	0.1		2.2	0.3
Estragole	0.9	4.1	2.6	-	-	1.7
E-Anéthole	**48.1**	**50.7**	**83.1**	**82.0**	-	**85.5**
β- Caryophyllène	9.7	6.1	1.0	1.7	0.7	-
Methyleugenol	**14.3**	**13.1**	-	-	-	**9.1**
Germacrène-D	5.0	4.3	-	2.9	-	-
E-Nerolidol	**10.2**	**12.0**	1.0	-	-	-

[a] Notre étude; [b]Uusitalo et al. 1999 [137]; [c] Tkachenko et al. 1993 [139]; [d]Rančić et al. 2005 [141]; [e]Hussain et al. 1990 [140].

Cette variation dans la quantité de composant peut paraître étonnante, mais pas impossible parce que le polymorphisme chimique est une caractéristique significative pour plusieurs plantes de la famille des ombellifères. Ainsi, cette thèse fournit une information actualisée sur la composition chimique de l'huile essentielle de *Myrrhis odorata* de deux origines différentes (Lituanie et France) qui n'avait pas été présenté auparavant. Et bien que les deux origines soient de bien différents, le pourcentage des composants principaux est assez similaire. Pourtant, en comparant avec des données obtenues dans études antérieures, les huiles essentielles ont révélé que sont plantes avec des chémotypes différentes. Par conséquent, des recherches ultérieures sont nécessaires pour obtenir des renseignements plus précis des huiles essentielles de différentes zones géographiques avec des conditions climatiques similaires afin d'atteindre les meilleurs résultats possibles. Cela permettrait d'identifier le potentiel génétique qui pourrait aider dans l'amélioration des programmes d'élevages et diversification de *Myrrhis odorata* pour l'utilisation industrielle.

Tussilago farfara

La quantité d'huile essentielle extrait a été de 0.07±0.01% (Lituanie) et 0.09±0.00% (France) (w/w). À notre connaissance, aucune information sur les composants chimiques de *Tussilago farfara* d'origine Française ou d'Europe du Sud n'était pas disponible. Les variations quantitatives et qualitatives dans la composition des plantes d'origines différentes (Lituanie et France) ont été analysées et les résultats sont présentés dans ce manuscrit. En bref, 1-nonene (34.1±0.0%) était le principal composé dans l'huile d'origine Française (tableau R.3).

Tableau R.3. Composition chimique (%) de *Tussilago farfara* de deux origines

No	Composés	R.I.[ap]	R.I.[p]	(%)	
				Lituanie	France
1	1-Nonène	890	922	8.1±0.3[b]	34.1±0.0[a]
2	*α*-Pinène	935	1024	3.7±01[a]	4.2±0.2[a]
3	Camphène	951	1068	-	tr
4	1-Decène	988	1040	-	2.1±0.0
5	*α*-Phellandrène	1004	1163	0.8±0.1[a]	1.4±0.3[a]
6	*p*-Cymène	1029	1270	0.1±0.0[b]	0.8±0.0[a]
7	*n*-Undecane	1100	1100	7.8±0.2[a]	4.8±0.1[b]
8	1-Dodecène	1191	1190	0.2±0.0[b]	0.5±0.0[a]
9	1-Decanol	1273	1275	0.1±0.0	-
10	1-Tridecène	1291	1365	0.1±0.0[b]	0.3±0.0[a]
11	*α*-Copaène	1376	1489	0.2±0.0	tr
12	*β*-Cubebène	1387	1522	0.2±0.0[b]	0.3±0.0[a]
13	1-Tetradecène	1389	1414	0.9±0.1	-
14	*β*-Caryophyllène	1419	1589	1.9±0.1[a]	0.3±0.0[b]
15	*γ*-Elemène	1439	1470	0.2±0.0[b]	0.4±0.0[a]
16	*α*-Humulène	1454	1665	0.4±0.0[a]	0.2±0.0[b]
17	Germacrène D	1485	1689	0.7±0.1[a]	0.6±0.0[a]
18	*β*-Selinène	1489	1696	0.7±0.0[a]	0.6±0.1[a]
19	*n*-Pentadecane	1500	1500	0.5±0.1	-
20	*β*-Bisabolène	1509	1715	1.2±0.1[a]	0.7±0.1[b]
21	*δ*-Cadinène	1522	1737	0.4±0.0[a]	0.2±0.0[b]
22	Spathulenol	1576	2128	2.1±0.1[a]	0.7±0.1[b]
23	Caryophyllène oxide	1585	1918	1.3±0.1[a]	0.2±0.0[b]
24	Tetradecanol	1680	2177	-	0.2±0.0
25	*n*-Nonadecane	1900	1900	0.4±0.0	-
26	Phytol	2112	2547	1.4±0.1[a]	0.4±0.0[b]
27	*n*-Tricosane	2300	2300	21.7±0.1	-
28	*n*-Tetracosane	2400	2400	0.7±0.0	-
29	*n*-Pentacosane	2500	2500	1.3±0.0	-
30	*n*-Hexacosane	2600	2600	5.1±0.1	-
	Total			62.2±0.1[a]	53.0±0.5[b]

[(ap)] Indices de rétention relatifs à C8-C28 alcanes sur colonne DB5
[(p)] Indices de rétention relatifs à C8-C28 alcanes sur la colonne Carbowax 20M
Valeurs avec des lettres différentes (a-b) sont significativement différentes à P <0,05; tr-trace <0.05%

En revanche, n-tricosane (21.7±0.1%) était le principal composé dans l'huile d'origine Lituanienne. De plus, sept composantes volatiles, voire tetracosane, n-tricosane, n-

pentacosane, n-hexacosane, n-pentadecane, 1-tetradecène et 1-decanol, ont été détectés seulement dans l'huile d'origine Lituanienne.

La quantité de 1-nonene dans l'huile d'origine française était quatre fois supérieure à celui d'origine lituanienne. De plus, les pourcentages de α-phellandrene, α-pinène, p-cymène et 1-dodecène ont été significativement supérieur que ceux d'origine Lituanienne. Enfin, il convient de faire remarquer qu'il existe une différence significative dans les conditions climatiques entre le deux régions étudiées (France et Lituanie). Par exemple, la moyenne de température et de précipitations pendant le mois de la récollette était supérieur dans le Midi-Pyrénées-France (10.5°C et 97.1 mm) qu'en Kaunas-Lituanie (6.9°C et 34.0 mm). Également, ces différences significatives dans la composition chimique des huiles essentielles de ses deux origines peuvent être associées aux conditions climatiques et aussi au génotype et chémotype.

Crushing Finger Device & Flash Aroma Dispenser: deux nouveaux outils pour l'étude des huiles essentielles natives

Les plantes à huiles essentielles désignent les végétaux odorants utilisés en parfumerie, cosmétique, cuisine et médecine douce dont des extraits aromatiques peuvent être obtenues par entraînement à la vapeur ou par hydrodistillation de leurs feuilles ou de leurs fleurs. Il est communément admis que l'emploi de ces méthodes d'extraction dur induit la formation d'artefacts du fait de réactions chimiques in situ (hydrolyse notamment) et en conséquence que l'extrait obtenu peut présenter une odeur sensiblement différente de celle des végétaux initiaux. L'accès à ces huiles essentielles natives (composés volatils odorants naturellement émis) est possible via l'écrasement de la feuille entre les doigts suivis du "sniffing" des effluves émis. Si cette action mécanique provoque bien l'éclatement des vésicules de stockage surfacique qui contiennent les huiles essentielles, la faible reproductibilité de la méthode ne permet pas de l'utiliser en vue d'une analyse instrumentale des effluves. Dans le cadre du programme AROMATIC, nous nous sommes intéressés à une instrumentalisation de l'évaluation rapide du potentiel aromatique de plantes à huiles essentielles basées sur l'analyse des effluves (HeadSpace) et sur un écrasement contrôlé de feuilles fraiches. Deux approches ont été envisagées, l'écrasement mécanique et l'écrasement par détente de gaz, toutes deux suivies du piégeage des composés volatils odorants émis en vue de leur analyse chromatographique ultérieure. Dans le premier cas, un appareillage novateur dénommé "Crushing Finger Device" (CFD) a été mis au point afin de mimer instrumentalement

l'écrasement de la feuille entre les doigts (avec contrôle de la température et de la force d'écrasement) (Fig. R.1).

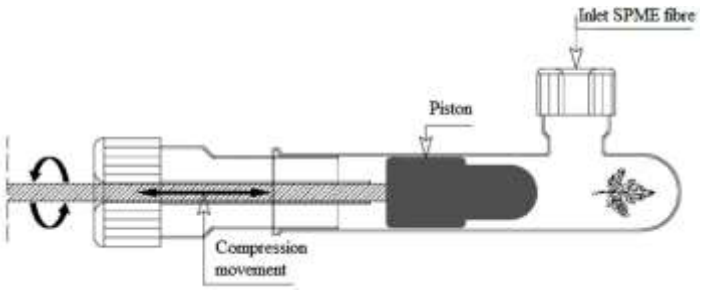


Fig.R.1. Crushing Finger Device" (CFD)

Dans le deuxième cas, un appareil dénommé « Flash Aroma Dispenser (FAD) » a été développé sur la base d'un siphon a crème chantilly et dans lequel la brusque détente d'un gaz (NO_2 ou CO_2) provoque également un éclatement partiel des vésicules de stockage surfaciques qui contiennent les huiles essentielles (Fig. R.2).

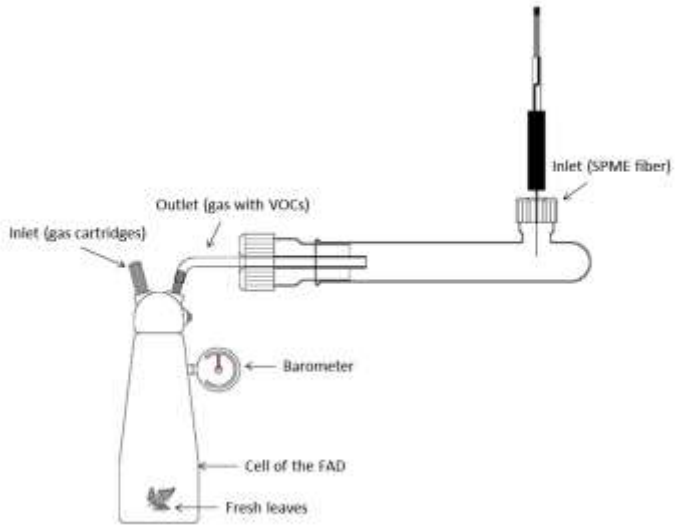


Fig.R.2. Flash Aroma Dispenser (FAD)

Dans le chapitre présenté, nous rapporterons la comparaison du profil chromatographique de l'huile essentielle de feuilles fraiches d'une plante modèle obtenue par hydrodistillation avec ceux des effluves de feuilles fraiches entières traitées via le CFD ou le FAD puis concentrées par SPME et analysées en GC-MS. Les paramètres expérimentaux de ces 2 techniques de génération d'huiles essentielles natives ont été optimisés via la technique des plans d'expériences tandis que l'observation microscopique des feuilles après traitement a

permis de valider l'efficacité du CFD et du FAD vis à vis de l'éclatement des poils sécréteurs et des vésicules à huiles essentielles (Fig. R.3)

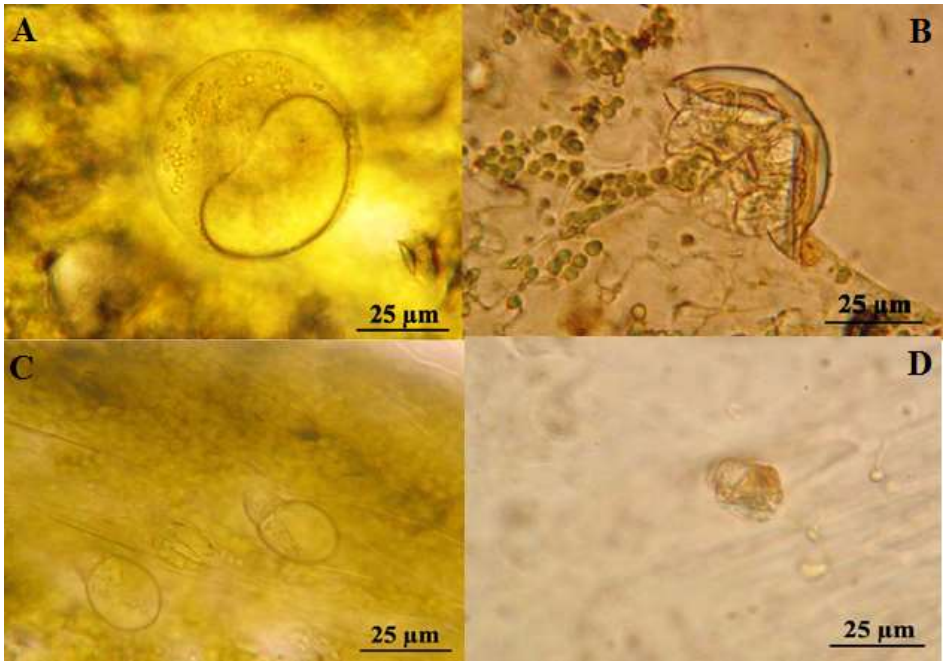


Fig. R.3. L'analyse microscopique de *C. grandiflora*. A et C avant l'expérience. B après CFD et D après FAD

L'activité antioxydante des résidus d'hydrodistillation des plantes aromatiques

La valorisation des résidus plantes aromatiques après distillation des huiles essentielles sont une tâche importante, car elle permet d'identifier des composants importants non utilisées dans les plantes qui sont encore utilisables. Par exemple, les produits à base de plantes sèches composées des glucides et de ses dérives présents dans les parois cellulaires et qui ne sont pas digestibles montrent peu d'intérêt dans l'application dans l'industrie alimentaire et/ou nutraceutique. Pour son utilisation, ces composants sont extraits au travers d'hydrodistillation. Pourtant, certains métabolites secondaires non volatils qui sont aussi de grands intérêts pour ces industries peuvent se trouver dans les résidus d'hydrodistillation et sont jetés. Ainsi, l'objectif principal de cette recherche était d'évaluer le potentiel antioxydant et la quantité de polyphénols dans les résidus d'hydrodistillation de *C. grandiflora*, *M. odorata* et *T. farfara*. Ce résidu qui est considéré comme un « soupe des herbes » qui consiste en une melange de solides et liquides. Il faut noter que dans certains cas, les extraits des plantes désodorisées qui sont produites des résidus des huiles essentiels étaient rapportés comme possédant d'un grand potentiel antioxydant supérieur à ceux des extraits obtenus des

matières premières. En effet, la composition des fractions obtenues par hydrodistillation dépendra de la solubilité des plantes dans l'eau bouillante, laquelle est un solvant de grandes polarités. Les résultats de la capacité à piéger les radicaux libres des extraits avec les méthodes DPPH, ABTS et FRAP sont listés dans les chapitres 3.3.1, 3.3.2 et 3.3.3. Il faut noter que les mesures de la capacité à piéger les radicaux libres avec les méthodes DPPH et ABTS sont facile, rapides et très sensibles et donc elles sont plus fréquemment utilisées pour une évaluation préliminaire du potentiel antioxydant des différentes substances naturelles. Mais, si bien que les princips de base sont similaires, la méthode ABTS est préférable pour sa capacité à piéger les radicaux libres dès les antioxydants lipophiles et hydrophiles.

Évaluation de l'activité antioxydante des résidus d'hydrodistillation de *C. grandiflora*

Pour l'évaluation de la capacité à piéger les radicaux libres des extraits de *C. grandiflora*, trois méthodes ont été utilisées: $DPPH^{\bullet}$, $ABTS^{\bullet+}$ et FRAP. Ces méthodes présentent des avantages, comme leur simplicité, leur utilisation généralisée pour la présentation rapide de résultats dans l'analyse des propriétés antioxydantes des plantes et des informations préliminaires fiables de la présence des composés antioxydantes actives dans les extraits.

La concentration nécessaire pour réduire la concentration initiale de $DPPH^{\bullet}$ à 50% (IC_{50}) a été choisi pour comparer la capacité à piéger les radicaux libres des différentes extraits (Fig. R.4). Ainsi, un faible IC_{50} est le résultat d'une capacité à piéger les radicaux libres.

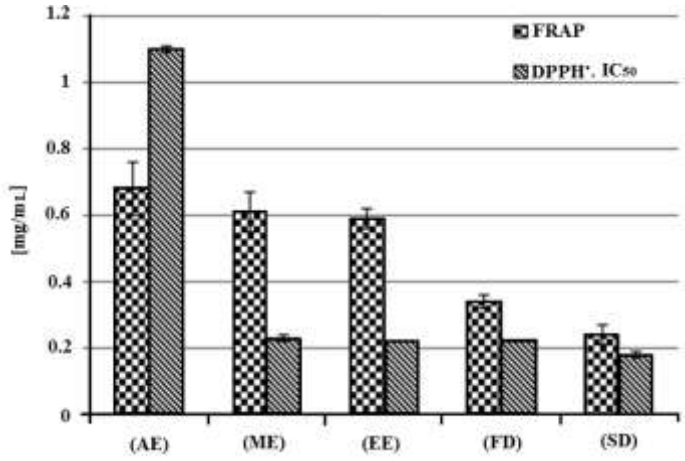


Fig. R.4. Activité antioxydante de *C. grandiflora*

Les valeurs de IC_{50} des extraits de *C. grandiflora* (résidus solides) dans $DPPH^{\bullet}$ ont été variable de 1.10 (AE) à 0.23 (ME) mg/mL (Fig. R.4) quand les valeurs de TEAC dans $ABTS^{\bullet+}$ ont été de 0.11(AE) à 0.66% (EE) (Fig. R.5). Il est évident que dans la polarité inférieure de l'extrait de l'acétone (AE) était significativement inférieur la capacité à piéger

les radicaux libres que dans l'extrait d'alcool (ME, EE). Ces résultats sont en accord avec les résultats publiés précédemment lesquels montrent que les solvants polars extraient plus de composants antioxydants que les solvants de polarité faible [86, 108, 185]. Le méthanol et l'éthanol, comme des solvants organiques sont relativement similaires, mais la capacité à piéger les radicaux libres dans l'extrait EE ont été plus élevés que ME dans la méthode $ABTS^{\bullet+}$. Néanmoins, l'éthanol est un solvant de qualité alimentaire donc son utilisation est préférable que le méthanol qui est toxique. Il faut aussi noter qu'en $DPPH^{\bullet}$, la réaction de la capacité à piéger les radicaux libres des extraits alcoolisés obtenus des résidus solides a été assez similaire à celui de la capacité à piéger les radicaux libres des extraits de l'eau, 0.23 (ME), 0.22 (EE), 0.22 (FD) et 0.18 (SD) mg/mL, respectivement. Ce qui est scientifique que certains composés actifs antioxydants ne se dissolvent pas dans l'eau bouillante et donc ils restent dans les résidus solides. Par ailleurs, au travers de la méthode FRAP, il est possible d'appliquer aux trois extraits aqueux, alcoolisé et de l'acétone de différentes plantes. Dans cette méthode, l'activité antioxydante est déterminée sur la base de la capacité à réduire de l'état ferrique (Fe^{3+}) à l'état ferreux (Fe^{2+}). Cette réduction est définie en mg de ferreux par mL d'échantillon.

Dans la méthode FRAP, un sel ferrique (Fe III) est utilisé comme un antioxydant et son potentiel d'oxydoréduction (0.70 V) est comparable à celui de $ABTS^{\bullet+}$ (0.68 V), et dont, par conséquent, il n'y a pas beaucoup de différences entre les méthodes TEAC et FRAP [186]. Pourtant, les résultats obtenus pour les extraits *C. grandiflora* d'avec ces deux méthodes étaient assez différentes. Cela montre que le type de test joue un rôle plus important dans la mesure de l'activité antioxydante. Ainsi, le pouvoir de réduction des différends extraits était entre 0.24–0.68 mg Fe (II)/mL (Fig. R.4). La différence la plus remarquable a été observée pour l'AE, lequel était plus faible antioxydant dans la méthode TEAC (Fig. R.5) à lorsque la méthode FRAP, AE l'agent réducteur ls plus forte. ME et AE produits avec les résidus solides de l'hydrodistillation des plantes ont été remarquablement élevée qu'avec les extraits séchées. Les différences observées entre les méthodes DPPH/ABTS et FRAP peuvent être expliqués pour les solvants avec une différence en sa polarité et un pH dans du milieu réactionnel [187].

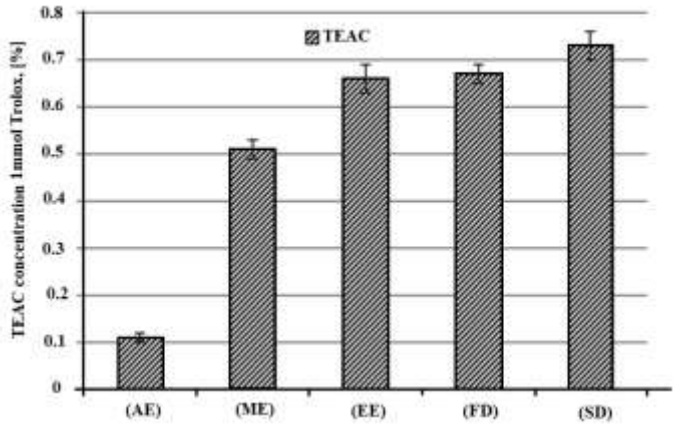


Fig. **R.5.** TEAC valeurs dans ABTS test de *C. grandiflora*

Les propriétés antioxydants des résidues de *M. odorata* de deux des zones géographiques différentes

Le climat, l'altitude et le sol peuvent être liés à l'activité antioxydante. Ainsi, l'objectif de cette recherche est de déterminer le lien entre les propriétés antioxydantes et l'origine des plantes. Pour ce faire, l'analyse antioxydante a été réalisée qui montre que dans tous les échantillons les résidus, l'activité antioxydante a été active, toutefois, la capacité à piéger les radicaux libres a varié significativement (Fig. R.6). En fait, la capacité à piéger les radicaux libres des extraits d'origine française (FR) ont varié entre 0.12±0.00 à 0.83±0.08 mg/mL dans le test DPPH$^{\bullet}$ et entre 0.02±0.00 à 0.79±0.07 mg/mL avec FRAP, alors que les extraits d'origine lituanienne (LT) ont varié entre 0.11±0.00 à 0.98±0.08 mg/mL dans le test DPPH$^{\bullet}$ et entre 0.14±0.01 à 0.99±0.02 mg/mL dans la méthode FRAP (Fig. R.6).

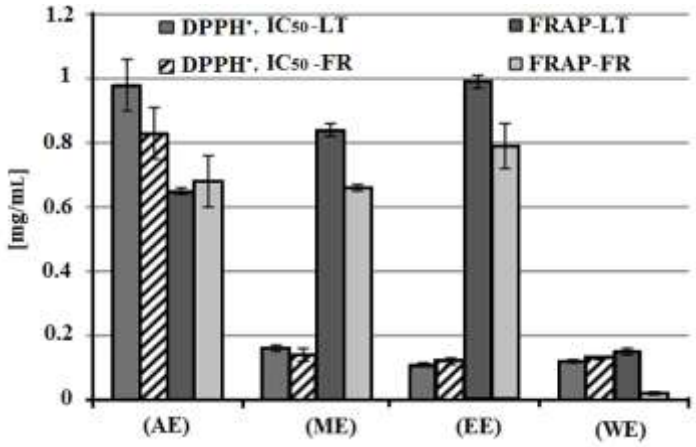


Fig. R.6. Activité antioxydant de *M. odorata* de la Lituanie et la France

L'activité antioxydante de *M. odorata* a été testée via la méthode DPPH$^{\bullet}$ qui a montré que les AE et ME extraits des plantes d'origine FR ont une activité supérieure à celles de la même famille botanique, mais de différentes origines (LT). De plus, la méthode FRAP a montré que les extraits LT ME, EE et WE ont une activité antioxydante plus élevée en comparaison avec les extraits FR. Ainsi, en considérant les résultats de l'analyse DDPH$^{\bullet}$, il est important de mentionner que n'existe pas une similitude significative entre les mêmes plantes de différents climats et origine (FR et LT). En revanche, les résultats obtenus de l'analyse FRAP montrent que les plantes localisées en LT une activité antioxydante supérieure à ceux localisés dans le Midi-Pyrénées.

Les informations précédentes ont démontré que la mesure de la capacité à piéger les radicaux libres dans ce modèle est une méthode fiable pour évaluer les propriétés antioxydante des plantes. Pourtant, il est bien connu que les antioxydants agissent très différemment avec les différents radicaux libres. En conséquence, afin d'obtenir plus d'informations sur la capacité à piéger les radicaux libres, nous avons présenté un deuxième modèle (ABTS$^{\bullet+}$) pour tester cette capacité. Ainsi, un test avec l'ABTS$^{\bullet+}$ de décoloration de cation radicalaire a montré un résultat similaire comparé avec ceux obtenus par DPPH$^{\bullet}$. Les extraits EE et ME ont une capacité à piéger les radicaux libres plus forts quelle extraits AE dans l'ABTS$^{\bullet+}$ teste. La concentration des extraits ont été similaires à l'activité de 1 mmol d'une solution de Trolox dans une réaction ABTS$^{\bullet+}$ (Fig. R.7).

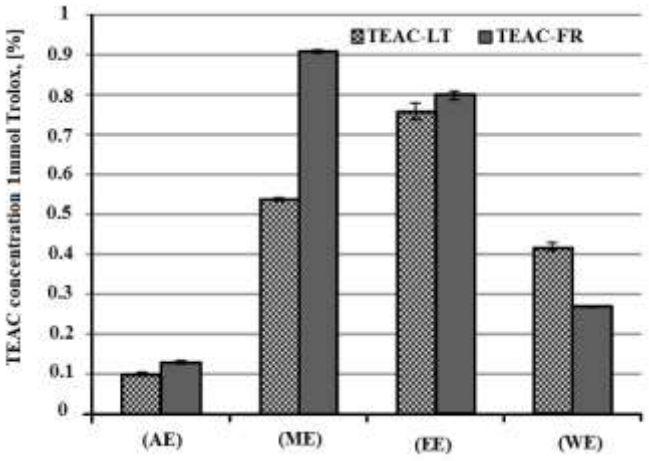


Fig. R.7. TEAC valeurs dans ABTS test de *M. odorata* de la Lituanie et France

L'activité plus élevée a été prouvée avec extrait d'EE (de 0.76 à 0.8 %) et de ME (de 0.54 à 0.91 %). Tous les extraits ont présente une activité antioxydante plus élevée que les extraits

d'AE dans les méthodes DPPH$^{\bullet}$, FRAP et ABTS$^{\bullet+}$. Donc, en comparant les différentes origines, la capacité à piéger les radicaux libres dans ABTS$^{\bullet+}$ à montrer que les extraits d'origine FR ont été plus élevé que les extraits d'origine LT, à exception de l'extrait WE. En outre, dans le test de DPPH$^{\bullet+}$, cette capacité dans les extraits EE et WE ont été légèrement plus élevé que ME, alors que les AE ont été une capacité de 6 à 9 fois plus faible que les extraits polaires (Fig. R.6). En ce qui concerne avec le fait que la majorité d'antioxydant sont décomposés phénoliques polaires, les résultats sont en accord avec les travaux déjà réalisés précédemment [188]. Un test préliminaire du potentiel antioxydant a indiqué que la fraction non volatile de *M. odorata* contient une quantité relativement élevée de composants phénolique qui pourrait être d'intérêt pour l'industrie alimentaire pour son isolation. De plus, le test a montré que la composition des métabolites secondaires des plantes dépendent de plusieurs facteurs, notamment la superficie de culture, les conditions climatiques, la phase végétative, les modifications génétiques. En conséquence, l'évaluation des propriétés des plantes de différentes régions géographiques sont une démarche intéressante qui a fait l'objet de nombreux travaux scientifiques [188-189].

L'activité antioxydante des organes anatomiques de T. farfara de deux différentes origines géographiques

Si bien que plusieurs composés comme produits phénoliques, mucopolysaccharides et des polysaccharides solubles dans l'eau ont été isolées de boutons de fleur chez *T. farfara* [151], aucun flavonoïdes n'ont pas été rapportés. Jusqu'à présent, aucune étude n'a été menée sur les résidus d'hydrodistillation. Pourtant, ces résidus peuvent contenir des molécules bioactives qui n'ont pas été antérieurement utilisées. Comme le résultat des récents développements dans la chimie verte et l'intérêt pour une utilisation complète des plantes dans l'industrie, la revalorisation des résidus de plantes après du processus d'extraction et transformation attire de plus en plus l'attention dans l'industrie chimique. Les dernières études ont prouvé que certaines espèces peuvent contenir une grande quantité des molécules bioactives telles que les antioxydants [188]. Pourtant, il n'existe pas d'information de l'activité antioxydante dans les résidus et des organes anatomiques de *T. farfara*. En conséquence, il a été intéressant d'analyser l'activité antioxydante des résidus des tiges des fleurs (FS), feuilles (L) et les racines (R) de *T. farfara* après hydrodistillation. Ainsi, l'analyse de ses différents parts de deux plantes de différentes origines ont été réalisés dans notre rechercher.

Les valeurs d'IC_{50} de DPPH$^{\bullet}$ pour l'analyse capacité à piéger les radicaux libres de *T. farfara* des organes anatomiques ont été le plus faible pour FR FS AE (0.88 mg/mL) et LT FS AE (0.54 mg/mL); l'activité plus élevée était dans l'extrait de FR L AE (0.05 mg/g) (Fig.R.8).

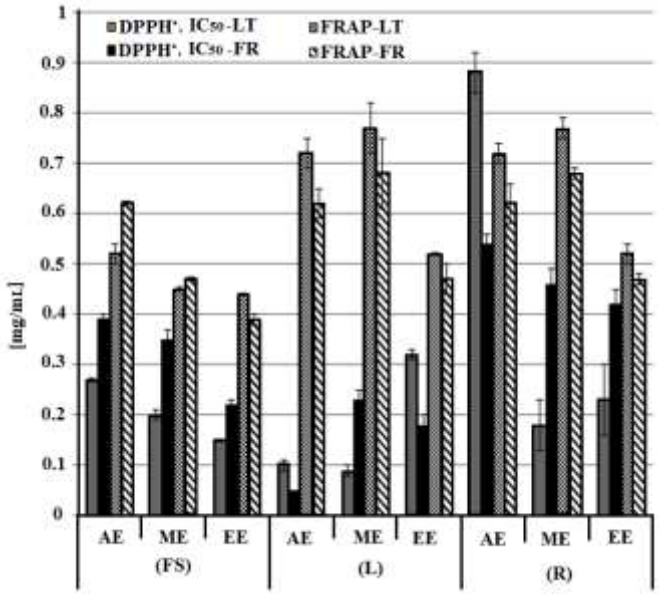


Fig. R.8. Activité antioxydant de *T. farfara* de la Lituanie et France.

Les valeurs d'IC_{50} des extraits de FS, L et R ont varié de 0.09 (L ME) à 0.88 (R AE) dans les extraits d'origine lituanienne et pour les extraits d'origine française 0.05 (L AE) à 0.54 (R AE) mg/mL. Comme prévu, la faible polarité AE des extraits FS et R ont été avec une plus faible capacité à piéger les radicaux libres que les extraits alcooliques polaires dans la méthode DPPH$^{\bullet}$. Ce résultat est en accord avec de nombreux rapports précédents [86, 108, 185]. En revanche, la méthode ABTS$^{\bullet+}$ a montré de résultats opposés à celui de DPPH$^{\bullet}$ (Fig. R.9). Cela implique que la méthode ABTS$^{\bullet+}$ peut être appliquée aux extraits lipophiles. Pour les valeurs TEAC dans la méthode ABTS$^{\bullet+}$, les extraits AE (FS) et R ont été les radicaux libres les plus actives que dans les extraits FS ME, R ME et FS EE, mais l'extrait R EE a montré une activité supérieure à R AE. La principale différence ce que DPPH$^{\bullet}$ peut être dissous seulement dans les solvants organiques (méthanol), alors qu'ABTS$^{\bullet+}$ est soluble en milieu aqueux et organique. En conséquence, le test ABTS$^{\bullet+}$ peut être réalisé dans systèmes hydrophiles et lipophiles [165].

Le test FRAP des extraits des différentes parties des plantes ont été entre le 0.18-0.77 mg Fe (II)/mL dans l'extrait LT et entre 0.15-0.68 mg Fe (II)/mL dans l'extrait FR.

L'activité antioxydante des extraits de FS mesurés avec le test FRAP a montré la même correspondance que $ABTS^{\bullet+}$. Dans le test FRAP le sel ferrique Fe (III) a été utilisé comme antioxydant et son potentiel redox est comparable à celui d'$ABTS^{\bullet+}$. En conséquence, les résultats des tests TEAC et FRAP ont montré de tendances similaires à celles indiqués dans les travaux précédemment [186]. Les extraits FS AE les antioxydants ont été remarquablement actifs que dans les autres extraits (FR ME et FR EE), mais pour les extraits de L, aucune relation existait entre $ABTS^{\bullet+}$ et FRAP, LT L EE a montré une activité élevée en $ABTS^{\bullet+}$ et une faible activité in FRAP, la différence observée peut-être explique pour la différence entre le pH dans la réaction dans le milieu media (pH_{FRAP}=3.6 et $pH_{ABTS^{\bullet+}}$=7.4).

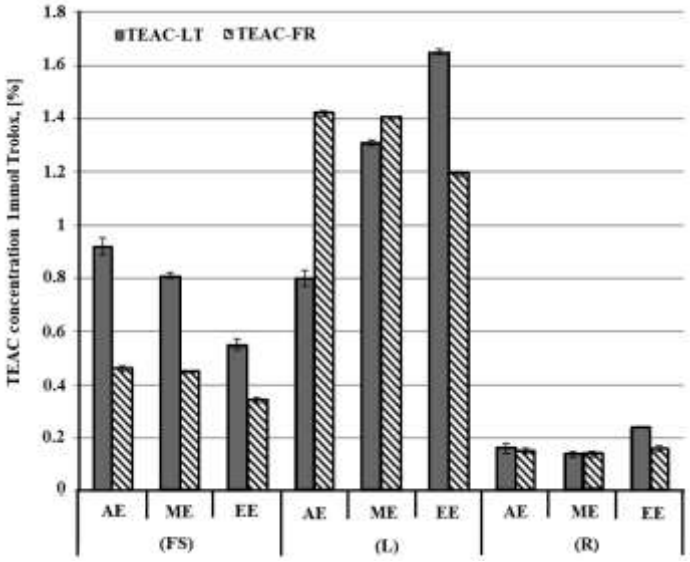


Fig. R.9. TEAC valeurs dans ABTS test de *T. farfara* de la Lituanie et France

En comparant les indicateurs de l'activité antioxydante, nous avons observé que les extraits isolés de *T. farfara* ont été différents. La différence entre les conditions climatiques, par exemple la quantité de précipitations pendant le développement de plante était presque trois fois moins abondante en Lituanie (340 mm) qu'en France (971 mm), ainsi qu'une valeur inférieure dans la température moyenne de la Lituanie qu'en France peut aussi avoir un 'impact de ces différences. De plus, autres facteurs comme le chémotype de la plante, le sol, le soleil peut aussi avoir un impact dans les propriétés et les composés des plantes. La

détermination dès les effets de ces facteurs dans les propriétés de *T. farfara* requerrait une autre démarche expérimentale qui dépasse l'étendue de la présente étude.

Évaluation de l'activité antioxydante sous-produit d'hydrodistillation en utilisant la méthode Oxipres

L'activité antioxydante des résidus de l'hydrodistillation ont été déterminées dans l'huile de tournesol au moyen de l'appareil Oxipres (Mikrolab Aarhus, Denmark). Cette méthode consiste à évaluer la consommation d'oxygène en mesurant la variation de pression dans une enceinte hermétiquement fermée et renfermant l'échantillon à doser ainsi que de l'oxygène.

La période d'induction (PI) de l'oxydation a été évaluée au moment où la pression commence à chuter. Le facteur de protection (FP) ainsi que l'activité antioxydante (AA) sont calculés à partir des formules suivantes:

$$PF = \frac{IP_X}{IP_K}; \qquad AA = \frac{IP_X - IP_K}{IP_{BHT} - IP_K}.$$

Où : IP_x est la période d'induction de l'échantillon avec additif ;

IP_K est la période d'induction de l'échantillon sans additif ;

IP_{BHT} est la période d'induction de l'échantillon contenant la référence BHT (hydroxy

toluène buthylé);

La méthode Oxipress est une procédure avantageuse et performante pour laquelle aucun

composé chimique n'a été utilisé.

Les effets des résidus de l'hydrodistillation évalués pour différentes concentrations de celles-ci (0,05% ; 0,1% et 0,2%) sont présentés dans le tableau R.4.

Tableau R.4. Activité antioxydante (AA) des résidus de l'hydrodistillation comparée à l'efficacité du BHT

Plant	Solvent	Conc %	Origine					
			Lituanie	France	Lituanie	France	Lituanie	France
			IP		**PF**		**AA**	
C. grandiflora	AE	0.20	-	2.68±0.01	-	1.34±0.00	-	0.35±0.00
		0.10	-	2.72±0.02	-	1.36±0.01	-	0.33±0.00
		0.05	-	2.48±0.00	-	1.24±0.00	-	0.23±0.00
	ME	0.20	-	2.81±0.01	-	1.41±0.01	-	0.39±0.00
		0.10	-	2.71±0.03	-	1.36±0.02	-	0.34±0.01
		0.05	-	2.58±0.02	-	1.29±0.01	-	0.28±0.00
	EE	0.20	-	2.98±0.01	-	1.49±0.00	-	0.47±0.00
		0.10	-	2.93±0.03	-	1.47±0.02	-	0.45±0.01
		0.05	-	2.78±0.00	-	1.39±0.00	-	0.38±0.00
	FD	0.20	-	2.21±0.01	-	1.31±0.00	-	0.29±0.00
		0.10	-	2.02±0.01	-	1.27±0.00	-	0.26±0.00
		0.05	-	2.01±0.00	-	1.15±0.00	-	0.14±0.00
	SD	0.20	-	2.78±0.03	-	1.39±0.02	-	0.38±0.01
		0.10	-	2.51±0.02	-	1.26±0.01	-	0.25±0.00
		0.05	-	2.37±0.01	-	1.19±0.00	-	0.18±0.00
M. odorata	AE	0.20	2.17±0.03	2.19±0.01	1.09±0.02	1.10±0.01	0.08±0.02	0.09±0.01
		0.10	2.09±0.02	2.11±0.01	1.05±0.01	1.06±0.01	0.04±0.01	0.05±0.01
		0.05	2.05±0.02	2.07±0.01	1.03±0.01	1.04±0.01	0.02±0.01	0.03±0.01
	ME	0.20	2.19±0.01	2.10±0.01	1.10±0.00	1.05±0.01	0.09±0.00	0.05±0.01
		0.10	2.07±0.00	2.02±0.01	1.04±0.00	1.01±0.01	0.03±0.00	0.01±0.01
		0.05	2.01±0.01	2.00±0.01	1.01±0.01	1.00±0.00	0.00±0.00	0.00±0.01
	EE	0.20	2.30±0.00	2.30±0.01	1.15±0.01	1.15±0.00	0.14±0.01	0.14±0.01
		0.10	2.10±0.01	2.20±0.01	1.05±0.01	1.10±0.01	0.05±0.01	0.10±0.01
		0.05	2.02±0.01	2.07±0.01	1.01±0.01	1.04±0.01	0.01±0.01	0.03±0.01
	WE	0.20	2.36±0.04	2.42±0.01	1.18±0.01	1.21±0.01	0.17±0.01	0.20±0.01
		0.10	2.21±0.01	2.34±0.01	1.11±0.01	1.17±0.01	0.10±0.01	0.16±0.01
		0.05	2.19±0.01	2.29±0.01	1.10±0.01	1.15±0.01	0.09±0.01	0.14±0.01

(*Continuation du Tableau R.4)*

Plant	Solvent	Conc %	Origine: Lituanie IP	France IP	Lituanie PF	France PF	Lituanie AA	France AA
T. farfara-FS	AE	0.20	2.72±0.02	2.54±0.01	1.30±0.01	1.22±0.00	0.32±0.01	0.23±0.00
		0.10	2.61±0.03	2.50±0.01	1.25±0.02	1.20±0.00	0.26±0.02	0.21±0.00
		0.05	2.56±0.02	2.34±0.03	1.22±0.01	1.12±0.02	0.24±0.01	0.13±0.02
	ME	0.20	2.57±0.01	2.67±0.01	1.23±0.01	1.28±0.01	0.24±0.00	0.29±0.00
		0.10	2.49±0.01	2.51±0.01	1.19±0.01	1.20±0.01	0.20±0.00	0.21±0.00
		0.05	2.46±0.01	2.38±0.01	1.18±0.01	1.14±0.01	0.19±0.00	0.15±0.00
	EE	0.20	2.59±0.01	2.75±0.01	1.24±0.01	1.32±0.01	0.25±0.00	0.33±0.00
		0.10	2.55±0.01	2.64±0.01	1.22±0.01	1.26±0.01	0.23±0.00	0.28±0.00
		0.05	2.48±0.00	2.53±0.01	1.19±0.00	1.21±0.00	0.20±0.00	0.22±0.00
T. farfara- L	AE	0.20	2.80±0.03	2.82±0.01	1.40±0.03	1.41±0.01	0.39±0.03	0.40±0.01
		0.10	2.62±0.01	2.64±0.01	1.31±0.01	1.32±0.01	0.30±0.01	0.31±0.01
		0.05	2.58±0.01	2.59±0.00	1.29±0.01	1.30±0.00	0.28±0.01	0.29±0.00
	ME	0.20	2.32±0.01	2.29±0.01	1.16±0.01	1.15±0.01	0.15±0.01	0.14±0.01
		0.10	2.30±0.01	2.21±0.01	1.15±0.01	1.11±0.01	0.14±0.01	0.10±0.01
		0.05	2.28±0.02	2.18±0.02	1.14±0.02	1.09±0.02	0.14±0.02	0.09±0.02
	EE	0.20	2.46±0.01	2.39±0.01	1.23±0.01	1.20±0.01	0.22±0.01	0.19±0.01
		0.10	2.40±0.01	2.36±0.01	1.20±0.01	1.18±0.01	0.19±0.01	0.17±0.01
		0.05	2.30±0.01	2.28±0.01	1.15±0.01	1.14±0.01	0.14±0.01	0.14±0.01
T. farfara -R	AE	0.20	2.62±0.00	2.53±0.01	1.31±0.00	1.27±0.01	0.30±0.00	0.26±0.01
		0.10	2.39±0.01	2.48±0.01	1.20±0.01	1.24±0.01	0.19±0.01	0.23±0.01
		0.05	2.26±0.02	2.30±0.01	1.13±0.02	1.15±0.01	0.13±0.02	0.14±0.01
	ME	0.20	2.41±0.01	2.45±0.01	1.21±0.01	1.23±0.01	0.20±0.01	0.22±0.01
		0.10	2.38±0.01	2.40±0.00	1.19±0.01	1.20±0.00	0.18±0.01	0.19±0.00
		0.05	2.31±0.01	2.33±0.01	1.12±0.01	1.17±0.01	0.15±0.01	0.16±0.01
	EE	0.20	2.58±0.03	2.60±0.01	1.29±0.03	1.30±0.01	0.28±0.03	0.29±0.01
		0.10	2.50±0.01	2.51±0.01	1.25±0.01	1.26±0.01	0.24±0.01	0.25±0.01
		0.05	2.42±0.01	2.46±0.01	1.21±0.01	1.23±0.01	0.20±0.01	0.22±0.01
BHT			0.02±0.00		4.07±0.00	2.04±0.00	1.00±0.00	
L'huile de tournesol			0.00±0.00		2.00±0.00	1.00±0.00	-	

Le résidu de l'hydrodistillation de *C. grandiflora* montre une activité antioxydante plus efficace, en comparaison des autres résidus de l'hydrodistillation. Les résidus de l'hydrodistillation de *C. grandiflora* et de *T. farfara* ont été les plus efficaces pour la stabilisation de l'huile de tournesol. L'efficacité des résidus de l'hydrodistillation de plante diminue en fonction de l'ordre suivant: *C. grandiflora* > *T. farfara* > *M. odorata*, pour de concentration de 0,2%.

Évaluation de l'activité antioxydante des radicaux libres (RSC) par une méthode HPLC—DPPH$^{\bullet}$

L'activité antioxydante des radicaux libres dans les extraits de *C. grandiflora*

La méthode HPLC-DPPH• peut être utilisée pour faire une analyse rapide des propriétés antioxydantes des radicaux libres des composées des extraits des plantes obtenues à travers une colonne chromatographique[199-200]. Les chromatogrammes HPLC/UV/DPPH• des extraits EE et FD (Fig. R.10) montrent que plusieurs composés séparés sont capables de piéger les radicaux libres. Prendre en compte que l'objectif de ce manuscrit est l'identification des différends composés dans les extraits des plantes, nous avons réalisé l'analyse de *C. grandiflora*. Les résultats ont montré que le composé le plus abondant identifié dans EE a été l'acide rosmarinique, qui est bien connu comme un antioxydant naturel. La spectrométrie de masse (LC-MS) de l'acide rosmarinique (RT=30.6 min.) en mode d'ionisation positive a observé à 359 m/z et m/z 161 de fragmentation, lequel possède une grande RSC (49.8% de la superficie totale de pics).

Une grande activité d'antioxydants des radicaux libres élues de *C. grandiflora* FD à environ 35.9 min: Il était identifié comme acide Salvianolique C. La spectrométrie de masse (LC-MS) observé à 491 m/z et m/z 311 de fragmentation, lequel possède une grande RSC (30.4% de la superficie totale de pics).

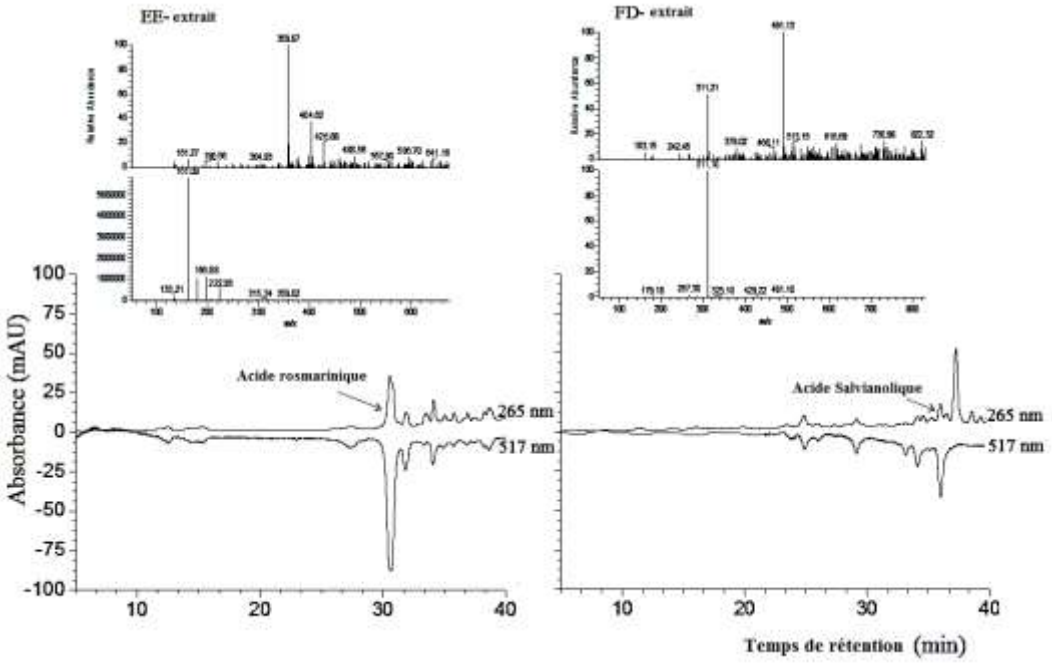


Fig. R.10. HPLC–DPPH• chromatogrammes des extraits de *C. grandiflora*

La composition phytochimique et l'activité antioxydante des radicaux libres (RSC) des composés de *M. odorata*

L'évaluation des propriétés antioxydantes de *M. odorata* avec la méthode TPC et RSC-DPPH$^{\bullet}$ suggèrent qu'il existait des différences remarquables entre les composés des extraits des plantes d'origine de FR et de LT, particulièrement dans le cas de WE et AE. Kaempferol-7-glucoside et cynarine ont été les composés antioxydants le plus active dans les extraits de *M. odorata*. Néanmoins, le premier composant a été le plus abondant dans tous les extraits d'origine LT alors que le deuxième composant a été le plus abondant dans le WE et FR. Au meilleur de notre connaissance, ces composants n'ont pas été signalés auparavant dans les différentes recherches de *M. odorata*. Le pic de Kaempferol-7-glucoside et cynarine dans EE et WE était compris entre 639.7-6038.2 et 0-849.1, a lorsque l'entrée dans le total RSC était 34.5-97.1 et 0-45.4%, respectivement.

Le profil chromatographique de WE (Fig. R.11) obtenue par la méthode HPLC–DPPH$^{\bullet}$ montre clairement que FR *M. odorata* contient une activité antioxydante plus élevée que LT *M. odorata*. Une quantité plus élevée du fort antioxydante cynarine était présente en FR *M. odorata* que dans LT *M. odorata*, toutefois, la quantité de Kaempferol-7-glucoside a été plus abondante en LTMO (Fig. R.11).

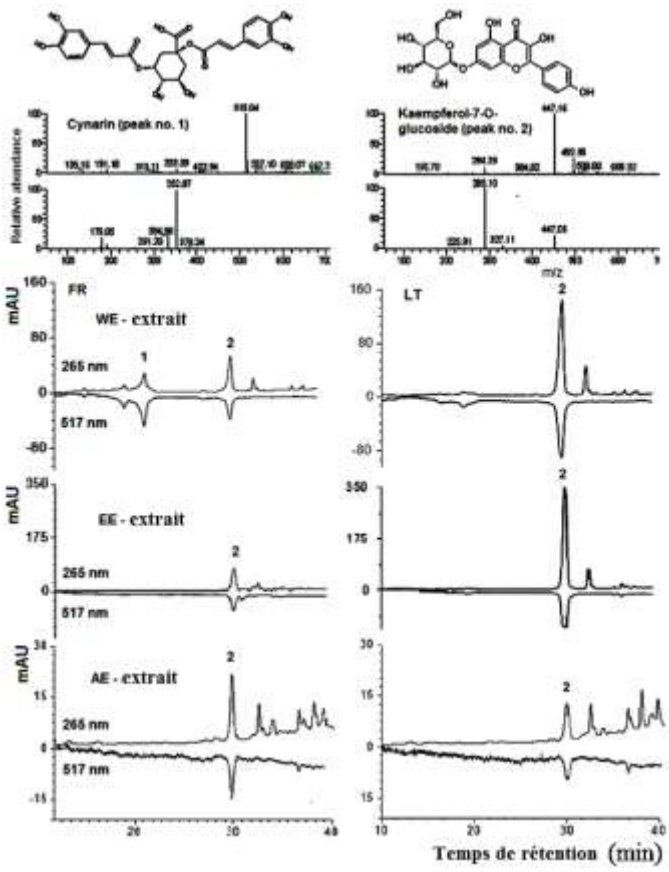


Fig. R.11. HPLC–DPPH• chromatogrammes des extraits de *M. odorata*

petits que ceux obtenues des fleurs (FS) et de feuilles (F) (Fig. R.12). L'analyses ont montré que les extraits de R, FS et F possèdent 5 composés similaires: acide quinique, acide

L'activité antioxydante des radicaux libres dans les extraits de *T. farfara*

Les extraits des tiges et des fleurs (FS) de *T. farfara* avaient eu une différence significative en comparaison avec les autres parties de la plante. Les racines (R) AE ont montré des pics plus dicaféylquinique, quercétine- pentoxyde et kaempfero-glucoside. Cependant, il existait aussi des composés différents, par exemple, l'ESI-MS d'AE, ME et EE de *T. farfara* des deux origines différentes étaient qualitativement différents. Les composés 3, 4 et 5 ont révélé que le signal [M-H] à m/z 515 avec une fragmentation à m/z 353 et 179 dont la formule moléculaire liée est $C_{25}H_{24}O_{12}$. L'ion à m/z 353 a indiqué un fragment d'acide chlorogénique obtenue pour la perdre d'un groupe de caféyl. L'ion à m/z 179 a indiqué des fragments d'acide caféique. Ces données MS indiquent que les composants 3-5 sont l'acide dicaféylquinique isomères.

Les extraits ESI-MS de LT T. farfara F présentent ions qui correspond à : 1-(m/z 191)- acide quinique, 2-(m/z 354) acide chlorogénique, 3-5 (m/z 515) acide dicaféylquinique, 6-(m/z

609)- quercétine-3- rutinoside, 7-(m/z 434)- quercétine- pentoxyde et 8-(m/z 447)- kaempfero-glucoside. Les extraits F d'origine FR étaitent qualitativement différents: l'extrait l'acide chlorogénique était absent.

Les donnes de MS des fleurs de *T. farfara* présentent ions qui correspond à m/z 463 quercétine- glucoside (6*) et m/z 678—acide tricaféylquinic (9), mais ces ions n'ont pas été trouvés dans les racines et tiges de fleurs (FS). D'un autre part, quercétine-3- rutinoside a été trouvé seulement dans l'extrait de FS.

Le RSC des extraits de *T. farfara* isolé des plantes origine de LT montrent une activité antioxydante légèrement plus élevée que les extraits d'origine FR. Cependant, d'après la méthode DPPH$^{\bullet}$ (Fig. R.8), il n'existe pas une différence importante dans les valeurs IC_{50} entre les plantes de différentes origines. Le profil des chromatogrammes avec la méthode in HPLC–DPPH$^{\bullet}$ (Fig. R.12) n'étaient pas semblables entre les deux plantes. En fait, les extraits de *T. farfara* FS obtenus de la plante d'origine LT ont montré des pics plus grands dans le chromatogramme négatif que dans les plantes d'origine FR. L'acide Dicaféylquinique et quercétine-pentoside étaient les composés majeure activité avec un total de RSC de 39.6%), (10%), (17.1%) et (16.9%), (13.8%), (13.1%) respectivement en extraits désodorisés FS de LT et FR AE.

Les composés avec le major activité de l'extrait LT AE F étaient acide dicaféylquinique et quercétine-pentoside, mais dans l'origine FR étaient dicaféylquinique et quercétine-pentoside, et l'acide tricaféylquinic, ces composés ont une activité RSC (47.7%), (9.2%), (11.5%) et (36.5%), (10.4%), (17.6%) respectivement.

Comme il a déjà été constaté, la composition phytochimique des métabolites secondaires des plantes dépendent de plusieurs facteurs, tels que la zone de culture, les conditions climatiques, la phase végétative, les modifications génétiques, les différents partis des plantes. Par conséquent, l'évaluation des propriétés des plantes de différentes régions géographiques étaient le du centre d'intérêt par de nombreuses études [147, 207].

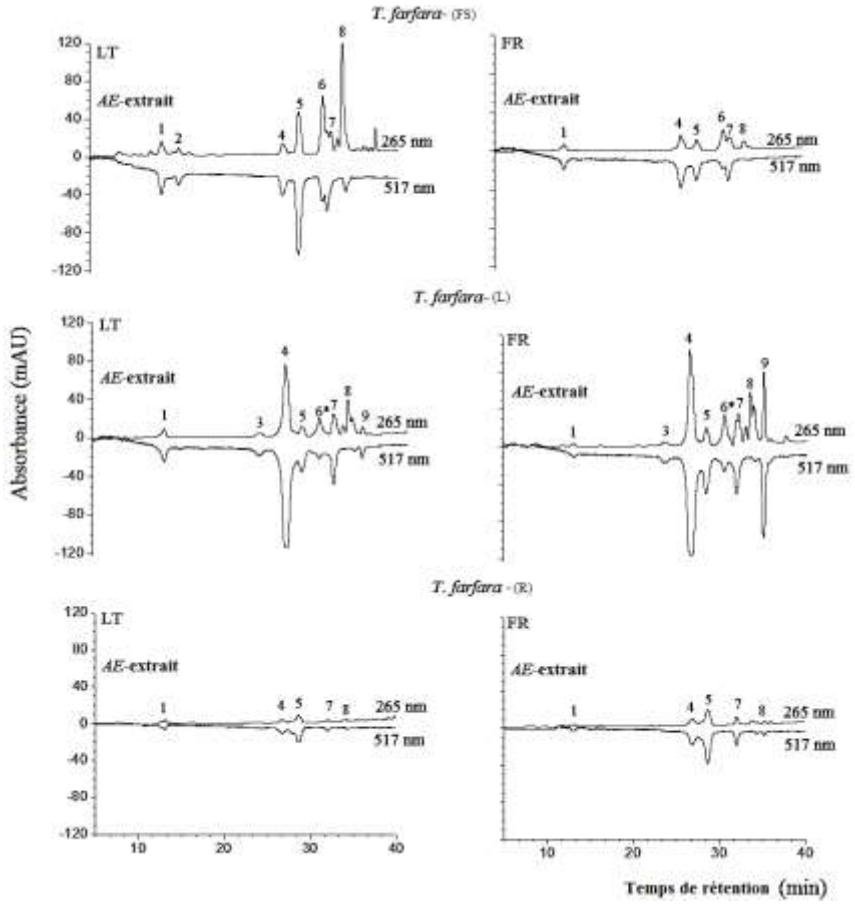


Fig. R.12 HPLC–DPPH$^{\bullet}$ chromatogrammes des (AE) extraits de *T. farfara*

Les possibilités technologiques d'application

Parallèlement à l'extraction des composées volatiles (huiles essentielles) qui peuvent être obtenues facilement dans les plantes aromatiques, l'extraction des composés d'une haute valeur ajoutée, voires antioxydant, dans les résidus serait une option économiquement réalisable dans l'avenir. Les études réalisées ont montré que les résidus solides et liquides après l'isolation d'huiles essentielles (hydrodistillation) peuvent avoir de grandes possibilités d'utilisation dans l'industrie chimique ou dans un autre par exemple. Les composés phénoliques obtenus de ces résidus peuvent être convertis en poudre avec une grande capacité antioxydante, laquelle peut être utilisée comme un additif naturel dans l'industrie alimentaire et nutraceutique.

En outre, toutes les expériences étaient réalisées avec de petites quantités de plantes à l'échelle de laboratoire. En conséquence, il serait intéressant la changer à une production à grande échelle, voire production industrielle. Pour ce faire, la première étape serait nécessaire de tester la technologie dans une usine pilote, puis la mise en œuvre au niveau industriel. Le

possible schéma technologique pour la valorisation de résidus des plantes aromatiques sont représentées dans la Fig. R.13.

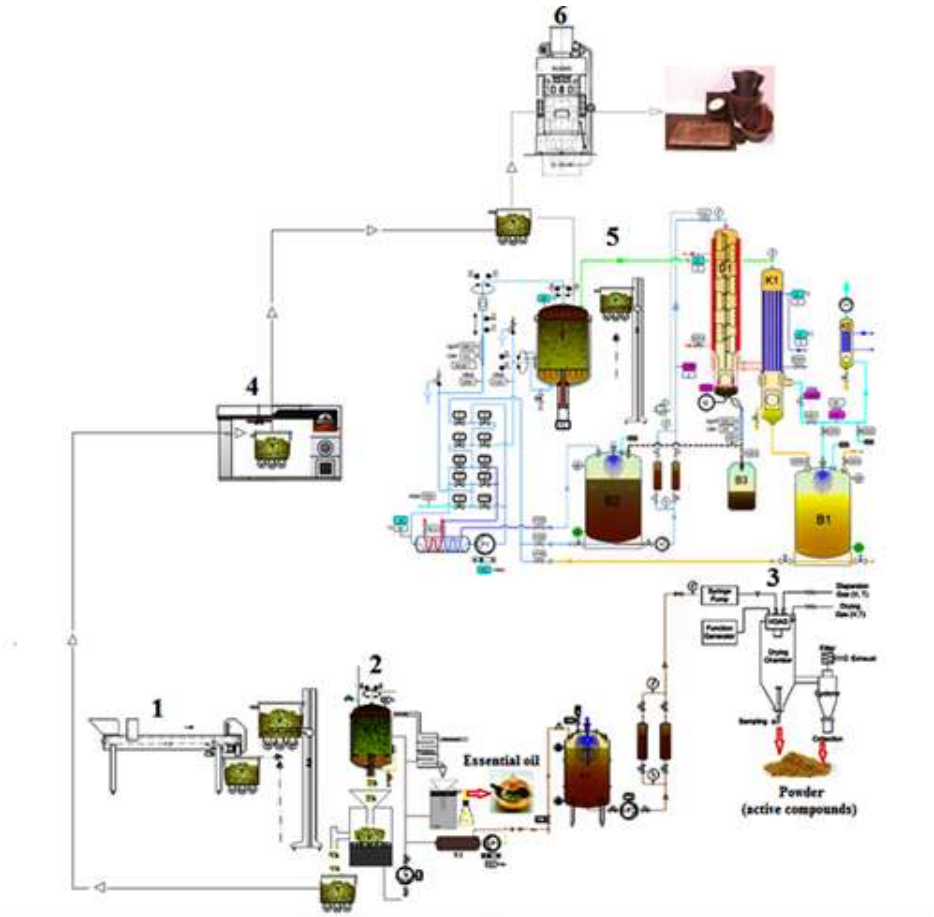


Fig. R.13. Le possible schéma technologique pour la valorisation de résidus des plantes aromatiques [205]

Les procédures utilisées en dans échelle de laboratoire de notre travail peuvent être mises en œuvre en utilisant des équipements commerciaux en couplage avec des nouveaux appareils. Par exemple, l'actuel schéma technologique développé par l'entreprise Dig-Maz [208] avec certaines modifications (Fig. 3.15) peut-être utilisé.

Conclusion

1. Basés sur les principaux composants de l'huile essentielle, *M. odorata* d'origine française et lituanienne peuvent être attribués au même E- anéthole, méthyleugénol et nérolidol chémotype. Pourtant, le pourcentage d'estragole était de 4 fois plus élevées dans l'huile essentielle française, tandis que les pourcentages de β-caryophyllène, germacrène-D, spathulenol et limonène étaient significativement plus élevées dans l'huile d'origine lituanienne. En raison de ces différences, l'huile française avait des arômes de réglisse, d'anis

et des notes florales et l'huile d'origine lituanienne avait des arômes d'épices accompagnées de notes boisées et fruitées. En autre, le pourcentage des principaux composés d'huile essentielle de *M. odorata* d'origine française et lituanienne collectés dans habitats éloignés étaient similaires. Pourtant, la comparaison avec les données étudiées auparavant montrent que les plantes sont de différent chémotype.

2. Les principaux composés volatils de *T. farfara* d'origine française et lituanienne étaient 1-nonène (34.1±0.0%) et n- n-tricosane (21.7±0.1%), respectivement. Pour la première fois, les composés volatils de *T. farfara* dans les fleurs et tiges étaient comparés l'analyse par microscopie ont révélé que les tiges contiennent « poches » de sécrétion (trichomes glandulaires), les organes pour l'approvisionnement des huiles essentielles suggèrent que toutes les parties aériennes de *T. farfara* pourraient être utilisées pour la production des huiles essentielles.

3. Deux nouvelles méthodes pour l'analyse des composées volatiles ont été développées : Crushing Finger Device (CFD) et Flash Aroma Dispenser (FAD). Ces méthodes pourraient être utilisées comme une alternative aux méthodes traditionnelles. De plus, ces méthodes peuvent permettre d'éviter certains inconvénients aux méthodes traditionnelles comme la dégradation thermique des composés volatiles. La composition des composés volatiles dépendait des méthodes d'extraction : l'existence de certaines différences quantitatives et qualitatives a été constatée entre les huiles essentielles hydrodistillées et les volatiles dans la plante. Dans *C. grandiflora*, hexanal, α-thujene, α-phellandrène, α-terpinène n'avaient pas été détectés dans l'huile hydrodistillée, tandis que ces composés ont été détectés dans les huiles volatiles, analysés par les méthodes CFD FAD. Linalool, p-menth-8-en-3-ol, pipéritone, l'acétate d'isomenthyle, bicyclogermacrene, l'acétate d'isoeugenyl et α-cadinol ont été détectés dans les huiles essentielles.

4. Tous les extraits des résidus hydrodistillés des plantes étaient des actifs antioxydants. Les extraits aqueux et alcoolisés de *M. odorata* et *C. grandiflora* avaient une activité relativement plus élevée que ceux en acétone (AE) avec la méthode $DPPH^{\bullet}$ et $ABTS^{\bullet+}$. La capacité à piéger les radicaux libres (RSC) des extraits obtenus des résidus solides était relativement similaire que dans les extraits aqueux, cela montre que certains composés antioxydants ne se dissolvent pas dans l'eau bouillante et donc ils restent encore dans les résidus. Quelques différences ont été déterminées dans l'activité antioxydante des plantes d'origine française et lituanienne. La zone de culture, les conditions climatiques, la phase

végétative, les modifications génétiques et les génotypes peuvent être quelques facteurs entre plusieurs qui ont une influence dans l'accumulation des composantes actives dans les plantes.

5. Les additifs des extraits de plantes réduisent l'oxydation d'huile de tournesol. L'extrait de *C. grandiflora* ajoutés à 0.2% avait l'effet plus fort suivi de *T. farfara* et *M. odorata*. Cependant, l'antioxydant l'hydroxytoluène butylé (BHT) dans l'huile avec une concentration dix fois plus inferieure (0.02%) était plus fort que les extraits. De plus, il faut souligner aussi qu'aucune corrélation positive n'était pas trouvée entre TPC et AA. Par exemple, l'extrait de *M. odorata* avec une haute valeur TPC était un antioxydant plus faible que l'extrait de racine de *T. farfara* avec une valeur faible de TPC.

6. Plusieurs antioxydants ont été identifiés dans nos travaux de recherche avec l'utilisation de nouvelles techniques chromatographiques et spectrométriques comme la méthode HPLC-DPPH$^{\bullet}$. Ainsi, l'acide rosmarinique était l'antioxydant le plus représentatif dans l'extrait par éthanol de *C. grandiflora* alors que l'acide salvianolique était le composé le plus représentatif dans l'extrait lyophilisé. Kaempférol-7-O- glucoside et cynarine ont été identifiés comme les composants avec l'activité antioxydante plus élevée dans les extraits de *M. odorata*. Ces composants n'ont pas été rapportés dans les travaux précédents de *M. odorata* et *C. grandiflora*. En autre, huit composés antioxydants ont été identifiés en *T. farfara* et deux d'entre eux n'ont pas été rapportés dans ce plante.

7. *C. grandiflora*, *M. odorata* et *T. fárfara* analysés dans ce manuscrit possèdent des composants très utiles, voire antioxydants et composés volatiles. Les résultats obtenus dans ce manuscrit peuvent être considérés comme un premier levier dans la valorisation des déchets afin d'obtenir de substances à haute valeur ajoutée présentes dans les fractions volatiles et non volatiles. Ces substances peuvent être ensuite utilisées pour la production d'additifs alimentaires ou des aliments-santé complémentaires. Enfin, une démarche d'agroraffinerie peut augmenter la faisabilité à la mise en œuvre pour résoudre des problèmes économiques, de traitement de déchets et le développement technologique dans le domaine agro-industriel.

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LIST OF NOMENCLATURES

Abbreviations:

AA − antioxidant activity

ABTS − 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

AE − acetone extract

B − bud

BHT − butylated hydroxytoluene

C. grandiflora − *Calamintha grandiflora* L. Moench

CFD − Artificial Crushing Finger device

CGA − chlorogenic acid

d.w. − dry weight

diCQA − dicaffeoylquinic acid

DPPH − 2,2-diphenyl-1-picrylhydrazyl hydrate

EE − ethanol extract

ESI − electrospray ionization

EO – essential oil

FAD − Flash Aroma Dispenser

FD − freeze-dried extract

F − flower

FID − flam ionization detector

FRAP − ferric reducing antioxidant power

FR − France

FS − flower and stem

GAE − gallic acid equivalents

GC − gas chromatography

HPLC − high performance liquid chromatography

IC_{50} − half maximal inhibitory concentration

IP − induction period

K-3-O-glc − kaempferol-glucoside

K-7-O-glc − kaempferol-7-O-glucoside

LC − liquid chromatography

L − leaf

LT − Lithuania

M. odorata − *Myrrhis odorata* L. Scop

m/z − mass-to-charge ratio

ME − methanol extract

MS − mass spectroscopy

Ni − not identified

P − significance level

PBS − phosphate buffered saline

PF − protection factor

Q-3-O-glc − quercetin-glucoside

Q-pent − quercetin-pentoside

R^2 − correlation coefficient

RE − rutin equivalents

RI − retention indices

R − roots

RSC − radical scavenging capacity

RT − retention time

S − stem

SD − spray-dried extract

SPME − solid-phase microextraction

T. farfara – *Tussilago farfara* L

TEAC − Trolox equivalent antioxidant capacity

TFA − trifluoroacetic acid

TPC − total phenolics content

Tr − trace levels

UV − ultraviolet

v/v − volume concentration (volume/volume)

VOCs − number of volatile organic compounds

WE − water extract

w/w − mass fraction (weight/weight)

GENERAL INTRODUCTION

Relevance of the work

Aromatic, medicinal and spice plants are immense and sustainable resources of natural compounds with various beneficial properties; they have been used for food flavouring, preservation, nearly all-medicinal therapies, and other applications since prehistoric times. Some of these plants are now grown commercially to produce a variety of valuable ingredients. However, some medicinal and aromatic herbs, *Calamintha grandiflora, Myrrhis odorata* and *Tussilago farfara* among them, for various reasons have not found wider application and sometimes are referred to as the "forgotten plants". Search of new natural bioactive compounds for nutraceuticals, pharmaceuticals, cosmetics and other products has led to the increase in bio-prospecting, i.e. collection, investigation and utilization of diverse biological resources; therefore, it is reasonable to revisit the "forgotten plants" in order to assess their applicability more systematically. Many medicinal and aromatic plants contain large amounts of antioxidants, such as polyphenols, which may play an important role in foods and human body in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing hazardous peroxides.

The occurrence of medicinal and aromatic plants and their composition depends on many factors, particularly on the climatic conditions and the type of soil. The biogenesis of biologically active compounds also depends on several factors: origin of the plant, geographical growing zone, its genotype, phenotype, and chemotype as well as annual climatic fluctuations. Most of natural compounds possess several biological activities: antioxidant, antimicrobial, anti-inflammatory, and others. Therefore, the comparison of the selected plant species grown in different geographical sites in order to assess their biological properties is an interesting and important task, particularly for the valorization of such plants for their commercial cultivation.

Another important task for the valorization of less widely used plants is their rational processing following the rule "more valuable products and less waste". The residues and/or by-products obtained during processing of raw plant materials may represent potential natural resources for antioxidants and other valuable substances. For instance, during distillation of aromatic plants for essential oil recovery the wastes may constitute up to 99.5% of the raw material, which currently are used rather inefficiently. There is increasing evidence that the nonvolatile compounds present in such residues may possess various activities, which might be applied in foods as antioxidant and antimicrobial additives and for preventing various diseases such as cancer, atherosclerosis and neurological disorders. Volatile fractions of plants, traditionally called essential oils, were also studied for their antioxidative properties [1–3] and were proposed as potential substitutes for synthetic antioxidants [4]. Considering that the production of essential oils of aromatic plants is very expensive, the rational use of by-products remaining after volatile oil removal by various distillation techniques could remarkably improve commercial feasibility of the production of bioactive compounds from such plants.

The by-products of *Calamintha grandiflora, Myrrhis odorata* and *Tussilago farfara* from different origins, as it may be judged from the available literature, have not been studied until now. Furthermore, the variation of bioactive compounds from different origins is an important task both from the chemical point of view and for optimization of the production of

bioactive functional ingredients. Therefore, these species, which sometimes are referred as 'Medieval forgotten plants' growing in Lithuania and south of France, were selected in this study as candidates for the production of valuable ingredients and characterisation of their composition and properties.

For this work, coltsfoot (*Tussilago farfara*) has been chosen because it's valuable functionality as antioxidant. Although there exits some complains about this plant because it contains pyrrolizidine alkaloids which have toxic properties, It is known that these toxics compounds are mainly accumulated in the coltsfoot buds. Thus, in this work, we selected different parts of the plant where these alkaloids can be found in very small quantities that were too difficult to identify. These parts are flowers, stems, young leaves and roots. However, even though the amounts of alkaloids could be insignificant, it is not recommendable to consume long time and in large quantities to prevent any damage. Additionally, to eliminate the pyrrolizidine alkaloids, in this work the aqueous fractions were not used considering that these alkaloids dissolve in water. Nevertheless, there is a new coltsfoot new genotype, created in Austria, which does not contain the toxic pyrrolizidine alkaloids. Therefore, this new *Tussilago farfara* genotype may be used and then the aqueous fraction which remains after hydrodistillation can also be used.

Aim and tasks of the work

The aim of the work was to evaluate the possibilities of the processing of *Calamintha grandiflora, Myrrhis odorata* and *Tussilago farfara* grown in Lithuania and south of France into aroma and antioxidant ingredients by applying the so-called agro-refinery concept.

The following tasks were raised to achieve the aim:

1. To determine the composition of essential oils isolated from aromatic plants collected in Lithuania (Kaunas) and France (Midi-Pyrénées).
2. To develop new analytical techniques for rapid extraction of volatile compounds and to compare the volatile compounds of *Calamintha grandiflora* leaves obtained by hydrodistillation and novel analytical methods (Artificial Crushing Finger device (CFD) and Design of Flash Aroma Dispenser (FAD).
3. To evaluate the antioxidant properties of hydrodistillation by-products prepared by using different solvents in different *in vitro* assays ($DPPH^{\bullet}$, $ABTS^{\bullet+}$, FRAP and Oxipres).
4. To assess antioxidant properties of hydrodistillation by-products *in situ* (sunflower oil) by comparing with commercial synthetic antioxidant (BHT).
5. To determine phytochemical composition of hydrodistillation by-products and to evaluate antioxidant properties of individual constituents by using chromatographic and spectrometric methods.
6. To adapt the technological scheme in the valorization of aromatic plants to obtain high-added value substances.

Scientific novelty

The following scientific novelty was achieved by fulfilling the above-listed tasks:

2. Two new analytical methods for the rapid evaluation of volatile oils from aromatic plants were developed and tested.
3. Deodorized residues of *Calamintha grandiflora, Tussilago farfara* and *Myrrhis odorata* were valorized as an antioxidant's bioresource for the first time; some compounds (rosmarinic acid, salvianolic acid C, kaempferol-7-O-glucoside, cynarin, quercetin-pentoside and kaempferol-glucoside) of these plants were not identified in the previously reported studies.

Practical significance

The by-products after hydrodistillation are promising resources of natural antioxidants. For example, in our study, we identified some compounds with an antioxidant activity such as rosmarinic acid, salvianolic acid C, kaempferol-7-O-glucoside, cynarin, quinic acid, chlorogenic acid, dicaffeoylquinic acid, quercetin-3-rutinoside (rutin), quercetin-pentoside and kaempferol-3-O-glucoside.

Additionally, the results obtained are important in the selection and evaluation of cultivation area of medicinal plants. These allow us to standardize and ensures the quality of raw materials to obtain a product with the desired compounds.

Finally, two new analytical methods for the rapid evaluation of volatile compounds were developed. They are useful for quick assessment of different types of aromatic and culinary plants.

Key points of the thesis

1. Valuable aroma and antioxidant ingredients are obtained from *Calamintha grandiflora, Myrrhis odorata* and *Tussilago farfara* whole plants and hydrodistillation residues by applying agrorefinery concept to their processing.
2. Rapid aroma profiling methods, namely, Artificial Crushing Finger device (CFD) and Design of Flash Aroma Dispenser (FAD) are used for the assessment of plant volatile compounds as alternative to the traditional extraction techniques.

Approbation of Results

The results of the research have been published in 3 scientific articles in the journals indexed by ISI Thomson Reuters (WOS) and 2 international conferences proceedings books. The results of the research have also been presented in the 8 international scientific conferences.

Structure and content of the dissertation

The thesis is written in English. It consists of the list of abbreviations; introduction; literature review; materials and methods; results and discussion; conclusions; references; and list of publications relevant to the subject of the dissertation. The dissertation has 106 pages, 18 tables and 27 figures. The list of references includes 208 bibliographic sources.

CHAPITRE 1

1 LITERATURE REVIEW

1.1 DIVERSITY OF AROMATIC AND MEDICINAL PLANTS

Medicinal and aromatic plants are very important sources of natural food additives, functional food products, dietary supplements, cosmetics, toiletries, domestic chemistry and herbal medicinal products for pharmacy [1-4]. Humankind has valued medicinal and aromatic plants since ancient times particulalry for their pleasant odours and healing properties. In addition, they had a knowledge how to use the scent of such plants in order to relax and stimulate their body, improve wellbeing and make skin look beautiful.

In the Middle Ages the herbs were used not only to flavour the food but for the medical treatment too. Herbal treatment originated from Ancient Greek medicine. For instance, Greek physicians used pine extracts to cure tuberculosis [5], the skills of Arab doctors, who preserved original Greek sources of treating different diseases with plants, assietd in preventing contagious diseases. These scientists passed their knowledge to the Europeans in the early Middle Ages. The influx of medical texts preserved in Arabic revived the Latin medical tradition from the tenth century on. Famous medicine centres were established in Italy; one of them was called Salemo where the famous tract *Tractatus de Herbis* was written in Latin. The mission of the tractate was to inform the townspeople about the plants. Those people used local herbs not only for treatment but also to flavour the food, as rich nobility in the Middle Ages used to do by various spices such as cinnamon and pepper, brought from the East that ordinary townspeople could not afford [6]. Moreover, medieval scientists taught the townspeople how to make ointments by themselves in order to cure wounds and burns. Also, they taught the people how to make various herbal elixirs and charms against incantations and ailments.

Plants which widely used in the medieval period recently have become more popular in the modern world too. Chefs of the modern ages have been using herbs which were known in the medieval ages not only to season fish, meat and vegetable dishes but also as additives enhancing nutritional value of the dishes. Plants used in the past return back to the modern world also due to highly trending vegetarianism. More and more humankind explores the properties of plants that may serve not only as spices but also as an individual food as well. Table 1.1 provides several medieval plants that have been widely used in modern cuisine because they contain active compounds (antioxidants, antimicrobials and flavourings) that both enhance the taste of food and demonstrate the value of medical effectiveness (e.g. ease coughs, help to relax and sleep well, etc.).

Table 1.1. *Some aromatic and medicinal plants widely used in medieval ages with therapeutic properties*

Plant name	Family
Achillea millefolium	Apiaceae
Vitex agnus-castus	Lamiaceae
Angelica archangelica	Apiaceae
Calamintha grandiflora	Lamiaceae
Chamaemelum nobile	Asteraceae
Crataegus monogyna	Apiaceae
Foeniculum vulgare	Apiaceae
Heracleum sphondylium	Apiaceae
Lavandula officinalis	Lamiaceae
Levisticum officinale	Apiaceae
Melissa officinalis	Lamiaceae
Mentha piperita	Lamiaceae
Myrrhis odorata	Apiaceae
Ocimum basilicum	Lamiaceae
Origanum majorana	Lamiaceae
Origanum vulgare	Lamiaceae
Tanacetum parthenium	Asteraceae
Thymus serpyllum	Lamiaceae
Tussilago farfara	Asteraceae

1.2 GENERAL CHARACTERIZATION OF ESSENTIAL OILS

1.2.1 Sources of essential oils

It is well known that plants are the richest source of bioactive phytochemicals, aromatic compounds and antioxidant nutrients [2]. Around 500,000 different species of plants are deemed to exist on Earth, however, people use only up to 10% of these species. Most of the aromatic plants and essential oil commodities in terms of world trade belong to the families of *Lamiaceae*, *Umbelliferae* and *Compositae* [7].

Essential oils are complex mixtures of volatile compounds characterized by strong odour and diverse biological properties. They are formed as secondary plant metabolites [7]. Biosynthesis of essential oils in plants proceeds in three basic pathways - the mevalonate pathway, the methyl-erithrytol-pathway and the shikimic acid pathway [7-8]. Commonly, the key components of essential oils are monoterpenes, diterpenes and sesquiterpenes. These terpenoids in plant are synthesized via mevalonate and deoxyxylulose phosphate pathways (Fig. 1.1).

Essential oils are isolated from different aromatic plants generally distributed in Mediterranean and tropical countries. Aromatic extracts may be isolated from different parts

of a plant, for instance, flowers, buds, stems, leaves, fruits, seeds, bark, fruits rinds, and roots [7-8] (Table 1.2), by using different methods such as water and steam distillation, supercritical fluid extraction, macerating distillation, extraction by solvents, cold pressing, destructive distillation [7, 9-13].

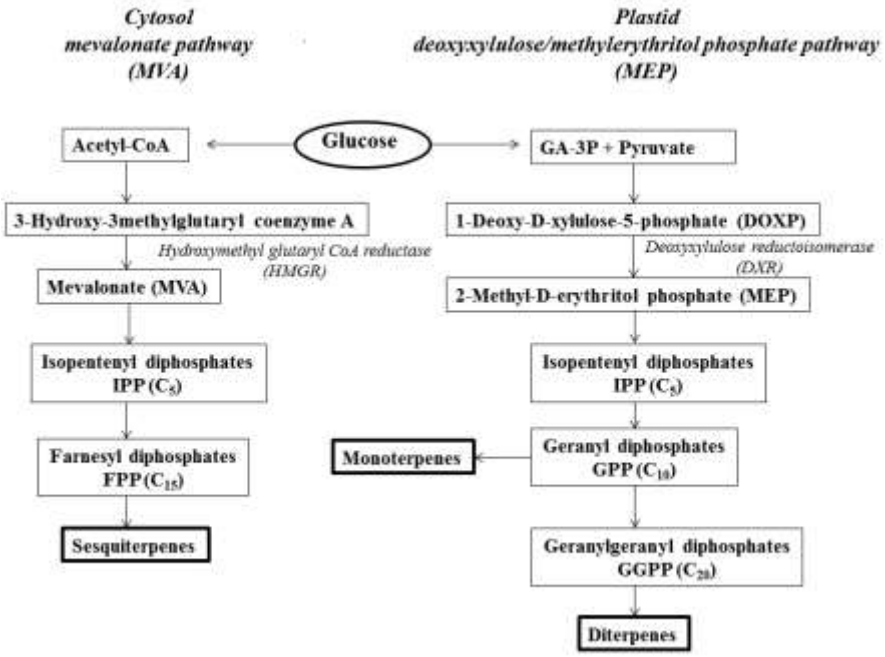


Fig. 1.1. *Biosynthetic pathways leading to terpenoids in plant cell – compartmentation of the biosynthesis between cytosol (mevalonate pathway, MVA) and plastid (deoxyxylulose/methylerythritol phosphate pathway, MEP) [7-8].*

The essential oils are used for food, animal feed as well as in pharmacy, perfumery or chemistry industry [14]. Due to the bactericidal and fungicidal properties of essential oils, their pharmaceutical and food uses are becoming increasing important as alternatives to synthetic chemical products to protect the ecological equilibrium [15]. Moerman analysed the use of different species of plants among the groups of Indians in America and found that up to 2564 different species of plants were used mostly for medical purposes, whereas for food it was used four times less [9, 14]. Presently, essential oils are produced from more than 2000 species of plants; however, only about 100 species have economical significance in the world of essential oils [9-11, 14-21].

Table 1.2. *Different parts of a plant which can be used for the isolation essential oil*

Leaves	**Flowers**	**Peel**	**Seeds**	**Wood**
Basil	Chamomile	Bergamot	Almond	Camphor
Bay leaf	Clary sage	Grape fruit	Nutmeg oil	Cedar
Cinnamon	Clove	Lemon		Rosewood
Eucalyptus	Geranium	Lime		Sandalwood
Lemon grass	Hyssop	Orange		
Melaleuca	Jasmine	Tangerine		
Oregano	Lavender			
Patchouli	Manuka			
Peppermint	Marjoram			
Pine	Orange			
Rosemary	Rose			
Spearmint	Ylang-ylang			
Tea tree				
Wintergreen				
Thyme				
Lovage				
Parsley				
Pimento				

Berries	**Bark**	**Resins**	**Root**	**Rhizome**
Allspice	Cassia	Frankincense	Valerian	Ginger
Anise	Cinnamon	Myrrh	Lovage	
Celery			Parsley	
Cumin				
Fennel				
Juniper				
Pimento				

Some essential oils or their bioactive components such as limonene from peel of lemon or orange, geranyl acetate, from leaves of lemongrass and carvone from seeds of caraway are also used as an important ingredients in tooth pastes and other hygienic products. They also act as antimicrobial agents in foods, and also have been employed for the treatment of different ailments in the folk medicine systems. Many species of the *Lamiaceae* have been used as culinary spices. For example, the leaves of oregano, rosemary, sage and thyme are typical seasonings in the Mediterranean region; oregano is consumed in particulalry large quantities all around the world as a part of pizza seasoning mix [10, 16-17, 19].

The sage (*Salvia officinalis*), one of the common species of this family is now widely cultivated in various parts of the world and is used as a culinary herb for flavoring and seasoning. Essential oil of sage was characterized by high concentrations of camphor, 1,8-cineole and thujone [16].

Essential oils of some spices and herbs such as sage, oregano, thyme, savory and others have shown their antioxidant potential [12] and thus can be used as natural antioxidants for the protection of fats/oils and related products.

1.2.2 Factors influencing the production and the quality of essential oil

The extracted oils or natural antioxidants can vary in quality, quantity and in the chemical composition depending upon the agroclimate, plant organ, age and vegetative cycle stage [7, 16]

Essential oils ussually conntain of a great number of chemical components mainly consisting of hydrogen, carbon and oxygen [7]. They can be essentially classified into two groups: **Volatile fraction:** Essential oil constituting of 90–95% of the oil in weight, containing the monoterpene and sesquiterpene hydrocarbons, as well as their oxygenated derivatives along with aliphatic aldehydes, alcohols, and esters. **Nonvolatile residue:** This comprises 1–10% of the oil, containing fatty acids, sterols, carotenoids, waxes and oxygen heterocyclic compounds [7].

However the composition of essentila oils isolated from the same species may vary in a wide range depending on different factors. For instance, Bonnardeaux investigated the oil composition and yield of different plant parts of basil (*Ocimum basilicum*) and found that lower oil yields were obtained from the distillation of basil with shorter flower spikes than from plants with long flower spikes [22]. Typical peppermint oil consists of about 50% of menthol, 20% of menthone and 8% of ester [23-24]; however, flower oil contains much higher amounts of menthofuran and pulegone (> 50%) than leaf oil, with remarkablylower amounts of menthol and menthone [23-24].

Chemical composition of essential oils also depends on such factors as plant chemotype (polymorphism), climatic conditions, geographical location, the type of soil, othre environmental and technological conditions [16, 25]. In addition, quality and quantity of the essential oils most commonly are influenced by several factors that are closely related together. It is difficult to segregate these factors from each other [26], therefore, in this chapter four main interdependent factors will be briefly reviewed.

Environmental and botanical (taxonomic) factors. Plants accumulate essential oil in different anatomical parts depending on their taxonomy. Polymorphism is characteristic for plants as well; consequently, plants of the same species can have different chemical composition which defines the quality of essential oils. Often producers of essential oils consider that the plants of the same species have the same main volatile compounds. However, essential oil composition highly depends on the plant's taxonomy. For example, main components of *Mentha* (species) plants are monoterpenes, however, *Mentha* × *Piperita* and *Mentha arvensis* ssp. *piperascen*s contain high amounts of menthol which is characteristic only for these two species of mint. On the other hand, eugenol, the compound which is characteristic to cloves plants (*Syzygium aromaticum*) is also found in large amounts in cinamon (*Cinnamomum zeylanicum*) and basil (*Ocimum basilicum*). Therefore, the producers should carefully of essential oils to take into account the chemotaxonomy of the plants through consider chemical polymorphism of various plant cultivars in order to guarantee the consistent quality of essential oil. In many cases plant species posses typical quantitatively major or in some cases even minor compounds which may be considered as their fingerprints. For instance, δ-3-carene mybe used to distinguish *Citrus grandis* from other *Citrus* species [27].

Climatic and environmental factors such as the length of the day, the activeness of the sun and radiation, the amount of water, the type of soil, the amount of emissions, the effect of vermins, etc. influence biosynthesis of essential oils in plants. However, in most cases the climate has the greatest influence on the quality of essential oils, as the temperature impacts the harvest of plants, while optimal interactions between humidity and temperature may increase the yield of essential oil. Humidity coupled with elevated temperatures produces conditions favorable to the proliferation of insect parasites and, most importantly, microorganisms. This sometimes causes plants to increase the production of essential oil for their own protection [28].

Seasonal and geographical origin factors. They are closely related and may have important influence on the quality of essential oils. Literary sources provide large amount of information about differences of essential oils of the same plant species harvested in different months and seasons [10, 21, 28-31]. For instance, Badi *et al*., concluded that spring is the most favourable time to collect thyme because its essential oil is richer in oxygenated compounds than collected in the summer, autumn or winter [17, 32]. On the other hand, thyme collected in the beginning of winter was higher in phenolic compounds [33]. The quality of essential oil may also greatly depend on their geographic location [34-38]. It was proved that chemical composition of *Mentha longifolia* (L.) and *Tagetes minuta* collected in various geographical areas differed significantly [38-41]. Some researchers hypothesized that chemical composition of plants varied due to different type of soil [38-41]. Besides, due to different growing site altitude above the sea level, the plants need to adapt and this also influenced the processes of biosynthesis in the plant. The same plant species collected in different regions with the same type of soil, yet often droughty or floody, showed different profiles of chemical composition. Therefore, it was stated that climate conditions due to which plant changes genetically and adapts have greater impact on plants biosynthesising essential oils [26, 42-44].

Other factors affecting yield and composition of essential oil. Composition and quantity of essential oils are as well influenced by the following factors: raw material used for distillation (dried or fresh), technique of postharvest drying (availability of sun and water), timing of plant sowing, plant resistance to fungous diseases, conditions of harvesting, distillation method, etc. [23, 45].

1.2.3 Extraction Methods for Recovery of Volatile Organic Compounds

The essential oils are produced from plant material by using different methods, such as hydrodistillation, water and steam distillation, direct steam distillation, solvent extraction, maceration, cold pressing, effleurage, super critical CO_2 extraction, etc. [7-8]. The extraction process generally depends on the rate of diffusion of the oil through the plant tissues to an exposed surface. The production of essentials oils for commercial use is based in three basic methods. Expression is used for the production of *Citrus* oils. The second method, hydrodistillation or steam distillation is the most commonly used one of the three methods and dry distillation is used very rarely in special cases [47].

Extraction of essential oils using hydrodistillation and steam distillation

Hydrodistillation is one of the simplest and oldest processes of essential oil isolation from the plants, which has been used mainly by small scale producers. Fresh or dried, whole or ground plant material may be hydrodistilled by boiling it in water at 100°C. During boiling the plant material soaks up the water and oil present within the plant cell diffuses via osmosis

[7]; volatile oil is carried over to the condenser along with the steam. The condenser cools the vaporized oil and steam and the condensates trickle down into the graduated tube where the oil is collected. In steam distillation the plant material is placed in the chamber and steam is allowed to pass through the material; it breaks the essential oil containing cells and carries the oil into the condenser similarly like in hydrodistillation, producing two–phases, essential oil and hydrosol [7-8, 46]. A number of factors determine the final quality of distilled essential oils. Apart from the plant material itself, most important factors are time, temperature and distillation equipment. The advantages of distillation are good oil yield, relatively cheap process; in case of steam distillation the compounds are better protected against oxidation. However, these methods have several disadvantages; the plant material becomes wet, which slows down distillation as the steam has to vaporize the condensed water. Hydrodistillation can thermally degrade and hydrolyze some of the oil components, which in some cases can lead to significant distortion of the native composition of the oils present in the ungtreted plants. The quality of oils is very variable and varied with each distillation. Monoterpenes are vulnerable to chemical changes under steam distillation conditions [7-8].

Solvent-extraction

Aromatic extracts can be extracted by using solvents such as dimethyl ether, petroleum ether, or hexane. All the extractable components from the plant are dissolved in the solvent [7-8, 46] and include volatile aroma molecules as well as non-volatile waxes, pigments and other constituents. The concentrates are usually further processed to remove the waxy materials which dilute the pure essential oil.

Maceration

Using maceration process plant material is soaked in vegetable oil thus obtaining the "infused oil" rather than the essential oil. This method is rarely used because product composition is largely different from natural oil.

Cold pressing

This method is used to extract the essential oils from citrus rinds. The rinds are separated from the fruit, are ground or chopped and then pressed, resulting in a watery mixture of essential oil and liquid [7-8]. Pressed oils retain high quality natural aroma, however their shelf life is rather short. This mechanical method, also called cold expression, is used in the industrial production of cold-pressed citrus oils. It has the advantage to protect the compounds as aliphatic and aldehydes from the thermal degradation as the formation of malodorous carboxylic acids. At the beginning, this process was an extremely laborious and characterized by substantial oil losses. Late, some improvements were development as the "scodella" method which was an apparatus with a metallic hemisphere lined inside with small spikes, with a tube attached at its center [48]. For extract the oils, the fruit is placed inside hemisphere which start to rotate and the fruit is squeezed against the spikes breaking the oils cells. The oil emulsion containing some wastes flowed into the central tube, thus the oils is separated by centrifugation. Nowadays, new machines are implemented to extract the oil: sfumatrice method and the FMC extractor which use high pressures, the Pellatrice and Brown process machine which use centrifugal forces [49-50].

Dry distillation-enfleurage

This process is applicable to plants, that have low content of essential oil and so delicate that heating would destroy the blossoms before releasing the essential oils. The principle of the enfleurage method is that fats dissolve essential oils and thereby absorb their aromas. Every day or every few hours after the vegetable or fat has absorbed as much essential oil as possible; the depleted petals are removed and replaced with fresh ones. Thereafter, the oil is extracted from the fat with alcohol and then isolated. This method is expensive and complicated. Enfleurage has the advantage that even the most delicate components of the flower oils are preserved.

Supercritical CO_2 extraction

Supercritical CO_2 extraction method is attractive because essential oils are being isolated at low temperatures. Supercritical CO_2 extraction involves carbon dioxide heated to 31 °C and pumped through the plant material at around 75 bar under these conditions; the CO_2 is likened to a 'dense fog' or vapor. Supercritical CO_2 extraction method has several advantages: low pressure and low temperature processing, there are no solvent residues left behind, and essential oil is pure also, there is no heat applied to the plant material or essential oil to alter it in any way [7-8, 46]. The essential oil is very accurate with respect to the original state of the plant. Despite these advantages, this method is expensive and this method cannot be used for dissolving polar molecules.

Modern methods of extraction of essential oils

Modern extraction methods have been developed using the latest inventions However, these methods are not designed for industrial use. These methods have been developed in order to minimize change the volatile compounds during the extraction process and these techniques are: solid phase micro-extraction, supercritical fluid extraction, microwave distillation, membrane extraction.

1.3 ANTIOXIDANTS

Synthetic antioxidants

Antioxidants are compounds that can retard the process of lipid oxidation; they can also block the propagation step of the radical chain reaction by destroying or binding free radicals, inhibition of catalysts or stabilization of hydroperoxides. Most common is to classify antioxidants to primary and secondary ones and to describe origin of antioxidants such as natural or synthetic. Synthetic antioxidants containing phenolic groups such as butylated hydroxylanisole (BHA), butylated hydroxyltoluene (BHT), gallic acid esters (propyl, octyl, dodecyl) and tert-butylhydroquinone (TBHQ) have been widely used as antioxidants in foods. TBHQ is the most effective antioxidant used by the food industry [51]. On the other hand, synthetic antioxidants are acceptable for their effectiveness and relatively low cost, but they may have negative side effects related to their toxicity. Because of negative aspects of synthetic antioxidants the interest in healthier and safer antioxidants from natural sources have increased. Therefore, the importance of the search for the exploitation of natural antioxidants, especially of plant origin, has greatly increased in recent years and natural antioxidants have gained popularity. Traditionally herbs and spices are added to food for improving organoleptic properties and they also may act as antioxidants and preservatives.

Natural antioxidants

Natural plant origin antioxidants are mainly phenolic or polyphenolic compounds. The structure of plant phenolics and polyphenols varies from simple molecules to polymerized compounds, such as proanthocyanidins; several thousands (among them over 8,150 flavonoids) different compounds have been identified so far. Several classes of phenolics have been categorized on the basis of their basic molecular skeleton (Table 1.3) [52-54]. Phenolic compounds are traditionally believed to play an important role in plant herbivore interactions and in disease resistance of plants. Flavonoids are one of the major subgroups of phenolic compounds wich mostly occur as their glycosylated derivatives in plants. Chemical structure of flavonoids are subdivided into flavones, isoflavones, anthocyanidins, flavan-3-ols, flavan-4-ols, flavan-3,4-diols, proanthocyanidins [55-56].

Table 1.3. *Classes of phenolic compounds in plants*

Class	Structure
Simple phenols, benzoquinones	C_6
Phenolic acids	C_6–C_1
Acetophenones, phenylacetic acids	C_6–C_2
Hydroxycinnamic acids, coumarins, phenylpropenes, chromones	C_6–C_3
Naphtoquinones	C_6–C_4
Xanthones	C_6–C_1–C_6
Stilbenes, anthraquinones	C_6–C_2–C_6
Flavonoids, isoflavonoids	C_6–C_3–C_6
Hydrolyzable tannins	$(C_6–C_1)_2$
Lignans, neolignans	$(C_6–C_3)_2$
Biflavonoids	$(C_6–C_3–C_6)_2$
Lignins	$(C_6–C_3)_n$
Catechol melanins	$(C_6)_n$
Condensed tannins	$(C_6–C_3–C_6)_n$

Tocopherols are other important antioxidants, which may be divided into tocols and trocotrienols. Tocopherols are considered as safe natural antioxidants; however they do not always provide effective protection against oxidation *in vitro*. Therefore, research of other natural antioxidants is highly desirable.

Carotenoids. Carotenoids are also considered as secondary antioxidants. They are yellow or orange-red pigments. The carotenoids are molecules with limited solubility properties and very sensitive to oxygen and light. The antioxidant activity of carotenoids is based on their singlet oxygen quenching properties and their ability to trap peroxyl radicals [56].

Recent studies have demonstrated that dietary plants are rich source of antioxidants and can contribute to the protection from age-related diseases Hinneburg *et al*. investigated antioxidant activities of extracts from selected culinary herbs and spices (basil, laurel, parsley, juniper, aniseed, fennel, cumin, cardamom, and ginger) [57]. The extracts from basil and laurel possessed the highest antioxidant activities and both extracts are promising alternatives to synthetic antioxidant. Yanishlieva *et al*. [51] in a review paper presented information about the antioxidative effects of rosemary, sage, oregano, thyme, ginger, summer savory, black pepper, red pepper, clove, marjoram, basil, peppermint, spearmint, common balm, fennel,

parsley, cinnamon, cumin, nutmeg, garlic, coriander, etc. Sage and oregano, which belong to the same family, have gained the interest of many research groups as potential antioxidants.

1.3.1 Plant residues

In the last decade the popularity of a healthy lifestyle has increased, therefore the consumers more often give preference to natural products. This tendency also increases the demand for essential oils. On the other hand, for enterprises producing essential oils distillation waste disposal becomes an increasing problem. Currently, the manufacturers of essential oils are using solid waste mainly for composting whereas fluid wastes are simply discarded. So far as essential oil content of the raw material is low, usually less than 5%, the waste consists of more than 95%; therefore, recently, the interest in the effective use of waste has been increasing However, this topic of research is relatively new, there is little information about active compounds in both liquid and solid residues of essential oil distillation; therefore, in this section brief survey on by-products formed from the processing of fruits and vegetables will be provided.

Processing of fruits and vegetables almost always results in some by-products, which are called waste in the case they are not utilized for further processing. The quantities of the waste are usually quite large (Table 1.4).

Table 1.4. *Amount of agro-industrial residues [58-62]*

Production process	**% of wastes or by-products**
White wine production	20-30
Red wine production	20-30
Fruit and vegetable juice production	30-50
Fruit and vegetable processing and preservation	5-50
Vegetable oil production	40-70
Essential oil production	85-99

Moure *et al.* suggested various ways of waste uses, one of these opportunities to recycle and use it as a source of phenol compounds, fiber and protein [59]. Other authors who studied the by-products such as the hulls of rice, buckwheat, and almond estimated that there is a large amount of minerals and active ingredients, possessing antioxidant properties; they have offered these active components to extract from the waste, thus increasing the complete recycling of primary raw material [63-65]. On the other hand, in many developed countries by-products of vegetables are burned as a fuel, instead of being discarded; however, there is the opinion, that such utilistion of processing of by-products is not suficiently rational. Therefore, purposeful and rational ways of waste recycling remains the challenge both for the scientists and industry.

1.3.2 Valorization of plant processing by-products

The quantities of solid and liquid waste formed in food industry mainly depend on the technological processes and the origin of raw material. However, the total formed waste in plants pose huge problems for their recovery, because there are complex technological and legal restrictions in waste recovery processes. Four recycling processes resulting in the formation of by-products in large quantities which could be further treated for isolating active

components will be shortly discussed [60, 65]. Large amounts of residues obtained after extraction of oil from the seeds of soybean, cotton, sunflower etc. are produced in the edible oil industry. Oilseed meals come from oil processing after the recovery of the oil by mechanical pressing or solvent extraction. They are significant protein sources due to high protein content, low price, and high availability.

Molasses and sugar beet pulp in the sugar industry are other abundant by-products. Sugar cane bagasse is one of the largest cellulosic agro-industrial by-products. Several processes have been used for sugarcane bagasse. These include electricity generation, pulp and paper production, and products based on fermentation. Various products, which have been obtained from the processes involving bagasse include chemicals and metabolites such as alcohol and alkaloids, mushrooms, protein enriched animal feed (single cell protein), enzymes, etc. One of the significant applications of the bagasse is the production of protein-enriched cattle feed and enzymes. Bagasse could also been used for the production of industrially important enzymes and biofuel.

By-products from fruit processing include citrus and pineapple pulps, reject apples, etc. can be processed for the production of alcohol or other products. Pectin is also produced from the peel by acid extraction, dietary fibers by mechanical processing, while the recovery of flavonoids and carotenoids are new potential applications. Citrus seeds can be used for oil extraction and recovery of terpenoids, while the meal remaining from the extraction is a good source of proteins.

The solid residue (leaves, flowers, stems, roots) and water residue obtained after hydrodistillation of essential oil in the essential oil industry can be processed into a powder with high content of phenolic compounds and strong antioxidant capacity; the product could be used as a natural additive or healthy food ingredient in foods and nutraceuticals.

The recycling processes of isolating organic compounds or natural molecules are usually much simpler than using the raw material, mostly because the waste is left of certain components which impede to isolate active compounds, such as oil materials and resin, which complicate the extraction of polyphenols. As an example, fruits, vegetables, oilseeds and hydrodistillation of the by-products processing schemes are shown in Fig. 1.2. Using simple technological processes can be distinguished valuable components such as antioxidants, protein, fiber, carotenoids and pectin, which later would be used for food, cosmetic or pharmaceutical products [60, 65]. The solid residue remaining after second recycling phase could be used for composting or for more specific applications, e.g. for producing biodegradable cups for plants.

Growing interest is pointing towards natural antioxidants obtained using „green“ processes. Natural antioxidants represented 38% of the market of antioxidants in 2004 and every year this market grows by 6–7% [66]. These products have a promising business perspective, for instance, in the formulation of functional foods when antioxidants can provide an important benefit for human health [67]. It is also important to emphasise that the amount of raw materials and energy is limited therefore they should be used as efficiently as possible [68]. The extraction of antioxidants from agricultural waste is thus an opportunity to obtain profit in a sustainable way from low-cost raw material. Studies related to the antioxidants recovery from residues remaining after extractions have achieved positive results regarding their potential use [58, 55].

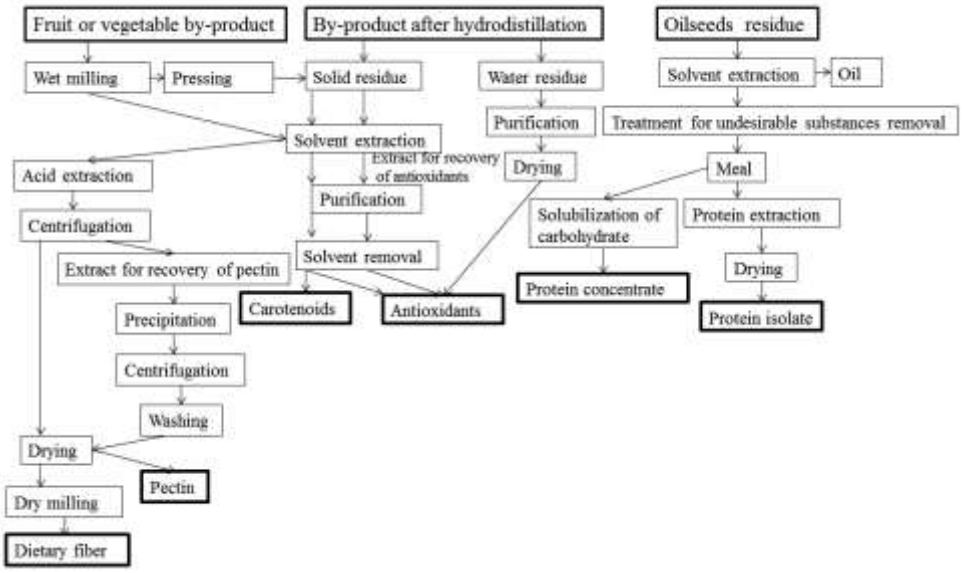


Fig. 1.2. *Flow diagram for the recovery of active componets from by-products*

1.3.3 Natural bioactive compounds from crop by-products

While in the developed countries of the world synthetic drugs replaced the majority of natural plant preparations used for treatment of various diseases a long time ago, the plants remain as the main source of treatment of various diseases in many developing countries. However, modern science have once again turned to the analysis of the plants for searching new active molecules, since the plants are the renewable resources; also a wide variety of plants enables finding a number of new previously unknown molecules possessing different functional properties [56]. It has long been known that the plants accumulate phytocomponents which can absorb and neutralize free radicals, inhibit the oxidation processes and slow down the formation of peroxides [69-77]. Increased consumption of plant-based products due to the pervasive vegetarianism significantly reduced some of the diseases and mortality, resulting in a greater interest in natural additives which might replace synthetic ones [78]. Natural ingredients made of by-products are also gaining popularity due to the developments in recycling procedures applied for the isolating active components. For instance, during last years pomegranate and cranberry wastes have been widely analyzed because they contain components that inhibit inflammatory processes. Pomegranate peel was found to contain a large amount of phenolic compounds including anthocyanins (derivatives of delphinidin, cyanidin and pelargonidin), hydrolysable tannins (catechin, epicatechin, punicalin, pedunculagin, punicalagin, gallic and ellagic acid esters of glucose) [79-80], and several lignans (isolariciresinol, medioresinol, matairesinol, pinoresinol, syringaresinol, and secoisolariciresinol). These phenolic compounds confer antioxidant, anti-mutagenic, anti-inflammatory and anticancer activities [80-82]. In recent studies husks of pomegranate were successfully used in the production of ellagic acid [77, 83]. The pomace of cranberry, a by-product of the cranberry juice, is a good source of ellagic acid and other phenol compounds as well [83-84]. Many literature sources reported that antioxidants isolated from by-products may possess higher activity than synthetic compounds; for example, polyphenols in the skin of pomegranate better inhibited lipid oxidation occurring in meat during cooking process than BHT [79, 85].

Povilaityte *et al*., reported the antioxidant effects of acetone and deodorized acetone extracts of Roman chamomile, purple peril and Moldavian dragonhead and compared it with synthetic antioxidant BHT [86]. Chen *et al*., studied extracts from rosemary leaves and determined that hexane and acetone extracts showed higher antioxidative activity than BHA and BHT [87], while methanol extracts had lower activity. By-products, like skins and seeds of grapes [88-89], peel and pulp of citrus [90], pomace of apples [91-92], waste of potatoes peel [93-94], by-products of onions [95], hulls of beans [96], plant residues after hydrodistillation [86] were reported to be rich in various classes of compounds such as coumarins, cinnamic acid derivatives and chalcones, phenolic diterpenes and phenolic acids, with promising nutritional and therapeutic applications [97-98].

By-products of fruit processing as it was shown for skin and seeds of citrus fruits consitute up 50% of the whole fruit mass; in addition, many studies demonstrated that the largest amount of phenolic compounds are being accumulated specifically in the not edible part of fruit part, which compose the waste [99-100]. The same patterns were found in processing apples, nectarines, peaches, pears, and pomegranate. Consequently it may be noted that the skin of the fruit should not be discarded because it contain at least two times more phenolic compounds than the peeled fruit. Therefore, the enterprises involved in fruits processing should also focus on recycling by-products, thus reducing the amount of waste and optimizing the effectiveness of the use of raw materials [52-54, 101-102]. The studies on by-products recycling also showed that the quantity of recovered phenolic compounds can depend on the process parameters. Jeong *et al*. found that exposure of lemon rinds at 150 °C for 40 min results in the increase of total phenolics from 71.8 to 171.0 μM [103]. On the other hand, Garrote *et al*. found that exposure of corn cobs at a higher temperature increased the amount phenolics compounds, however their activity decreased, leading to the hypothesis that heat treatment may change the structure of phenolic compounds, which determines the activity of compound [104]. The ability of phenolic compounds from by-products to act as antioxidants *in vitro* depends on the number and the arrangement of the hydroxyl groups and the extent of structural conjugation. Many studies established very important relationships between structures and activities of polyphenols [103-104].

The changes of phenol compounds structure can significantly alter the selected method of extraction or solvent, so the recycling of waste should evaluate these factors.

1.3.4 Structure-activity of phenolic compounds

Phenolic compounds may demonstrate both primary and secondary antioxidant activities as chain breaking antioxidants able to donate hydrogen atoms to lipid radicals (radical scavenging) or by preventing initiation reactions by such means as chelating catalyst metals [105-107]. Moreover, the fact that a molecule of phenolic compound is capable to donate a hydrogen atom to a reactive molecule thus transforming it into a non-radical compound with lower activity demonstrates it being a typical radical scavenger [108]. Cai *et al*. investigated the relationship between the structure and radical scavenging activity of phenolic compounds and confirmed that the position and degree of hydroxylation and the related glycosylation, methoxylation and other substitution determine the antioxidant activity of phenolic compounds [109]. The effectiveness of phenolic antioxidants depends on several factors:

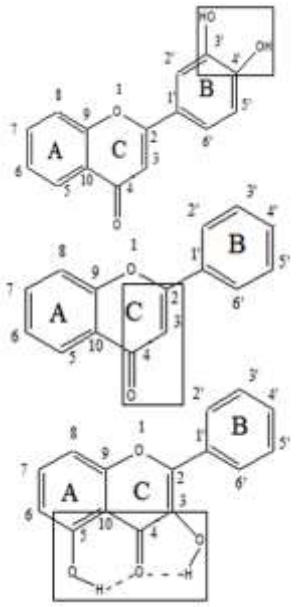


Fig. 1.3. *Antioxidant*

The hydroxyl group in ortho or para positions of phenolic acid increases antioxidant activity while the second hydroxyl group in meta position change antioxidant capacity by decreasing the stability of radical. Glycosilation and esterification of phenolic compounds also decrease antioxidant activity if comparing with the corresponding aglycones [110]; for example, the esterification of phenolic acids by quinic acid decreases antioxidant activity of the compound [111].

1.3.5 Polyphenol extraction from by-products

The objective in extracting phenolic compounds from by-products of plant sources is to liberate these compounds from the vacuolar structures where they are found, either through rupturing plant tissue or through a process of diffusion; they are commonly extracted by alcoholic or other organic solvents. Usually before extraction plant samples are treated by milling, grinding and homogenization [112].

Many methods are described in the literature for extracting phenolic compounds such as Soxhlet extraction, solid-liquid, liquid-liquid, supercritical fluid, accelerated solvent, ultrasound-assisted, microwave-assisted and other methods; however, a single extraction protocol which can be considered optimal for all types of residues does not exist. By-products of plant materials may be composed of variable amounts of phenolic acids, phenylpropanoids, anthocyanins and tannins, among others. This interaction might cause the formation of hardly soluble complexes, which has an effect on the extraction procedure and its efficiency [113-115]. Consequently, in many cases it is not easy to develop an universal method for all plant phenolics. In this chapter some widely used solvent extraction methods for phenolic compounds will be briefly reviewed.

The most popular extraction method is solid-liquid extraction, which efficiency depends on many factors, first of all plant origin and polarity of extraction solvent; however, temperature, solvent to materials ratio, number of extraction cycles, properties of antioxidant active components present in the by-products may also play an important role [116-117]. Nonpolar solvents (hexane, petroleum ether) can be used for the recovery of tocopherols and

certain phenolic terpenes. Ethyl ether and ethyl acetate are very efficient for the recovery of flavonoid aglycons, low-molecular-weight phenols, and phenolic acids. Solvents of higher polarity (ethanol or ethanol–water mixtures) additionally can extract flavonoid glycosides and higher molecular weight phenols, resulting in higher yields of total extracted polyphenols. In particular, methanol has been generally found to be more efficient in extraction of lower molecular weight polyphenols while the higher molecular weight flavanols are better extracted with aqueous acetone [118-121]. Ethanol is another good solvent for polyphenol extraction and is safe for food applications [122]. In preparing anthocyanin-rich phenolic extracts from by-products of plant materials, an acidified organic solvent, most commonly methanol or ethanol is used. Methanol was also used to extract phenolic acids from Lamiaceae family plants [123-124]. However, a dittany water extract was found to be richer in polar phenolic compounds and therefore more effective in retarding lipid oxidation and in scavenging free radicals than acetone, ethanol and methanol extracts of the same plant material [125]. It the study of Moller et al. [126] also has been shown that the aqueous extract of dittany is more effective than the methanol, ethanol and acetone extracts in scavenging hydroxyl radicals as generated by the Fenton reaction and in reducing oxygen consumption when initiated by metmyoglobin. Chen *et al*. analysed the amount of carnosol and carnosic acid from rosemary extracted by different solvents [87]. In contrast to the aforementioned results, they observed lower antioxidant activity for methanol extracts than for either hexane or acetone extracts. They concluded that acetone was the most efficient solvent for the antioxidant substances of rosemary [87]. The efficiency of phenolic compounds extraction greatly depends on origin of plant material but also on the extraction time and temperature [127].

An increase in the extraction temperature can promote higher analyte solubility by increasing both solubility and mass transfer rate. In addition, the viscosity and the surface tension of the solvents are decreased at higher temperature, which helps the solvents to reach the sample matrices, improving the extraction rate. However, increasing the temperature is limited by the nature of compound of interest: high temperatures can cause denaturation of these compounds; therefore the optimum temperature should be carefully selected taking into account several factors [58, 122].

Duration of extraction is another important factor for exhausting extraction. In general, when shorter extraction time is applied, more extraction cycles are used isolate phenolic compounds more completely. It should also be taken into account that long extraction time at high temperature increases the risk of oxidation of phenolics.

According to Wettasinghe *et al*., to reduce the risks of damage of antioxidant compounds the temperatures lower than 60–70 °C are suggested for drying of the raw material and extraction of antioxidants from by-products for both extraction and [128-129].

1.4 REVIEW ON SELECTED AROMATIC PLANTS

The soil and climatic conditions is good for growing of a lot of aromatic and medical herbs in Lithuania and France. However, nowadays many herbs, which were widely used in medieval ages, are underinvestigated and therefore being used in small amounts, if any. This chapter is focused on 3 such aromatic plants, namely *Calamintha grandiflora, Myrrhis odorata* and *Tussilago farfara*, which may be referred to some extent as „forgotten medieval plants“ (Fig. 1.4).

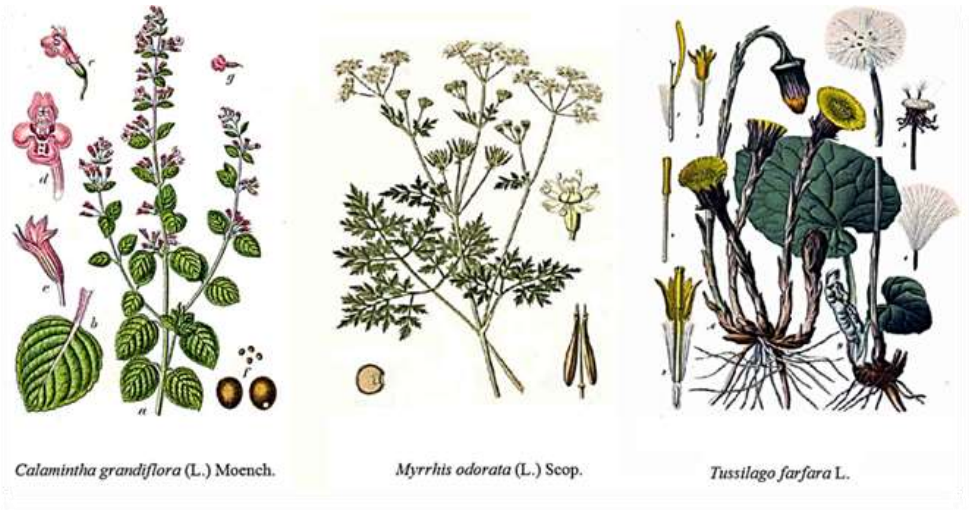


Fig. 1.4. *The selected plants used in the present study*

1.4.1 Calamintha grandiflora (L.) Moench

C. grandiflora (L.) Moench is an aromatic plant of Lamiaceae family possessing strong and penetrating mint odour, which is actually used in fresh state as a condiment in French "Nouvelle Cuisine". *C. grandiflora* is a shrubby plant with spreading rootstock presented form southern Europe to north Iran. Common names of *Calamintha grandiflora* are greater calamint, large-flowered calamint, showy calamint, mint savory. Dark-green, downy, toothed leaves are covered in tiny pale-purple flowers that seen from a distance look like a mist. The plant has a long-flowering season. A sweet and aromatic herb tea is made from the leaves [130]. The leaves are harvested when the plant begins flowering; they are dried for later use [131]. They possess a pleasant mint-like odour [130]. A warm poultice of the leaves may help soothe bruises and rheumatic pains [131]. Volatile compounds of this herb from southern European regions were investigated previously; however the publications on *C. grandilfora* properties and chemical composition are very scarce. Pulegone was reported as the major compound in *C. grandiflora* from Greece and France, whereas isopinocamphone was dominating constituent in the plants collected in Turkey [132-134]. The essential oil obtained from Romanian *C. grandiflora* contained high amounts of volatile compounds germacrene D being the most important component (57.92 %), followed by β-caryophyllene (19.68 %) (Fig. 1.5) [135]. The reports on antioxidative activity of *C. grandiflora* by-product remaining after hydrodistillation were not found in the available literature sources, whereas one paper about the antioxidant potential of the total extract and fractions was reported by Conforti *et al*. [136].

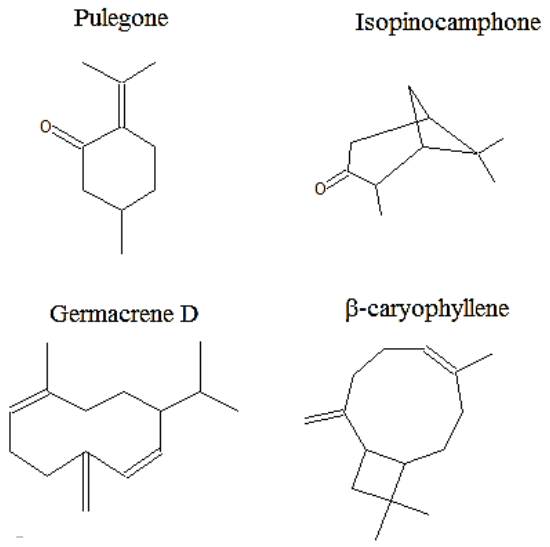


Fig. 1.5. *Chemical structure of major components of* C. grandiflora *essential oils isolted from plant leaves*

Literature data showed that the ethyl acetate fractions (EtOAc) possessed highest antioxidant activity, probably due to major content of polyphenols. The lowest radical scavenging activity was exhibited by petrol ether fraction (Fig.1.6).

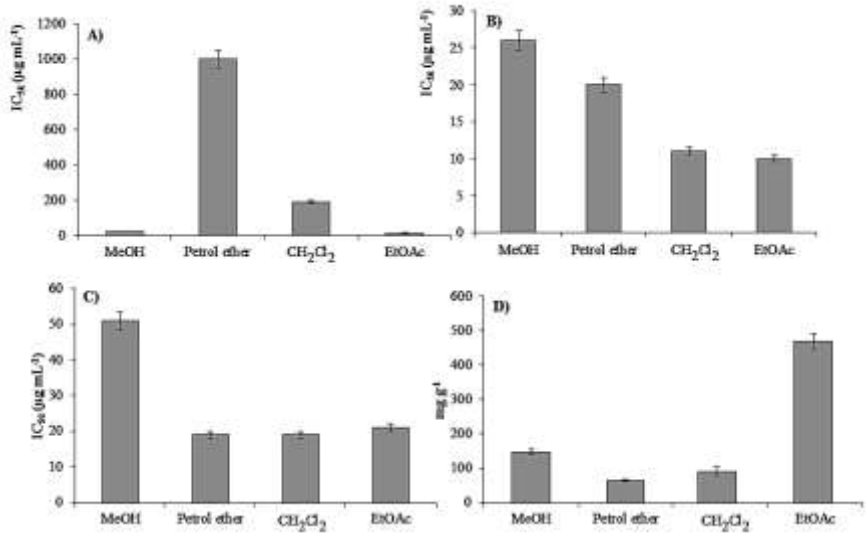


Fig. 1.6. *IC_{50} values of antioxidant activities and total phenolics content of MeOH extract and fractions from* Calamintha grandiflora*: (A) DPPH (ascorbic acid, IC_{50}=2 mg mL-1, was used as positive control); (B) β-carotene bleaching test after 30 min of incubation; (C) β-carotene bleaching test after 60 min of incubation (propyl gallate, IC_{50}=1 mgmL-1, was used as positive control); (D) Total phenolics content using Folin-Ciocalteau method. Values expressed as milligram of chlorogenic acid/g of extract [136].*

However, the authors of the study did not analyse the antioxidant composition of the extracts obtained. Furthermore, considering many factors influencing the composition and the concentrations of bioactive constituents in *C. grandiflora* as well as the remarkable importance of these plants to their health-promoting potential, studies should be continued by focusing on the identification of active compounds and comprehensive characterization.

1.4.2 *Myrrhis odorata (L.) Scop.*

***M. odorata* (L.) Scop.** is an underrated plant deserving more attention as a culinary herb and as a sugar saver. Common names of *M. odorata* are sweet cicely, anise cicely, Spanish chervil, sweet chervil, anise chervil, garden myrrh, sweet scented myrrh. Sweet cicely is widely spread in whole Europe. It grows in very moist forests and mountainous regions. Sweet cicely is being cultivated in Lithuania too. Also, feral types of this species might be found. As it is related to chervil, it may also be called by this name. *M. odorata* is a perennial plant and it might reach the height of 90 cm. It blooms early in the spring up to the middle of summer. Blossoms are tiny and white; leaves resemble the fern, though the smell of anise might be felt after grinding it. A sweet cicely is very easy cultivated plant because it grows almost everywhere: both in sunny or shadowy places. This plant may be sowed either in autumn or in the end of winter. The leaves of sweet cicely produce essential oil which has the aroma of anise [137].

Previously this plant was widely used in cuisine; however, as a potherb, its cultivation dercreased. Fresh, sometimes frozen or dried leaves are usually used to season the food. Green or dried seeds and roots that are collected in autumn may be used too. It is worth mentioning that dried sweet cicely's aroma is noticeably weaker than that of fresh herb. Young stems and leaves can be cut all the time. They are especially soft. Green sweet cicely has the strongest taste; therefore, the leaves may be frozen. The taste of the fruits is best while they are still green and unripe. The roots can be dug out in autumn and used as vegetables. In cuisine the leaves of *M. odorata* may be used as a replacement for sugar in the sweet dishes as using this plant for desert decreases the use of sugar twice. Fresh leaves are used to season the salad, soup, sauce or omelette because the leaves of sweet cicely make the taste more delicate. Only a little of the leaf is enough to give a dish a certain taste of sweet anise. On the other hand, the stems are also used as the replacement for celery. Roots are useful to make wine or salad may also been consumed boiled. The seeds are used as a fragrant material which is also usable for making cream, ice cream or candy as it improves the texture too. In addition, the seeds may be used as a replacement for cloves while baking cakes. Children eat it as a snack. Blossoms are edible or used for decorations. In cuisine, this plant is also used because it has a lot of nutrients and is fat free. It has a sweet taste; therefore, diabetics might use it in their diet for replacing saccharose.

Sweet cicely is not very popular in nowadays cuisine. But it is still used in the Scandinavian countries as the people make liquor or spice out of it to make meat, fish and vegetables taste better.

Previously, the oaken furniture used to be polished by the leaves of sweet cicely. The stalk of the plant was used to make the orange-brown paint for the textile. The seeds were used to make the varnish for the furniture to make the textile better and redolent.

A few centuries ago, all parts of *M. odorata* were used in the medicine. This plant was thought to be harmless, thus, a lot of scientists recommended it. Sweet cicely revs up the digestive system. It reduces the caught, tones the organism and acts as an antidepressant. *M. odorata* presents expectorant, diuretic and was proposed for malaria treatment [138]. Essential oils and flavonoids that are present in the plant act as anticeptic agents. It stabilizes the appetite and cleans the blood too. The essential oil gathered from sweet cicely is used in perfumery.

In some literature sources is mentioned that *M. odorata* contains *E*-anethole up to 80% in the total oil [137, 139-140]. *p*-Cymene was dominating constituent (62.0%) in *M. odorata* oils from Serbia (Prokletije region) (Fig.1.7) [141].

Flavonoids, apigenin- and luteolin-*7-O-β*-glucopyranosides [142], some coumarins [143] and several phenolic acids [144] were reported previously in *M. odorata* from Poland, however the information about antioxidative activity of *M. odorata* extracts has not been found in the available literature sources.

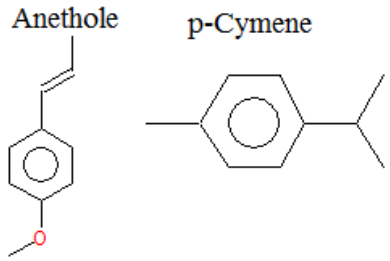


Fig. 1.7. *Chemical structure of major components of* M. odorata *essential oils*

M. odorata species has been introduced into the collection of medicinal plants at Kaunas Botanical Garden of Vytautas Magnus University. The average vegetation period of *M. odorata* is 190 days. The quality of raw material was characterised analysing the bioactive compounds. The differences in phytochemical composition of *M. odorata* plant's secondary metabolites depend on plant's geographical location, chemotype, meteorological factors, raw material preparation time and conditions. Therefore, it is very important to evaluate those predisposing factors. On the other hand, no information is available on the antioxidant activity and chemical constituents of *M. odorata* essential oils from the plants grown in France and Lithuania

1.4.3 Tussilago farfara L.

***T. farfara* L.**, commonly called coltsfoot, is an Asteraceae family plant, which traditionally has been used as a medicinal herb. Coltsfoot which is native from Europe to Asia is also a common plant in North and South America where it has been introduced, most likely by settlers as a medicinal item. Early coltsfoot is not big herbaceous perennial plant with the creep branched underground rhizomes. It blooms of bright yellow blossoms before issuing leaves – in March-April. In the beginning of spring from rootstock grows scaly, leafless, 10–25 cm tall stem of blossom with one bud at the top. Before flowering and blooming the buds are pendulous. After flowering grow large, round heart-shaped, angular leaves. The upper surface of leaf is dark green, the bottom - white gray, because abundantly covered with white nap. The Latin name of coltsfoot "*Tussilago*" is derived from the words "tussis – cough" and "Agere – budge". This medicinal plant is found throughout Lithuania. Only one kind of coltsfoot - early grows in Lithuania. It prefers loamy and sandy soils; the plant also grows very well in wet soil areas. Most often it is found on the fields, on coasts of water's bodies, near ditches, on meadows. It can be cultivated and grown artificially.

In Europe early coltsfoots are some of the most popular medicinal plants used in folk medicine for treatment of various diseases; the dried leaves and already made preparations can be purchased in pharmacies. The flowers have been wider used in European countries, while in the Eastern countries the buds, containing pyrrolizidine alkaloids prevail in the

market. Rhizomes accumulating various nutrients also have been used for disorders treatments. Coltsfoots have bneen mostly used for treatment of respiratory diseases: bronchitis, cough, and asthma [145]. Crushed flowers supposedly cured skin conditions. Coltsfoot is not commonly used as a food but it is listed by the Council of Europe as a source of natural food flavouring [146-147].

Very small quantities of pyrrolizidine alkaloids, which severely damage the liver, are being detected in the early coltsfoots; therefore the preparations of this plant are dangerous when used in large quantities. It was found that very small quantities of pyrrolizidine alkaloids harmful to health, therefore, it was proposed to completely abandon the use of early coltsfoots for therapeutic purposes. Pyrrolizidine alkaloids break during decoction preparation. Consequently, the preparations of these plants can be used just under medical advice, not exceeding the treatment more than 4–6 weeks. The fresh squash leaves can place on sores, bruises, rose and other painful areas of the body. In folk medicine with dry shredded leaves fumigated the sick persons, suffering from shortness of breath, difficulty in breathing. With the juice of coltsfoot's fresh leaves can treat runny nose, wounds. Was thought to be sufficient to include the juice of coltsfoot into the nostrils and runny nose will pass. The leaves of coltsfoot contain 2.6% bitter, up to 10% of tanning materials, up to 8% mucilage, essential oil, organic acids, vitamin C and carotene [145-147].

The buds of early coltsfoots before blossoming or at the beginning of blossoming may be consumed fresh or stewed; also they can be added to salads. These plants have barely perceptible smell of anise. Very young leaves can be used in vinaigrettes, salads and soups. Fragrant tea can be prepared from fresh or dried leaves; wheras young shoots may be cooked and consumed as sweets. For food these plants are mostly used in the Scandinavian countries.

The extraction and composition of *T. farfara* essential oils has been widely studied [148-149]. The extracts of *T. farfara* was reported as exhibiting various activities, such as antioxidant effect, antimicrobial activities and inhibitory effects on NO synthesis in LPS-activated macrophage and diacylglycerol acyltransferase activity [147, 150]. Some pure compounds such as a number of phenolics, mucopolysaccharides and water-soluble polysaccharides were isolated from the flower buds of *T. farfara* [151]. Quercetin *3-O-β-L*-arabinopyranoside and quercetin *3-O-β-D*-glucopyranoside were reported previously in Korean *T. farfara* [152] and 3,4-dicaffeoylquinic, 3,5-dicaffeoylquinic, 4,5-dicaffeoylquinic acids and rutin were isolated from Chinese *T. farfara* [153].Two sugar conjugates of quercetin, quercetin *3-O-β-L*-arabinopyranoside and quercetin *3-O-β-D*-glucopyranoside exhibited higher antioxidative activity than their aglycone quercetin in the NBT superoxide scavenging assay (Fig. 1.8) [152].

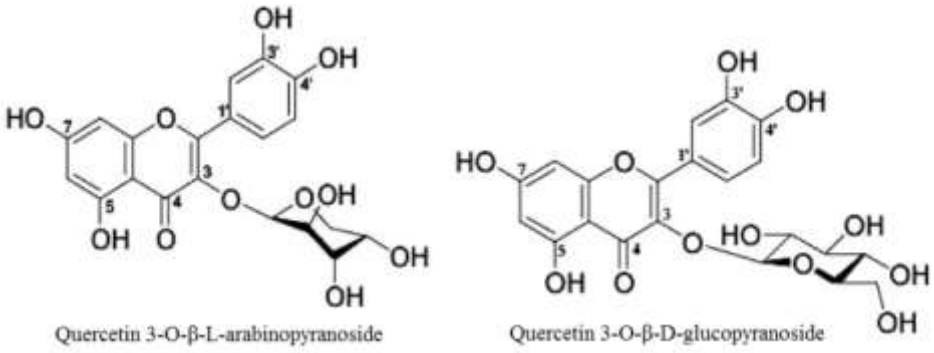


Fig. 1.8. *Two glycosides of quercetin from Korean of T. farfara*

The study reported by Gao *et al*. showed the methanolic extract from flower buds of *T. farfara* possessed the highest maltase inhibitory activity using maltose as a substrate [153]. 3,4-dicaffeoylquinic, 3,5-dicaffeoylquinic and 4,5-dicaffeoylquinic acids showed comparative maltase inhibitory activities with the IC_{50} values of 0.91 mM, 0.90 mM and 0.89 mM, respectively. In the same study it was also suggested that the number of caffeoyl groups attached to a quinic acid core were important for the activities of dicaffeoylquinic, chlorogenic, quinic and caffeic acids [153]. Kokoska *et al*. reported that ethanol extract of *T. farfara* aerial parts did not show antibacterial activity against *E. coli* [154], while in the study of Turker *et al*. water extract of *T. farfara* leaves inhibited the growth of these bacteria [155].

T. farfara is a good source of proteins, lipids, essential oils and a wide range of polyphenolic phytochemicals. The amounts of these compounds in *T. farfara* from Kosovo reported by Faikul *et al*., varied of 2.3-2.7% (proteins), 1.6-2% (lipids) and 0.1-0.2% (essential oil).

T. farfara is also considered as a source iron, calcium, potassium, magnesium, coper, sodium and zinc. The biological activity of this plant depends on the minerals and on the composition of the essential oil [156].

The literature survey shows that plant biodiversity is very high; however due to increasing consumer's demand it is important to look for the new bioactive components and their sources. Despite several published reports dealing with bioactivity of isolated compounds from flower or buds of *T. farfara*, very little information was found about antioxidant activity of extracts from *Calamintha grandiflora*, *Myrrhis odorata* and *Tussilago farfara* leaves. On the other hand, by-products obtained during distillation of aromatic plants for essential oil were not studied. Considering the high content of wastes, it is reasonable to search for ways of a most effective use of the wastes with the aim to process the whole plant material of flavours and antioxidants.

CHAPITRE 2

2 MATERIALS AND METHODS

2.1 SOLVENTS AND REAGENTS

The reference compounds for *β*-myrcene (≥ 95.0%), estragole (≥ 95.0%), *E*-anethole (≥ 98.0%), methyleugenol (≥ 98.0%), *E*-nerolidol (≥ 98.0%) (Fluka, Steinheim, Switzerland), *n*-alkanes C_8-C_{28} (Sigma Chemical, USA) were used in the GC analysis. Acetone (analytical grade) was from Stanchem (Lublin, Poland), ethanol (rectified spirit 95%, Stumbras, Lithuania) and methanol (analytical grade) were from Fluka Chemie (Buchs, Switzerland) were used isolated non-volatile extracts from hydrodistillation residue.

Acetonitrile (HPLC grade), methanol (HPLC grade), acetic acid (HPLC grade), gallic acid, rutin, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH$^{\bullet}$, 95%), 2,6-di-tert-butyl-4-methylphenol (BHT), anhydrous sodium sulphate and phosphate buffered saline (PBS) were from Sigma-Aldrich (Steinheim, Germany). Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), (97 %) was from Acros Organics (Geel, Belgium). Folin-Ciocalteu's phenol reagent, methanol (analytical grade), 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS$^{\bullet+}$) were from Fluka Chemie (Buchs, Switzerland). Commercial refined sunflower oil "Vilnius" (Vilniaus AB "Aliejus", Lithuania). KCl, NaCl, NaH_2PO_4, $K_2S_2O_8$ (Merck, Darmstadt, Germany). KH_2PO_4 (Jansen Chimica, Beerse, Belgium) were used to evaluate antioxidant properties in the residues extracts.

2.2 PLANT MATERIAL

The leaves of large flowered calamint (*Calamintha grandiflora* (L.) Moench) were collected in Midi-Pyrenées (south-west of France, 43° 36′ 16.2″ N, 1° 26′ 38.4″ E) in May, 2009 at flowering vegetation phase, which is considered as a preferable time in terms of phytochemical composition and functional properties of herbs.

The leaves of sweet cicely (*Myrrhis odorata* (L.) Scop.) were collected after flowering in Saint-Cyr-en-Val, region of France (47° 49′ 9.09″ N/1° 56′ 51.3″ E) during July, and in Kaunas Botanical Garden of Vytautas Magnus University, Lithuania (54° 52′ 14″ N/ 23° 54′ 40″ E), during August, 2009.

The flowers and peduncle of coltsfoot (*Tussilago farfara* L.) were collected in Midi-Pyrenées (south-west of France 43° 36′ 16.2″ N/ 1° 26′ 38.4″ E) in April, 2011 and in Kaunas Botanical Garden of Vytautas Magnus University, Lithuania (54° 52′ 14″ N/ 23° 54′ 40″ E), during April, 2010 at flowering vegetation phase.

The leaves and roots of coltsfoot (*Tussilago farfara* L.) were collected in July, 2011 and August, 2010 after flowering vegetation phase.

Table 2.1. *Investigated plants*

Plant name	Family	Anatomical part	Hervesting stage	Origin
Calamintha grandiflora	Lamiaceae	Leaves	Full flowering	France
Myrrhis odorata	Apiaceae	Leaves	After flowering	France, Lithuania
Tussilago farfara	Asteraceae	Flowers, stems, leaves, roots	Flowering, after flowering	France, Lithuania

2.3 PREPARATION OF PLANT EXTRACTS AND ISOLATION OF ESSENTIAL OILS

Volatile extracts

The collected and separated from non-used parts plants were dried at 30±2°C in a ventilated oven "Vasara" (Utena, Lithuania). Dried plant leaves, roots, flowers and stems were ground in a Moulinex D53 grinder (Moulinex, France).

The essential oils were obtained by hydrodistillation from 40 to 80 g grounded plants in a Clevenger type European Pharmacopoeia apparatus over a 3 h period. The obtained oil was separated from the water and dried over anhydrous sodium sulphate. Isolation of oil was performed in duplicate and the samples were stored in a freezer prior to further analysis. The yields of *M. odorata*, *T. farfara* and *C. grandiflora* essential oils were expressed in % (w/w) of dry weight (Fig. 2.1).

Non-volatile extracts for isolating antioxidants

For the preparation of deodorized acetone (AE) and methanol (ME) or ethanol extracts (EE) the essentials oils were removed (Fig. 2.2). The residues of hydrodistillation were separated into the solid and liquid fractions. The solid residue was dried at 30°C in a flake "abc Bio Dörrer" (Gengenbach, Germany). 12 g of dry residue were extracted with 120 mL of acetone, methanol or ethanol with automatic extractor IKAWERKE RET control visc (Staufen, Germany). The solvent was evaporated in a R114 vacuum rotavapor equipped with a B480 water bath (60°C) and B169 vacuum pump (Büchi, Switzerland). The liquid fraction (water extract) was divided in two parts. One of it was spray-dried (SD) in a Büchi Mini Spray Dryer 190 (Donau, Switzerland) and the other one freeze-dried (FD) using Maxi-Dry Lyo (Heta, Germany) for *C. grandiflora*, and evaporated in a vacuum rotavapor (W) for *M. odorata.* The extractions were performed in triplicate, and the dry extracts were stored in a freezer below -18°C prior to further analysis.

Acetone (AE), methanol (ME) or ethanol (EE) of *T. farfara* leaves and roots extracts were obtained by extracting 15 g of dry crushed leaves and roots with 200 mL of acetone, methanol or ethanol during 3 h in a Soxhlet apparatus. The extracts were concentrated in a R114 vacuum rotavapor equipped with a B480 water bath (60°C) and B169 vacuum pump (Büchi, Switzerland).

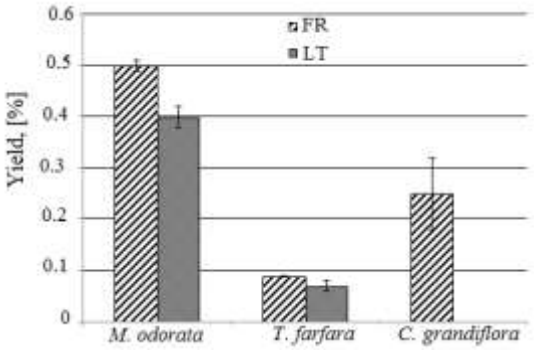


Figure 2.1. *Yields of* M. odorata, T. farfara *and* C. grandiflora *essential oils*

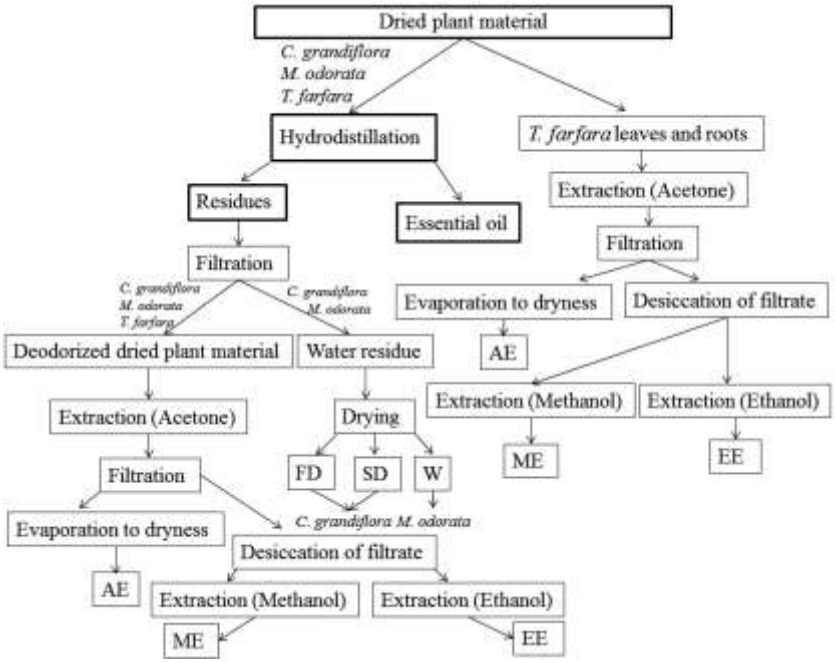


Figure 2.2. *Scheme of* C. grandiflora, M. odorata *and* T. farfara *extraction procedure*

2.4 ANALYSIS OF ESSENTIAL OILS (VOLATILE EXTRACTS)

2.4.1 GC-FID and GC-MS analysis of oil constituents

The oil was analyzed by GC using a Hewlett-Packard 6890 apparatus (Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and electronic pressure control (EPC) injector. DB-5 column (30 m × 0.25 mm, 0.25 µm film thickness) was used. The flow of the carrier gas (N_2) was 1.0 mL/min at a column pressure of 42 kPa. The analysis was performed using the following temperature program: oven temperature isothermal at 50°C for 5 min, increase at a rate of 5°C/min and isothermal at 300°C for 5 min (for *M. odorata* samples), for *C. grandiflora* samples GC oven temperature was programmed from 30°C (5 min hold) to 200°C (10 min hold) at 5°C/min, and for *T. farfara* samples the column temperature was programmed from 50°C (5 min hold) to 280°C (10 min hold) at a rate of 3°C/min Injector's and detector's temperatures were held at 280°C and 300°C, respectively.

The GC-MS analyses were performed on a gas chromatograph HP 5890 (II) interfaced with an HP 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with

electron ionization (EI) 70 eV. An HP-5MS capillary column (60 m × 0.25 mm, 0.25 µm film thickness) was used. The column temperature was programmed the same as the GC-FID method. The carrier gas was He with a flow rate of 1.2 mL/min. Scan time and mass range were 1 s and 50-550 m/z, respectively. The injected volume was 1 µL, and the total run time was approximately 63 min. Identification was based on a comparison of peak retention indices relative to (C_8-C_{22} (Sigma Chemical, St. Louis, USA)) n-alkanes with literature data or authentic compounds available in our laboratory. Further identification was confirmed by matching recorded spectra with Wiley/NBS mass spectral library and other published mass spectra [157]. Quantitative data were obtained from the electronic integration of the FID peak areas.

2.5 DIRECT EVALUATION OF VOLATILE COMPOUNDS IN FRESH PLANTS LEAVES

2.5.1 Design of Flash Aroma Dispenser (FAD) method

Some entire fresh leaves of *C. grandiflora* weighting about 500 mg were placed in the cell of the FAD, thermostated from 8 to 25°C. N_2O and CO_2 gas cartridges were used for the explosion of the vesicles of storage of essential oil and the release of the main volatile compounds of the plant in atmosphere (Fig. 2.3). Volatiles emitted after explosion were absorbed during 10 min. using a SPME syringe equipped with a Carboxen/PDMS/DVB fibre (Supelco, Bellefonte, USA). In order, the temperature and pressure were controlled. For each sample three replicates were performed.

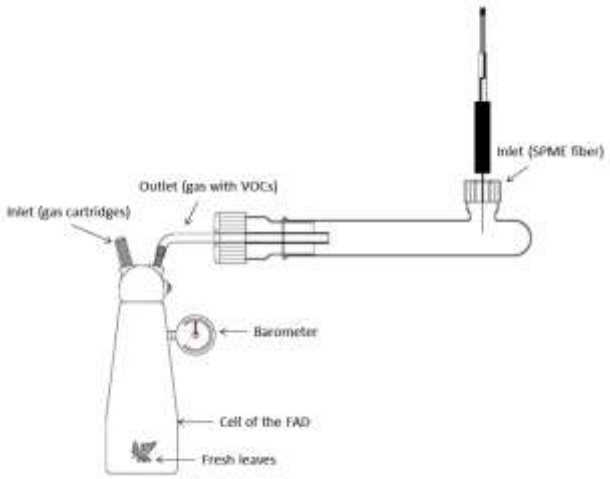


Figure 2.3. *Instrumental Flash Aroma Dispenser system*

2.5.2 Crushing Finger Device (CFD) method

Some entire fresh leaves of *C. grandiflora* weighting about 400 mg were placed in the cell of the CFD; piston was used for the explosion of the vesicles storage of essential oil, and the release the main volatile compounds of the plant in atmosphere (Fig. 2.4). Power changes and number of crushing were controlled for the optimization. Volatiles emitted after crushing were absorbed during 10 min. using a SPME syringe equipped with a Carboxen/PDMS/DVB fibre. For each sample three replicates were performed.

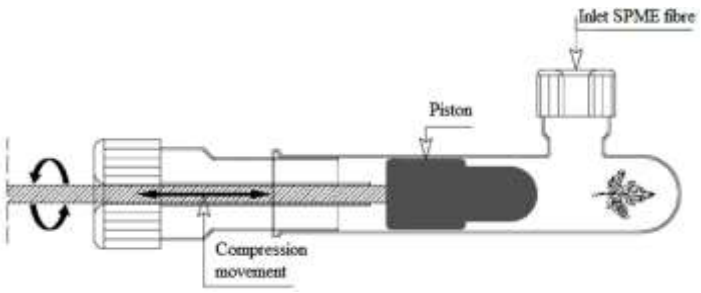


Figure 2.4. *Instrumental Crushing Finger Device system*

2.5.3 Methodology of experimental design for FAD and CFD techniques

A single-factor-at-a-time method studies the phenomenon by varying one factor while keeping all other conditions constant. However, the effect of each factor is not necessarily additive. It is, therefore, necessary to take into account the influence of each factor and the interaction between these factors which may be synergistic or antagonistic [158]. The most influential factors on the characteristics of volatiles compounds are temperature (U_1) and pressure (U_2) in FAD method, and power changes (U_1) and number of crushing (U_2) in CFD method, respectively. The statistical significance of the model was determined by the application of Fisher's F test. A Doehlert matrix was used to represent the responses studied in all experimental regions of these factors (temperature, pressure, power changes and number of crushing) [159]. The factors are given in the form of coded variables (X) with no units in order to permit comparison of factors of different natures. The transformation of natural variables (U) into coded corresponding variables (X) is made on the basis of the following equation:

$$X_{ij} = \frac{(U_{ij} - U_j^0)}{\Delta U_j}; \qquad (1)$$

where: X_{ij} is the value of coded variable j in experiment i;

U_{ij} is the value of natural variable j in experiment i;

U_j is the value of natural variable j in the center of the domain of interest; it corresponds to X_j=0; ΔU_j is the variation of the natural variable j corresponding to a variation of the coded variable j equal to +1.

Doehlert's experimental matrix is given in Table 2.2.

This combination is chosen in such a way that the factors are not correlated with each other and that modelling can be done with uniform accuracy over the whole parameter space [160]. This analysis was achieved by response surface methodology which gave satisfactory results. Each response (Y) can be described by a second order model adequate for predicting the responses in all experimental regions:

$$Y = a_0 + a_1 X_1 + a_2 X_2 + a_{11} X_2^2 + a_{22} X_2^2 + a_{12} X_1 X_2; \qquad (2)$$

where: X_1, X_2 are the coded variables related to the natural variables U_1, U_2,; a_0 is the intercept term, a constant that corresponds to the response when X_i is zero for each factor; a_1 determines the influence of X_1; a_2 determines the influence of X_2; a_{12} is the interaction effect between the X_1 and X_2; a_{11}, and a_{22} can be regarded as a curve 'shape' parameter.

For each response (Y), the coefficients (a_i, a_{ij}) of the postulated model were calculated on the basis of the experimental responses by least squares regression using the NEMROD software (New Efficient Methodology for Research using Optimal Design) [161].

Table 2.2. *Doehlert's experimental matrix and experimental domain*

No. exp.	X^1_{1ij}	X^2_2ij
1	1	0
2	-1	0
3	0.5	0.866
4	-0.5	-0.866
5	0.5	-0.866
6	-0.5	0.866
7	0	0
8	0	0
9	0	0

2.6 SAMPLE PREPARATION (FREE HAND SECTIONING METHOD)

To confirm the presence of essential oil on leaves, flower, and stem, anatomical study has been used (free hand sections). This method allowed examining the samples in a few min. It is also suitable for a variety of plant materials, such as soft herbaceous stems and small woody twigs [162]. Fresh tissues of leaves, stem, and flower were harvested, and free finest hand section was prefomed, and placed for one min in the Gazet du Châtelier reagent [163].

Then the sections were mounted on clean glass slide with a help of glycerin-water (50/50, v/v), and covered by a glass cover slip. Then the slides were observed directly on a light microscope (Leica DM-LB, magnification 100-400; Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) and photographed using a digital camera (Canon Power Shot S40; Canon, New York, U.S.A).

2.7 ASSESSMENT OF NON-VOLATILE EXTRACTS AND ANTIOXIDANT ACTIVITY

2.7.1 DPPH• scavenging assay

Radical scavenging capacity (RSC) of plant extracts against stable 2.2-diphenyl-2-picrylhydrazyl hydrate (DPPH•) was determined spectrophotometrically by a slightly modified method of Brand-Williams and co-workers [164]. The solution of DPPH• in methanol (6.5×10^{-5} M) was prepared daily before measurements on a UV/visible light spectrophotometer Spectronic Genesys 8 (Thermo Spectronic, Rochester, NY) at 515 nm. Extract solutions were prepared by dissolving dry extract in methanol. Two mL of this solution were mixed with 50 µL of different concentration (3, 2.5, 2, 1.5 and 1 mg/mL) extract solution in 1 cm path length disposable microcuvette. The samples were kept in the dark for 30 min at room temperature and then the decrease in absorbance was measured. The absorbance of a blank sample containing the same amount of methanol and DPPH• solution was prepared and measured daily. The experiment was carried out in triplicate. The RSC was calculated by the following formula:

$$I = \frac{A_B - A_A}{A_B} \times 100\ \% \qquad (3)$$

where: I is DPPH$^{\bullet}$ inhibition, %; A_B is the absorbance of a blank sample (t=0 min); A_A is the absorbance of extract solution (t=30 min).

The amount of extract required to decrease the initial DPPH$^{\bullet}$ concentration in the reaction by 50% is referred as an effective concentration; the lower the IC_{50} the higher radical scavenging effect.

2.7.2 ABTS$^{\bullet+}$ decolourisation assay

The RSC of extracts was also measured by ABTS$^{\bullet+}$ assay [165]. Stock solution of (2 mM) ABTS was prepared by dissolving in 50 mL of phosphate buffered saline (PBS) obtained by dissolving 8.18 g NaCl, 0.27 g KH_2PO_4, 1.42 g Na_2HPO_4 and 0.15 g KCl in 1 L of ultra-pure water. If the pH was lower than 7.4, it was adjusted with NaOH. Ultra-pure water was used to prepare 70 mM solution of $K_2S_2O_8$. ABTS$^{\bullet+}$ was produced by reacting 50 mL of ABTS stock solution with 200 µL of $K_2S_2O_8$ solution and allowing the mixture to stand in the dark at room temperature for 15–16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the assessment of extracts, the ABTS$^{\bullet+}$ solution was diluted with PBS to obtain the absorbance of 0.800±0.030 at 734 nm. Three mL of ABTS$^{\bullet+}$ solution were mixed with 30 µL ethanol solution of leaf extract in 1 cm path length microcuvette. The absorbance was read at ambient temperature after 1, 4, 6 and 10 min. PBS solution was used as a blank sample. All determinations were performed in triplicate. The percentage decrease of the absorbance at 734 nm was calculated by formula:

$$I = \frac{A_D - A_C}{A_C} \times 100\ \% \qquad (4)$$

where: I is ABTS$^{\bullet+}$ inhibition, %; A_D is the absorption of blank sample (t=0 min); A_C is the absorption of extract solution (t=10 min).

The RSC was expressed as a percentage of inhibition of Trolox (6-hydroxy-2.5.7.8-tetramethylchroman-2-carboxylic acid), mM equivalent per 1000 g using prepared calibration curve. The extent of quenching of ABTS$^{\bullet+}$ by extract was compared with standard amounts of Trolox. The concentrations of Trolox standard used for calibration curve were 0.001, 0.01, 0.1, 0.5, 1, 1.5 mM.

2.7.3 Ferric reducing/antioxidant power (FRAP) assay

The ability of plant extracts to reduce ferric ion to the ferrous one, (FRAP assay) is another indicator frequently used for assessing antioxidant power [166]. Ferrous ion (Fe^{2+}) produced in this assay forms a blue complex (Fe^{2+}/TPTZ) absorbing at 593 nm. Briefly, the reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), a solution of 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ at 10:1:1 (v/v/v). Firstly 300 µL of freshly prepared FRAP reagent was heated to 37°C and an absorbance (A_0) of a blank reagent was read at 593 nm in a Biotek EL808 microplate reader (Vermont, USA). Then 10 µL of 0.1% extract solution in water and 30 µL H_2O were added (final dilution of

samples in the reaction mixture was 1:34) and the absorbance (A) was recorded every 1 min during the whole monitoring period which lasted up to 30 min. The change in the absorbance (ΔA593 nm) between the final reading and A_0 was calculated for each sample and related to the ΔA593 nm of a Fe^{2+} reference solution which was measured simultaneously.

2.7.4 Analysis of total phenolic compounds (TPC)

The content of TPC in extracts was determined with Folin–Ciocalteu reagent [167]. Calibration curve was prepared by using 1 mL reference gallic acid solutions in ethanol (0.025, 0.075, 0.100, 0.175 and 0.350 mg/mL), which were mixed with 5 mL of a standard Folin-Ciocalteu reagent and diluted with distilled water (1:10) and 4 mL of 7.5% sodium carbonate solution in distilled water. The absorption was read after 30 min at 765 nm. The concentration of TPC was expressed in mg of gallic acid equivalents (GAE) per g of plant extract. The TPC was calculated by the following formula:

$$C = \frac{c \times V}{m}; \qquad (5)$$

where: C is concentration of the total phenolics, in gallic equivalents (GAE); c is the concentration of gallic acid, determined form the calibration curve (mg/mL); V is the volume of plant extract (100 mL); m is the weight of pure plant extract (g).

2.7.5 Determination of flavonoids

The content of flavonoids was measured using rutin as a reference [168]. One mL of plant extract solution in 95% ethanol (10 g/L) and 1 mL of aluminium trichloride solution in 95% ethanol (20 g/L) were pipetted into a 25 mL volumetric flask and made up with 95% ethanol. The absorbance was read at 415 nm after 40 min at 20°C. Blank samples were prepared from the mixture of 1 mL of plant extract and 1 drop of diluted acetic acid. The absorbance of a reference solution, which was prepared by using 1 mL of rutin solution instead of plant extract, was read simultaneously. Rutin solution was prepared from 0.05 g of dried at 130-150°C for 3 h rutin, which was diluted in 100 mL of 95% ethanol. All determinations were performed in triplicate. The concentration of flavonoids in plant extracts was calculated by formula:

$$X = \frac{A \times m_0 \times 10}{A_0 \times m}; \qquad (6)$$

where: X is concentration of the total flavonoids in rutin equivalents (RE); A is the absorbance of extract; A_0 is the absorbance of rutin; m is the weight of pure extract (g); m_0 is the weight of rutin (g).

2.7.6 Determination of flavonols

The content of flavonols was determined by the previously reported method [168]. Series of reference rutin solutions containing 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 mg/mL of rutin were prepared. Two milliliters of such reference were mixed with 2 mL of aluminum trichloride solution (20 g/L) in 95% ethanol and 6 mL of sodium acetate solution in ethanol (50 g/L) were added. The absorbance was read at 440 nm after 2.5 h at 20°C and the calibration curve on the dependence of the absorbency on the concentration of rutin was drawn. Plant extract samples were prepared under the same conditions by using 2 mL of

extract (10 g/L) in 95% ethanol instead of rutin. All determinations were performed in triplicate. The concentration of flavonols was calculated by the formula:

$$Y = \frac{C \times V}{m}; \qquad (7)$$

where: Y is concentration of the total flavonols in rutin equivalents (RE); C is the concentration of rutin, determined form the calibration curve (mg/mL); V is the volume of plant extract (mL); m is the weight of pure plant extract (g).

2.7.7 Measurement of the inhibition effect of plant extracts on oil oxidation in Oxipres apparatus

The samples were prepared by mixing commercial refined sunflower oil "Vilnius" with 0.05, 0.1 and 0.2% of extracts. Five g of oil were placed in a reaction vessel and thermostated at 110°C under oxygen atmosphere (5 bars) in Oxipres apparatus (Mikrolab, Aarhus, Denmark). Pressure changes which occur due to the absorption of oxygen consumed for oil oxidation were recorded. The protection factor (PF) value of sunflower oil in case of using plants extracts and their antioxidant activity (AA) were calculated by the following formulas:

$$PF = \frac{IP_X}{IP_K}; \qquad (8)$$

$$AA = \frac{IP_X - IP_K}{IP_{BHT} - IP_K}.; \qquad (9)$$

where: IP_X is induction period of sample with additive (h); IP_K is induction period of sample without additive (h); IP_{BHT} is induction period of sample with synthetic antioxidant BHT (h).

2.7.8 On-line HPLC–DPPH assay

The extracts were analysed on a HPLC/UV system supplemented with DPPH$^{\bullet}$ scavenging detector. In this system two chromatograms were recorded simultaneously, one of which representing UV absorbance of effluent at 265 nm prior to the reaction, while a mirror chromatogram was obtained recording absorbance at 517 nm after reaction of effluent with DPPH$^{\bullet}$ solution in the reaction coil. Mobile phase was supplied to the column by a model 9012 HPLC gradient pump (Varian. USA) at flow rate 0.75 mL/min. The samples of 20 µL were injected into the HPLC system by means of Cheminert C1 injector (Valco Instruments, USA). Reverse-phase LiChroSpher RP-18e 5 µm 12.5 × 0.4 cm column and 0.5 × 0.4 cm precolumn (Merck, Germany) were used for separation. The DPPH$^{\bullet}$ reagent was prepared by dissolving 0.01 M DPPH$^{\bullet}$ in 0.1 M sodium citrate buffer (pH=7.6), methanol and acetonitrile (50:25:25 v/v). It was continuously supplied into a reaction coil (3 m of 0.25 mm ID fused-silica capillary) by a model 2200 HPLC pump (Bischoff, Germany) at a flow rate of 0.75 mL/min. Signals were acquired at 265 and 517 nm wavelengths by means of Linear 206 PHD and Linear UVIS 200 UV-VIS detectors, respectively. Solution A (bidistilled water with 0.05% TFA) and B (methanol with 0.05% TFA) were used as the mobile phase components for gradient elution. Extracts were separated using the following gradient: 10% of B at 0 min, 25% of B at 5 min, 40% of B at 25 min, 95% of B at 40 min, 95% of B at 43 min and 10% of B at 44 min. Clarity chromatography software (DataApex. Czech Republic) was used for data acquisition.

2.7.9 HPLC and LC-MS analysis

LC system consisted of a Thermo-Fisher Spectra System (TFSP, San Jose, CA) P1000XR pump, a TFSP 6000LP Photodiode Array Detector and a TFSP AS 3000 autosampler. Separation of compounds was performed on a Varian Pursuit XRs 5 C18 column (250 mm × 4.6 mm ID, 5 μm) using a linear gradient of 0–30 min, 3%–97% of acetonitrile (A) to bidistilled water with 0.1% TFA (v/v) (B), 30–35 min, 20%–80% of A to B, 35-45 min, 3%-97% of A to B. Flow rate was 1 mL/min and 10 μL of the sample was injected.

Mass spectra were acquired using a Thermo-Fisher LCQ mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an atmospheric pressure chemical ionization source (APCI) using both positive and negative ion mode. The APCI source operating parameters were as follows: the capillary and APCI vaporizer temperatures were set to 250°C and 450°C, respectively, and the spray was stabilized with nitrogen sheath and auxiliary gas (80 and 25 arbitrary units, respectively). Discharge current was 5 μA and capillary voltage was +15 V and −15 V in the positive and negative ion mode, respectively. The mass spectra were acquired in the data dependent mode with wideband activation (i.e. the most intense ion obtained for each scan in the full mass spectrum is further submitted to 38% collision energy for MS/MS).

2.8 STATISTICAL ANALYSIS

The values are expressed as a means of 3 replicates with standard deviations (SD). RSC tests and content of TPC, flavonoids and flavonols were calculated using MS Excel software (CORREL statistical function). Statistical analysis of the data was performed by using one-way analysis of the variance (ANOVA), followed by the Duncans' post hoc test to compare the means that showed significant variation ($p < 0.05$). All the data were subjected to variance analysis using the GLM procedure of SAS [169]. Analyses were performed using MSStat (ANALYT MTC, Muehlheim, Germany, MS Statistical Software Version 3.02u); the software was looking for differences in the plant origins.

CHAPITRE 3

3 RESULTS AND DISCUSSION

3.1 INVESTIGATION OF AROMATIC PLANTS COLLECTED FROM TWO GEOGRAPHICAL LOCATIONS

Chemical composition of aromatic and medicinal plants depends on cultivation area, climatic conditions, vegetation phase, genetic modifications and other factors; therefore characterization of flora present in different growing sites, countries and geographical zones is an important task. Factors that determine the composition and the yield of essential oil are numerous and in some instances it is difficult to segregate these factors from each other, since many are interdependent and influence one another [37]. These variables may include seasonal and maturity variation, geographical origin, genetic variation, growth stages, part of plant and post-harvest drying, and storage [15, 37]. There are many reports in the literature showing the variation in the yield and chemical composition of the essential oil with respect to geographical regions. For instance, variations in the yield and chemical profile of essential oils were reported for *Rosmarinus officinalis* and *Tagetes minuta* populations, collected from different geographical locations [10, 39-40]. Such differences could be linked to the soil textures and possible adaption response of different populations, resulting in different chemical products being formed, without morphological differences being observed in the plants [40].

The aims of this part of study were to examine the composition of *M. odorata, T. farfara* and *C. grandiflora* essential oils of two different origins and to compare to previously published results on these species.

3.1.1 Essential oil composition of Myrrhis odorata (L.) Scop. leaves grown in Lithuania and France

The yields of the oils were 0.40±0.02% (Lithuania) and 0.50±0.01% (France) (w/w) which was in accordance with literature [140, 142]. Chemical composition of the oils of two origins is presented in Table 3.1. Twenty and twenty-six components were detected in Lithuanian and French oils, respectively. Six volatile compounds, namely camphor, *iso*-menthone, *cis*-thujone, pinocarveol, carvacrol and *p*-anisaldehyde which were identified in *M. odorata* French oil, were not detected in Lithuanian oil. Volatile constituents identified in the sweet cicely belong to the following classes of organic compounds: phenylpropanoids, terpenoids, alcohols and aldehydes.

Phenylpropanoids were the most abundant components in *M. odorata* leaves constituting 63.3±0.7%–68.2±0.6% of total oil, followed by terpenes (33.3±0.2%–29.3±0.1%). Hydrocarbon monoterpenes constituted 3.7±0.1%–4.1±0.1%; while oxygenated monoterpenes (1.2±0.1%) were detected only in French origin plant. Higher percentage of hydrocarbon sesquiterpenes was determined in *M. odorata* from Lithuania (16.9±0.1%), than in French origin oil (11.7±0.1%), while the content of oxygenated sesquiterpenes was almost similar, 12.3±0.4% and 12.7±0.3%, respectively. The essential oil of sweet cicely contained higher content of hydrocarbon sesquiterpenes than hydrocarbon monoterpenes.

Table 3.1. *Essential oil composition (%) of* Myrrhis odorata *(L.) Scop. leaves from Lithuania and France.*

No	Compounds	RI^{th}	$RI^{\blacklozenge}$	Content GC area (%)		Odor perception[c]
				Lithuania	France	
1	α-Pinene	939	936	0.1±0.0[a]	0.1±0.0[a]	fresh, camphor, sweet pine, earthy, woody
2	β-Myrcene[*]	992	986	1.7±0.1[a]	1.8±0.1[a]	peppery, terpene, spicy, balsam, plastic
3	*p*-Cymene	1027	1022	0.2±0.0[a]	0.5±0.1[a]	fresh, citrus, terpene, woody, spice
4	Limonene	1029	1030	1.7±0.0[a]	0.4±0.0[b]	citrus, fresh, peppery
5	*E*-β-Ocimene	1038	1032	0.3±0.1[b]	0.8±0.0[a]	sweet, herbal, green, celery spicy, floral
6	γ-Terpinene	1074	1058	0.3±0.0[b]	0.6±0.1[a]	oily, woody, terpene, lemon, tropical, herbal
7	*cis*-Thujone	1102	1092	-	0.2±0.0	thujonic, cedarleaf
8	Nonanal	1104	1103	0.2±0.0[b]	0.4±0.0[a]	aldehydic rose, fresh, orange peel, fatty peely
9	Camphor	1139	1131	-	0.3±0.0	medicinal, camphoreous
10	*iso*-Menthone	1162	1168	-	0.2±0.1	powerful, refreshing cleanminty, cool, peppermint
11	Pinocarveol	1169	1169	-	0.5±0.0	herbal, camphor, woody, pine, balsam
12	Carvacrol	1192	1192	-	0.4±0.0	spice, woody, camphor, thymol
13	Estragole[*]	1200	1197	0.9±0.1[b]	4.1±0.4[a]	sweet, licorice, anise spice, green herbal fennel
14	*p*-Anisaldehyde	1263	1271	-	0.3±0.0	sweet, powdery, mimosa, floral, hawthorn, balsam
15	*E*-Anethole[*]	1285	1290	48.1±1.4[a]	50.7±1.1[a]	sweet, green, anethole herbal
16	Methyleugenol[*]	1407	1401	14.3±0.5[a]	13.1±0.4[a]	sweet, fresh, warm, spicy, clove, carnation, cinnamon
17	β-Caryophyllene	1419	1409	9.7±0.3[a]	6.1±0.3[b]	sweet, woody, spice, clove dry
18	α-Bergamotene	1431	1430	0.9±0.1[a]	0.5±0.0[b]	woody, warm, tea
19	α-Humulene	1467	1459	0.9±0.2[a]	0.5±0.0[a]	woody, herbal
20	Germacrene-D	1487	1485	5.0±0.1[a]	4.3±0.3[a]	woody, spice
21	β-Bisabolene	1498	1496	0.1±0.0[a]	0.1±0.0[a]	balsamic, citrus, myrrh, opoponax, spicy
22	α-Farnesene	1500	1504	0.3±0.0[a]	0.2±0.0[b]	citrus, herbal, lavender, bergamot, myrrh, neroli, green
23	*E*-Nerolidol[*]	1563	1559	10.2±0.8[a]	12±0.3[a]	floral, green, waxy, citrus, woody
24	Spathulenol	1619	1618	2.1±0.5[a]	0.7±0.0[a]	earthy, herbal, fruity
25	Hexadecanol	1870	1864	1.2±0.2[a]	0.7±0.0[a]	waxy, floral
26	Phytol	2128	2125	1.4±0.1[a]	0.4±0.0[b]	delicate, floral, balsam, powdery, waxy
Total				99.6±0.2	99.9±0.1	
Phenylpropanoids (%)				63.3±0.7[a]	68.2±0.6[a]	
Monoterpene hydrocarbons (%)				4.1±0.1[a]	3.7±0.1[a]	
Oxygenated monoterpenes (%)				-	1.2±0.0	
Sesquiterpene hydrocarbons (%)				16.9±0.1[a]	11.7±0.1[b]	
Oxygenated sesquiterpenes (%)				12.3±0.4[a]	12.7±0.3[a]	
Others (%)				3.0±0.1[a]	2.4±0.1[b]	

$RI^{\blacklozenge}$**:** Retention indices relative to C8-C22 alkanes on AnHP-5MS column
RI^{th} : index presented from from database http://www.flavornet.org
[*]authentic standards were co-injected for the compounds identification
[c]Odour description adapted from www.thegoodscentscompany.com
Values with different letters (a–b) are significantly different at $P < 0.05$

It is known that the parent sesquiterpenes are biosynthesized from geranyl diphosphate by its condensation with isopentenyl diphosphate. It can

be suggested that monoterpenes are consumed by the plants faster at late vegetation phases as compared to sesquiterpenes. The major components in both *M. odorata* oils were *E*-anethole, methyleugenol, *E*-nerolidol, and the differences in their percentages were not remarkable. The odor of these compounds were preliminary assessed and is described as sweet, green, anethole herbal anise, licorice (*E*-anethole), clove, spicy (methyleugenol) and floral, green, waxy, citrus, woody (*E*-nerolidol), and they are found in such important spices as *Illicium verum, Foeniculum vulgare*, *Piper claussenianum* [170-172]. From this point of view *M. odorata* from France and Lithuania may be preliminary assigned to the same *E*-anethole, methyleugenol and *E*-nerolidol chemotype. However, some qualitative and significant quantitative differences in oil composition of two remote origins may be observed (Table 3.1). *Cis*-thujone, camphor, *iso*-menthone, pinocarveol, carvacrol and *p*-anisaldehyde were detected only in *M. odorata* oil of French origin; the content of estragol in it was more than 4 times higher than in the oil of Lithuanian origin plant material. The odor of estragol is described as anise, licorice so the French oil had a stronger anise, licorice flavor than Lithuanian essential oil. On the other hand, the percentage of *β*-caryophyllene, germacrene-D, spathulenol and limonene was significantly higher in the oil from Lithuania and this oil had fresh, sweet, woody, spice, clove dry, earthy, herbal, fruity odor.

Table 3.2. *Comparison of main essential oil constituents in* Myrrhis odorata *(L.) Scop. leaves from different geographic origins*

Compound	Lithuania[a]	France[a]	Finland[b]	Russia[c]	Serbia[d]	North USA[e]
	(%)	(%)	(%)	(%)	(%)	(%)
β-Myrcene	1.7	1.8	1.1	-	-	-
p-Cymene	0.2	0.5	-	-	**62.0**	-
Limonene	1.7	0.4	0.1		2.2	0.3
Estragole	0.9	4.1	2.6	-	-	1.7
E-Anethole	**48.1**	**50.7**	**83.1**	**82.0**	-	**85.5**
β-Caryophyllene	9.7	6.1	1.0	1.7	0.7	-
Methyleugenol	**14.3**	**13.1**	-	-	-	**9.1**
Germacrene-D	5.0	4.3	-	2.9	-	-
E-Nerolidol	**10.2**	**12.0**	1.0	-	-	-

[a]Our study; [b]Uusitalo et al. 1999 [137]; [c] Tkachenko et al. 1993 [139]; [d]Rančić et al. 2005 [141]; [e]Hussain et al. 1990 [140].

Comparison of the percentages of the main components in *M. odorata* oils analyzed in our study and previously reported in literature are

summarized in Table 3.2. *E*-Anethole was dominating constituent (82–85%) in *M. odorata* oils from Finland [137], Russia [139] and North USA [140], and it may be assumed that the plants from these countries were purely *E*-anethole chemotype. It should be noted that the plants from North USA contained reasonable amount of methyleugenol, which was not found in Finnish and Russian origin sweet cicely, while *E*-nerolidol was not detected in Russian and Serbian chemotypes. Completely different *M. odorata* chemotype was reported in Serbia (Prokletije region) with *p*-cymene as the major compound constituting 62.0% in the total oil [141]. This variation in component level it is surprising, but not impossible because chemical polymorphism is significantly characteristic of many Umbelliferae family species [173]. The oil reported from Serbia may be would not have the typical sweet, anise, licorice odor of *M. odorata* because *p*-cymene had a stronger fresh, citrus, terpene, woody, spice smell. These differences may be dependent on climatic conditions prevailing during plant development and their genetic peculiarities. Indeed, the composition of essential oils as well as other chemical molecules of plants may vary considerably depending on annual climatic changes and genotype [146, 174]. These parameters influence the total content of essential oils and their composition. Unfortunately, weather conditions were not reported in studies performed in Finland, Serbia and USA.

The present study provides new information about essential oil chemical composition of *M. odorata* from two geographical origins (Lithuania and France), not studied before. The percentage of the main oil compounds in the studied *M. odorata* collected in remote habitats was quite similar; however comparison of the obtained data with previously studied *M. odorata* oils revealed that there are different plant chemotypes. Therefore, further investigations are necessary to ascertain our results by studying a collection of accessions from different geographic regions cultivated under same climatic conditions. This could allow identifying the genetic potential which could help for improvement *M. odorata* breeding programs and diversification of it industrial uses.

3.1.2 *Essential oil composition of* Tussilago farfara *L) from Lithuania and France*

The yield of *T. farfara* flower essential oil was 0.07±0.01% (Lithuania) and 0.09±0.00% (France) (w/w). A close essential oil yield 0.10% (w/w) from *T. farfara* buds grown in China was obtained by Liu *et al*. [148].

However, in this study aerial biomass (flower and peduncle) was used. This result highlighted the possibility and the interest to used whole plant for essential oil extraction instead of buds. Moreover, European Food Safety Authority (EFSA) have addressed in 2009 an opinion to the scientific substantiation of health claims in relation to *T. farfara* and normal function of the upper respiratory tract and "immune health" [175]. The report of EFSA have provided information on the conditions uses of flower and leaf of *T. farfara* plant as infusion, powder, tincture and fluid extract. However, no information was provided on the conditions of use of stem part. In order to prove the potential interest of the peduncle as provider of essential oil, we performed anatomical study. The result emphasized clearly the presence of glandular trachoma in stem of *T. farfara* similar to those showed in flowers (Fig. 3.1) which can be a useful organ for essential oil supply. This result reinforces the fact of using the aerial part (flower and peduncle) of *T. farfara* plant for essential oil production. It should be noted that up to date, no sufficient scientific information on *T. farfara* specie was conducted in literature as noted in EFSA report [175]. Moreover, stem represented an important percentage of the aerial part (50±3.5%) of plant dry matter; while those of the bud and flower were two times lower (28.6±2.4% and 21.4±3.7%, respectively). Accordingly it could be suggested the use of peduncle as potential and additional part of *T. farfara* specie for essential oil supply.

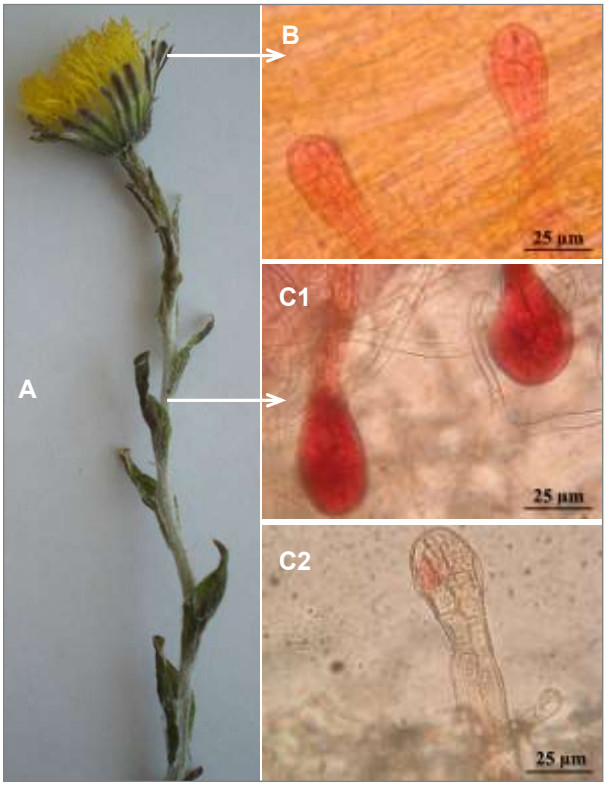


Fig. 3.1. *Microscopic photographs of glandular trachoma from two parts of (A)* Tussilago farfara *(L.) plant; (B) flower; (C1 and C2) peduncle. Magnification 400×*

GC-MS analysis exhibited 27 and 22 components were detected in Lithuanian and French oil, respectively, accounting for 62.2±0.1% and 53±0.5% of the total intedgarted GC area. Chemical composition of *T. farfara* essential oil previously studied from different origin and reported in several works is presented in Table 3.4. Up to now, no information is available on the chemical constituents of *T. farfara* essential oils from the plants grown in France or other Southern European countries. There is only one study on volatile oil of coltsfoot from Eastern European origin [149]. Qualitative and quantitative differences in oil composition of two origins (Lithuania and France) are presented in Table 3.3. 1-Nonene (34.1±0.0%) is the major compound in French oil; however, *n*-tricosane (21.7±0.1%) is the major compound in Lithuanian oil. Moreover, 7 volatile compounds, namely tetracosane, *n*-tricosane, *n*-pentacosane, *n*-hexacosane, *n*-pentadecane, 1-tetradecene and 1-decanol were detected only in Lithuanian *T. farfara* oil.

Table 3.3. *Chemical composition (%) of flower and peduncle essential oils of Tussilago farfara from two origins*

No	Compounds	R.I.[ap]	R.I.[p]	Content (%)	
				Lithuania	France
1	1-Nonene	890	922	8.1±0.3[b]	34.1±0.0[a]
2	*α*-Pinene	935	1024	3.7±01[a]	4.2±0.2[a]
3	Camphene	951	1068	-	tr
4	1-Decene	988	1040	-	2.1±0.0
5	*α*-Phellandrene	1004	1163	0.8±0.1[a]	1.4±0.3[a]
6	*p*-Cymene	1029	1270	0.1±0.0[b]	0.8±0.0[a]
7	*n*-Undecane	1100	1100	7.8±0.2[a]	4.8±0.1[b]
8	1-Dodecene	1191	1190	0.2±0.0[b]	0.5±0.0[a]
9	1-Decanol	1273	1275	0.1±0.0	-
10	1-Tridecene	1291	1365	0.1±0.0[b]	0.3±0.0[a]
11	*α*-Copaene	1376	1489	0.2±0.0	tr
12	*β*-Cubebene	1387	1522	0.2±0.0[b]	0.3±0.0[a]
13	1-Tetradecene	1389	1414	0.9±0.1	-
14	*β*-Caryophyllene	1419	1589	1.9±0.1[a]	0.3±0.0[b]
15	*γ*-Elemene	1439	1470	0.2±0.0[b]	0.4±0.0[a]
16	*α*-Humulene	1454	1665	0.4±0.0[a]	0.2±0.0[b]
17	Germacrene D	1485	1689	0.7±0.1[a]	0.6±0.0[a]
18	*β*-Selinene	1489	1696	0.7±0.0[a]	0.6±0.1[a]
19	*n*-Pentadecane	1500	1500	0.5±0.1	-
20	*β*-Bisabolene	1509	1715	1.2±0.1[a]	0.7±0.1[b]
21	*δ*-Cadinene	1522	1737	0.4±0.0[a]	0.2±0.0[b]
22	Spathulenol	1576	2128	2.1±0.1[a]	0.7±0.1[b]
23	Caryophyllene oxide	1585	1918	1.3±0.1[a]	0.2±0.0[b]
24	Tetradecanol	1680	2177	-	0.2±0.0
25	*n*-Nonadecane	1900	1900	0.4±0.0	-
26	Phytol	2112	2547	1.4±0.1[a]	0.4±0.0[b]
27	*n*-Tricosane	2300	2300	21.7±0.1	-
28	*n*-Tetracosane	2400	2400	0.7±0.0	-
29	*n*-Pentacosane	2500	2500	1.3±0.0	-
30	*n*-Hexacosane	2600	2600	5.1±0.1	-
	Total			62.2±0.1[a]	53.0±0.5[b]

[(ap)]Retention indices relative to C8-C28 alkanes on DB5 column
[(p)]Retention indices relative to C8-C28 alkanes on Carbowax 20M column
Values with different letters (a–b) are significantly different at $P < 0.05$
tr-trace <0.05%

The content of 1-nonene in French oil was four times higher than in the Lithuanian oil. On the other hand, the percentage of *α*-phellandrene, *α*-pinene, *p*-cymene and 1-dodecene was higher in the French oil, than in Lithuanian oil (Table 3.3). It is important to note that the climatic conditions from two studied areas countries are clearly different; mean temperature and rainfall amount during the month of collection were higher in Midi-Pyrenées-France (10.5°C and 97.1 mm) than in Kaunas-Lithuania (6.9°C and 34.0 mm).

Comparison of the percentages of main components in *T. farfara* oils analyzed in our study and those previously reported in literature are

summarized in Table 3.4. Comparing *T. farfara* oil of Lithuania origin analyzed in our study and that previously reported by Judzentienė and Budienė [149] it may be observed that it have roughly the same chemical profile with *n*-tricosane (22.7-21.7%) as a major compound. However, 1-nonene (8.1±0.3%) was the second major compound in our study, while in previous studies this compound was absent. Also *n*-undecane and *n*-pentacosane were found at the concentrations of 7.8±0.2% and 1.3±0.0% respectively; however their percentage was different comparing to published data [149] reporting *n*-undecane and *n*-pentacosane 3.9% and 9.4%, respectively. In addition, the organs and the collection area of *T. farfara* plant investigated in our study (flower-stem from Kaunas area) were not the same as those studied by Judzentienė and Budienė (flower or stem from Vilnius and Kėdainiai area) [149].

Table 3.4. *Comparison of main essential oil constituents of* Tussilago farfara *(L.) from different plant parts and different geographic origins*

Compound	Lithuania					France	China	Romania
		flower					buds	leaves
	Ka-FS	V-F	V-S	Ke-F	Ke-S	M-FS	Ku-B	C-L
1-Nonene	8.1	-	-	-	-	**34.1**	0.1	-
α-Pinene	3.7	tr	-	-	1.4	4.2	-	-
n-Undecane	7.8	-	1.8	-	3.9	4.8	4.8	-
n-Tricosane	**21.7**	**21.6**	**22.7**	**19.4**	**7.2**	-	-	-
β-Bisabolene	1.2	0.6	-	0.8	-	0.7	**13.9**	-
n-Pentadecane	0.5	0.7	0.4	1.0	0.4	-	4.6	-
Spathulenol	2.1	2.0	1.6	3.8	2.2	0.7	3.4	-
n-Hexadecanoic acid	-	-	-	-	-	-	3.1	-
E-Cycloundecene	-	-	-	-	-	-	8.5	-
α-Bisabolol	-	-	-	-	-	-	-	**20.7**
n-Hexacosane	5.1	4.4	5.8	4.5	4.9	-	-	-
n-Pentacosane	1.3	9.4	2.0	9.0	4.0	-	1.0	
n-Tetracosane	0.7	2.1	-	1.8	-	-	0.16	-
Chamazulene	-	-	-	-	-	-	-	7.2
Phytol	1.4	1.8	2.1	2.5	2.4	0.4	0.3	-
References	This study	[149]				This study	[148]	[176]

Collection area: [Ka] Kaunas, [V] Vilnius, [Ke] Kedainiai, [M] Midi-pyrénées, [Ku] Kuandonghua, [C] Câmpeni.
Plant part: [FS] Flower and stem, [F] Flower, [S] Stem, [B] Bud, [L] Leave.
tr-trace <0.05%

On the other hand, significant differences were found between the main constituents of *T. farfara* oils studied in this work and reported by Liu *et al.* [148] and Hãdărugã *et al.* [176] from China and Romania origin, respectively. Chinese *T. farfara* buds oil contained reasonable amount of *E*-cycloundecene (8.5%), which was not found in studied oils from Lithuania and France. Moreover, *β*-bisabolene was the major constituent (13.9%) in

Chinese essential oil, which does not exceed 0.7% and 1.2% in French and Lithuanian oil, respectively. Romanian *T. farfara* leaf oil contained two main compounds, α-bisabolol (20.7%) and chamazulene (7.2%), which were not found in Lithuanian and French oils. These significant differences can be related to climatic conditions or studied part of plant. Also, genotype and chemotype may be important factors responsible for this difference [146, 174] Together with other factors such as climatic conditions, soil characteristics, harvesting time.

The present study provides new information about flower and stem essential oil chemical composition of *T. farfara* from two geographical origins. Microscopy analysis revealed that stem contain secretory pockets (glandular trichoma) which can be a useful organ for essential oil supply which reinforce the fact of using all the aerial part of *T. farfara* plant for essential oil production. Also, these new data can be used for the assessment of plant taxonomy. The percentage of the main oil compounds in the studied *T. farfara* collected in remote habitats was significantly different; however comparison of the obtained data with previously studied *T. farfara* oils revealed that there are different plant parts. Therefore, further investigations are necessary to advance our results by studying a collection of accessions from different geographic regions cultivated under same climatic conditions. This could allow identifying the genotypic difference which could help for improvement *T. farfara* breeding programs and diversification of it industrial uses. Also, for an optimal valorization of aerial parts of *T. farfara* plant, it is useful to investigate the chemical composition and the yield of essential oil of each plant part (flower, stem, bud, leaf) taken separately from the same origin.

3.2 RESPONSE ANALYSIS AND INTERPRETATION OF CFD AND FAD DATA

Experimental design (Doehlert matrix) has been used to optimize the experimental conditions for the CFD and FAD analysis. Concerning the design analysis, two-factor model was selected for each response Y. The Doehlert matrix consists of N experiments with $N=K^2+K+1$, where K is the number of variables studied. In this case $K=2$ and, therefore, the matrix was comprised of seven experiments (Table 3.5 and 3.6).

These 7 points are scattered uniformly in the space of the coded variables (X_i) and are located in the corners of a hexagonal polyhedron. The responses studied were the number of volatile organic compounds (VOCs) using two new CFD and FAD methods (Y_1, Y_2). Following the model

established for response, we can represent graphically the surface of the corresponding responses. The values of the coefficients provide information on the influence of the factors and the curves obtained show the extreme, or at least privileged, directions (zones of interest of the factors). The main objective of the optimization was to determine the optimal conditions of power and number of crushing for the new CFD method and for FAD method was to determine temperature and pressure. So far as these two new methods are intended to be used in a rapid analysis of medicinal and aromatic plants, it was necessary to establish the domains of variation of response (the number of VOCs) in relation to the required characteristics.

From an economical point of view, the total number of VOCs (Y_1, Y_2) is a positive parameter. The total VOCs measured using CFD and FAD methods is an important value, because the number of VOCs is very similar compared with essential oil obtained by hydrodistillation.

Table 3.5. *Doehlert's experimental matrix and the corresponding experimental conditions and experimental responses*

			CFD		
No. exp.	Number of crushing X^1_1	Power of piston X^2_2	Number of crushing	Power of piston, W	VOCs Y_1[a]
1	1	0	20	2.6	22
2	-1	0	0	2.6	0
3	0.5	0.866	15	4.3	18
4	-0.5	-0.866	5	0.9	5
5	0.5	-0.866	15	0.9	10
6	-0.5	0.866	5	4.3	12
7	0	0	10	2.6	22
8	0	0	10	2.6	22
9	0	0	10	2.6	22
			FAD			
No. exp.	Temperature X^1_1	Pressure X^2_2	Temperature, °C	Pressure, bar	VOCs Y_1[c]	VOCs Y_2[d]
1	1	0	26	6	19	12
2	-1	0	8	6	5	5
3	0.5	0.866	22	12	20	12
4	-0.5	-0.866	13	0	0	0
5	0.5	-0.866	22	0	0	0
6	-0.5	0.866	13	12	17	8
7	0	0	17	6	18	10
8	0	0	17	6	18	10
9	0	0	17	6	18	10

[a] Number of volatile organic compounds (VOCs) using CFD method.
[1] Number of crushing in coded variable.

[2] Power of piston in coded variable.

[c] Number of VOCs using FAD with N_2O.

[d] Number of VOCs using FAD with CO_2.

Table 3.6. *Experimental domain*

			CFD	
	Factors	Units	Centre	Step
U_1	Number of crushing	-	10	10
U_2	Power of piston	W	2.6	2
		FAD		
U_1	Temperature	°C	17	9
U_2	Pressure	bar	6	7

The number of VOCs (Y_1) in CFD analysis is shown in Fig. 3.2. These results indicate that the number of VOCs is very low at low power (around

0.6 W) and high at high power (around 4.6 W) and high number of crushing. The effect of power on these characteristics is noticeable only at high number of crushing, especially in the case of number of VOCs. Thus, at power below 0.6 W the number of crushing did not affect the capacity of the VOCs, whereas at high number of crushing, a small increase in power brings about a major increase in a number of VOCs.

The optimal point using CFD indicated by the model corresponds to a power of 2.6 W and at number of crushing 10 (Fig. 3.2). The response values of number of VOCs calculated from the model for these experimental conditions are given in Table 3.6.

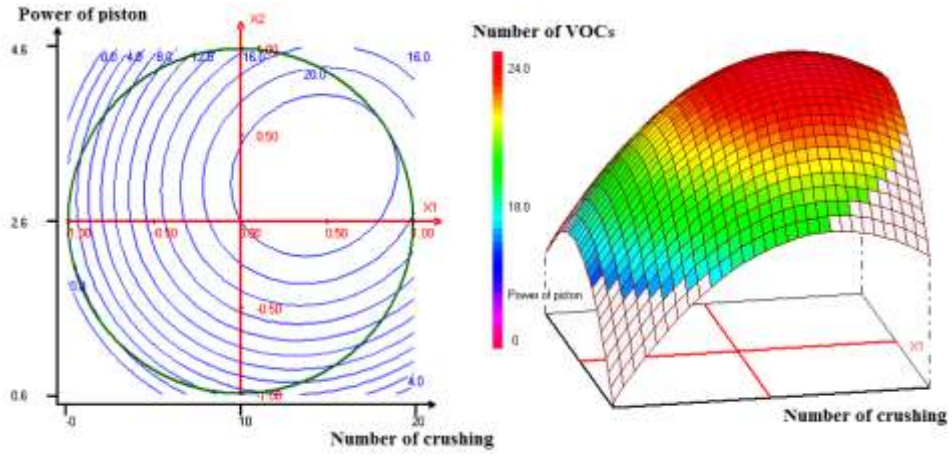


Fig. 3.2. *Variation of the number of VOCs (Y_1) versus (X_1, X_2) in CFD method*

The optimum conditions are schematized as an ellipse. The center represents the optimum conditions in terms of power and number of crushing. The maximal efficiency is obtained for power and number of crushing at the 0 points in coded variable, i.e., 2.6 W and 10 number of crushing in real variable. The analysis of the isoresponse curves at the chosen experimental field delimited by a circle show that the maximum number of VOCs was obtained when the power and the number of crushing are increased. According to these obtained results, the coefficients of the polynomial model were calculated using the NEMROD Software [161].

Number of VOCs $=22 + 9.17X_1 + 4.33X_2 - 11X^2_1 - 10.67X^2_2 + 0.58X_1X_2$.

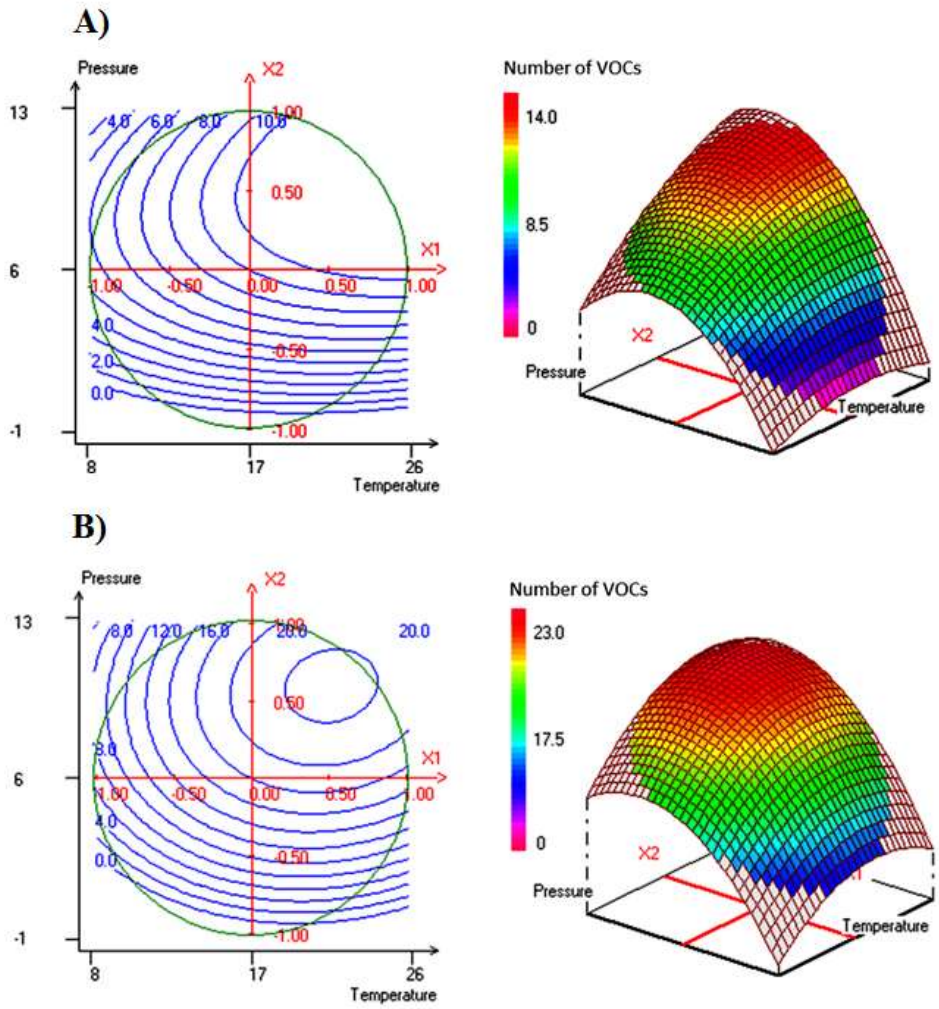


Fig. 3.3. *Variation of the number of VOCs (Y_1, Y_2) versus (X_1, X_2) in FAD method. A) - using FAD with CO_2, B) - using FAD with N_2O*

Two types of gases, N_2O and CO_2 (Y_1, Y_2) were used to obtain the responses for FAD method. Following the model established for response, we can represent graphically the surface of the corresponding responses. The values of the coefficients provide information on the influence of the factors and the curves obtained show the extreme or at least privileged directions (zones of interest of the factors). The number of VOCs (Y_1) and (Y_2) analysis of this response are presented in Fig. 3.3. These results indicate that the number of VOCs is very low at low temperatures (around 8 ºC) and high at high temperatures (around 26 ºC). The effect of pressure on these characteristics is noticeable only at high temperatures, especially in the case

of number of VOCs. Thus, at temperatures below 8 ºC the pressure did not affect the capacity of the VOCs, whereas at high temperatures, a small increase in pressure brings about a major increase in number of VOCs.

The optimal point using FAD with N_2O indicated by the model corresponds to a temperature of 19 to 26 °C and a pressure of 9 to 12 bar (Fig. 3.3). The response values of number of VOCs calculated from the model for these experimental conditions are given in Table 3.6. The center represents the optimum conditions in terms of temperature and pressure. The maximal efficiency is obtained for temperature 0.2 and pressure 0.5 in coded variable, i.e., 19 ºC and 10 bar in real variable. The analysis of the isoresponse curves at the chosen experimental field delimited by a circle show that the maximum of number of VOCs was obtained when the temperature and the pressure are increased.

The optimal conditions using FAD with CO_2 is obtained for temperature (-0.09-1) and pressure (-0.07-1) in coded variable, i.e., 16-26 ºC and 6-12 bar in real variable. Using FAD method with N_2O gas we obtained more volatile compounds, than in case of CO_2 (Table 3.5); consequently the release of VOCs in a new FAD method would be more efficient when N_2O gas is used, because the gas is aggressively attacking the cuticle of the multicellular headed glandular trichomes of the leaf.

3.2.1 Rapid evaluation of volatile compounds in fresh plants leaves by the two novel techniques Crushing Finger Device (CFD) and Flash Aroma Dispenser (FAD)

A decade ago, several attempts were performed by the companies of aromatic industry sector both in South-America and West-Africa in order to investigate the aroma potentials of rare exotic plants as a possible source of novel flavors and fragrances formulations. As Midi-Pyrenees region (Southwest of France) has a rich and poorly valorized wild vegetal heritage, especially regarding aromatic and medicinal plants, the studies were carried out in order to collect and analyze various regional so-called ‘medieval’ aromatic plants, e.g. *Achillea millefolium, Myrrhis odorata.* So far as many of such plants are rather rare or protected (their harvesting is problematic), the sampling of such plants becomes a limiting factor in their phytochemical investigations [177]. One of such plants is *C. grandiflora*, which can be

found in France, whereas in Lithuania it is very rare. The quantities of the available plants material are very important for the comprehensive analysis of their volatile compounds and further valorization. Therefore, new methods (techniques) for the release and collection of volatile compounds from small amounts of raw material are quite important. The essential oil components are being secreted by glandular trichomes present in the leaves. Therefore, before the analysis of aromatic extracts of plants, they have to be extracted and concentrated using a number of different methods for that purpose such as traditional hydrodistillation, steam distillation and solvent extraction techniques and some new methods. However, these extraction methods usualy require comparatively large amounts of raw materials. In addition, monoterpenes are known to be vulnerable to chemical changes during steam distillation conditions, and even conventional solvent extraction is likely to involve the losses of more volatile compounds during removal of the solvent [178].

During extraction process essential oil storage vesicles were exploded due to temperature and mechanical factors and consecutively aromatic compounds could be more easily extracted by water vapor. Florists and farmers are simulating this treatment when they crush between their fingers a couple of leaves or flowers followed by the sniffing of the generated odor in order to perform a rapid evaluation of the aromatic potential of such plants. Air dried leaves are usually subjected to hydrodistillation or steam distillation for about 3-8 hours. The losses of volatile compounds, long extraction times, degradation of unsaturated compounds and toxic solvent residue in the extract may be encountered with the current extraction methods. Hydrodistillation can thermally degrade and hydrolyze some of the oil components, which in some cases can lead to significant distortion of the composition of the oils naturally found in the intact plants.

In order to avoid degradation of volatile compounds a simple extraction technique, called Crushing Finger Device (CFD) method has been developed. The method developed is easy to perform using common laboratory equipment. In this method the sample is being crushed with an artificial finger. The advatages of CFD in comparison with the more traditional approaches are its high efficiency, low energy requirements and no use of solvent or boiling water. Another simple extraction method, so called Flash Aroma Dispenser (FAD) uses two types of gas, N_2O and CO_2. A strategic approach based either on the use of a specific/high effective sampling system with a classic detection (GC or GC-MS) was developed or tested in this study. CFD and FAD methods may become an important alternative to the

traditional extraction methods, since the above mentioned limitations can be overcome using this new techniques.

Previously published results indicate that the isolated and naturally present aromatic extracts may vary depending on their isolation and preparation method [179-181]. Therefore, it was interesting to compare the composition of *C. grandiflora* by using hydrodistillation and two new techniques, namely CFD and FAD.

The yield of *C. grandiflora* essential oil was 0.25±0.07%. It was reported previously that the yield of oil obtained by steam distillation from dried leaves of *C. grandiflora* was approximately 0.35% [134]. Table 3.7 lists the composition of *C. grandiflora* oil obtained by different methods according to the results of GC-MS. Twenty eight compounds in the *C. grandiflora* aromatic extracts have been identified, pulegone, isomenthone and neo-isomenthol being the main volatile components of *C. grandiflora* (Table 3.7).

Table 3.7. *Chemical composition of essential oil of* C. grandiflora *and volatile compounds isolated from the leaves by CFD and FAD methods*

No	Compounds			Peak area			
		RI[a]	RI[th]	Essential oil%	CFD%	FAD-N_2O%	FAD-CO_2%
1	Hexanal	802	802	-	1.48±0.00	0.69±0.00	-
2	*α*-Thujene	930	931	-	4.99±0.08	10.50±1.00	16.78±0.75
3	*α*-Pinene	934	939	0.03±0.00	0.94±0.00	0.63±0.00	1.14±0.00
4	Sabinene	973	975	0.19±0.01	5.69±0.65	3.50±0.05	10.90±0.90
5	*β*-Pinene	980	979	0.21±0.02	0.95±0.00	0.95±0.00	1.44±0.30
6	Myrcene	986	991	0.09±0.00	3.33±0.03	0.61±0.00	-
7	3-Octanol	993	991	0.25±0.00	0.35±0.00	0.07±0.00	0.80±0.00
8	*α*-Phellandrene	1004	1005	-	0.07±0.00	3.26±0.00	4.95±0.07
9	*α*-Terpinene	1018	1019	-	3.42±0.01	0.33±0.00	-
10	Limonene	1029	1029	0.71±0.03	1.14±0.00	1.11±0.06	1.14±0.00
11	*cis*-*β*-Ocimene	1033	1037	0.08±0.00	-	-	-
12	Terpinolene	1070	1089	0.03±0.00	0.75±0.00	1.62±0.10	2.81±0.00
13	Linalool	1099	1091	0.04±0.00	-	-	-
14	*p*-Menth-8-en-3-ol	1150	1150	0.24±0.01	-	-	-
15	Menthone	1158	1153	4.40±0.05	1.36±0.09	0.92±0.00	-
16	Isomenthone	1169	1162	34.07±0.00	15.99±0.15	8.35±0.70	4.61±0.60
17	Isomenthol	1181	1183	7.65±0.10	1.85±0.01	1.62±0.06	-
18	Neo-isomenthol	1193	1187	19.83±0.09	1.82±0.00	15.82±0.85	11.05±0.09
19	Pulegone	1243	1237	19.54±0.50	51.75±0.60	12.07±0.65	7.63±0.03
20	Piperitone	1255	1253	1.28±0.01	-	-	-
21	Isomenthyl acetate	1304	1305	0.03±0.00	-	-	-
22	Copaene	1362	1377	0.04±0.00	0.13±0.00	-	-
23	*β*-Bourbonene	1369	1388	0.13±0.00	0.24±0.00	-	-
24	*β*-Caryophyllene	1397	1409	1.99±0.04	1.23±0.02	0.42±0.00	0.42±0.00
25	Santalene	1443	1447	0.37±0.01	-	-	-
26	Germacrene-D	1477	1485	3.55±0.15	0.32±0.03	0.31±0.00	-
27	Bicyclogermacrene	1495	1500	1.78±0.03	-	-	-
28	*α*-Farnesene	1501	1506	0.11±0.00	0.55±0.00	-	-
29	*γ*-Cadinene	1518	1514	0.12±0.02	0.67±0.00	0.10±0.00	-
30	Isoeugenyl acetate	1578	1568	0.93±0.00	-	-	-
31	Caryophyllene oxide	1584	1583	0.43±0.00	-	0.20±0.00	-
32	*α*-Cadinol	1655	1654	0.2±0.00	-	-	-
	Total			98.32±0.00	99.02±0.00	63.08±0.00	63.67±0.00
Monoterpene hydrocarbons (%)				1.34	21.28	22.25	39.96
Oxygenated monoterpenes (%)				87.05	72.77	38.78	23.28
Sesquiterpene hydrocarbons (%)				8.09	3.14	0.83	0.42
Oxygenated sesquiterpenes (%)				0.63	-	0.20	-
Others (%)				1.21	1.83	1.02	-

a – Retention indices relative to C8–C22 alkanes on polar DB-5column.
th- relative retention indices (literature values [157]).

In general, the qualitative composition of *C. grandiflora* oils extracted by different methods is quite similar, whereas relative concentrations of the identified compounds are apparently different. Volatile constituents identified in the *C. grandiflora* belong to the terpenoid class of organic compounds. Hydrocarbon monoterpenes constituted in essential oil 1.34%; while

oxygenated monoterpenes were dominating (87.05%). Remarkably higher percentage of hydrocarbon monoterpenes was determined in *C. grandiflora* oil isolated by FAD (22.25-39.96%) and CFD (21.28%) techniques, than in the hydrodistilled essential oil (1.34%), whereas the content of oxygenated monoterpenes was higher in essential oil (87.05%) than in the volatiles isolated by FAD and CFD; 72.77%, and 38.78- 23.29%, respectively. The essential oil of *C. grandiflora* contained higher content of oxygenated monoterpenes than hydrocarbon monoterpenes, while the volatiles isolated using new FAD method contained almost similar amount of oxygenated monoterpenes and hydrocarbon monoterpenes, 23.28, 38.78% and 22.25, 39.96% respectively.

Some qualitative and quantitative differences between hydrodistilled oils and the products isolated by FAD and CFD were observed. Four volatile compounds, hexanal, *α*-thujene, *α*-phellandrene and *α*-terpinene which were identified in the volatiles isolated by CFD and FAD techniques were not detected in *C. grandiflora* hydrodistilled essential oil. On the other hand, 8 volatile compounds which were present in the hydrodistilled oil, namely linalool, *p*-menth-8-en-3-ol, piperitone, isomenthyl acetate, santalene, bicyclogermacrene, isoeugenyl acetate, *α*-cadinol, were not detected in the oils obtained by CFD and FAD methods. The differences might be due to the water and the thermal effect during hydrodistillation, because water is a polar solvent, which accelerates many reactions, especially reactions via carbocations as intermediates; hydrodistillation is also more aggressive method (Fig. 3.4). Another possible reason might be too little amounts of these compounds for GC/MS detection, which were released in CFD and FAD devices at the used parameters.

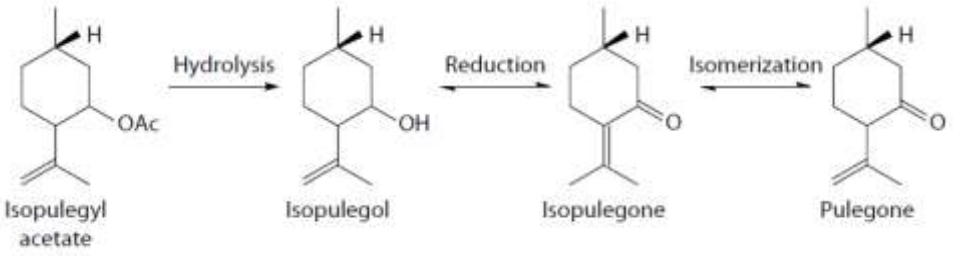


Fig. 3.4. *Hydrolysis of isopulegyl acetate*

In general, based on the composition of hydrodistilled and FAD-CFD-isolated oils the chemotype of analyzed *C. grandiflora* may be characterized

by the oxygenated *p*-menthane skeleton monoterpenes, namely menthone, isomenthone, isomenthol, neo-isomenthol and pulegone. Isomenthone and pulegone were the major constituents in *C. grandiflora* oil. The same structures were most abundant in Greek and previously studied French origin plants, however their percentages were different; pulegone in Greek *C. grandiflora* constituted 35.2%, menthone 20.2% and isomenthone 15.2% [134], while in the oil from France the contents of pulegone, isomenthone, neo-isomenthone and menthol were 27.6%, 24.7%, 23.7% and 10.8%, respectively [133]. Completely different *C. grandiflora* chemotype was reported in Kütahya Domaniç-Daritepe region of Turkey; isopinocamphone was the major compound constituting 52.6% in the total oil [132]. It is not surprising because chemical polymorphism is characteristic for many Lamiaceae family species. For instance, 5 chemotypes were identified for another Lamiaceae family species *Thymus pulegioides* previously; some of these chemotypes produced completely different terpene compounds, such as geraniol/geranial/neral, thymol and linalool [182]. In addition, the composition of essential oils varies significantly depending on annual climatic changes; therefore, even within the same chemotype, the remarkable variations in essential oil composition are possible. Each of two techniques developed (CFD and FAD) can be coupled with Fast-GC or GC in order to identify different chemotypes, to identify the key aromas compounds and to control the quality of plants.

3.2.2 Microscopic analysis of samples after CFD and FAD extraction

The investigation of the essential oils by direct sampling from secretory glands is of fundamental importance in studying the true essential oil composition of unharvested aromatic plants, since the usual applied techniques such as hydrodistillation and extraction are known to produce in some cases several artifacts. Essential oil is deposited on the surface of the plant inside fragile glandular trichomes. The plants emit volatiles from all tissues, although certain volatiles are evolved only from particular anatomical structures. Most volatile compounds are not emitted by all plant parts. Volatile emission rates are influenced by the resistance within the diffusive pathway from biosynthetic sites to the atmosphere. The physical path probably involves diffusion through cells, intercellular air spaces and passage through the stomata [183]. The secretion and subsequent volatilization of essential oils, which consists largely of terpene hydrocarbons, is generally associated with specialized secretory structures such as glandular trichomes of leaves.

In distillation processes, the fresh or dried aromatic plant material is contacted with vapour or boiling water, a process that results in the disruption of the glandular trichomes and, consequently, in the release of the volatile substances contained therein [184].

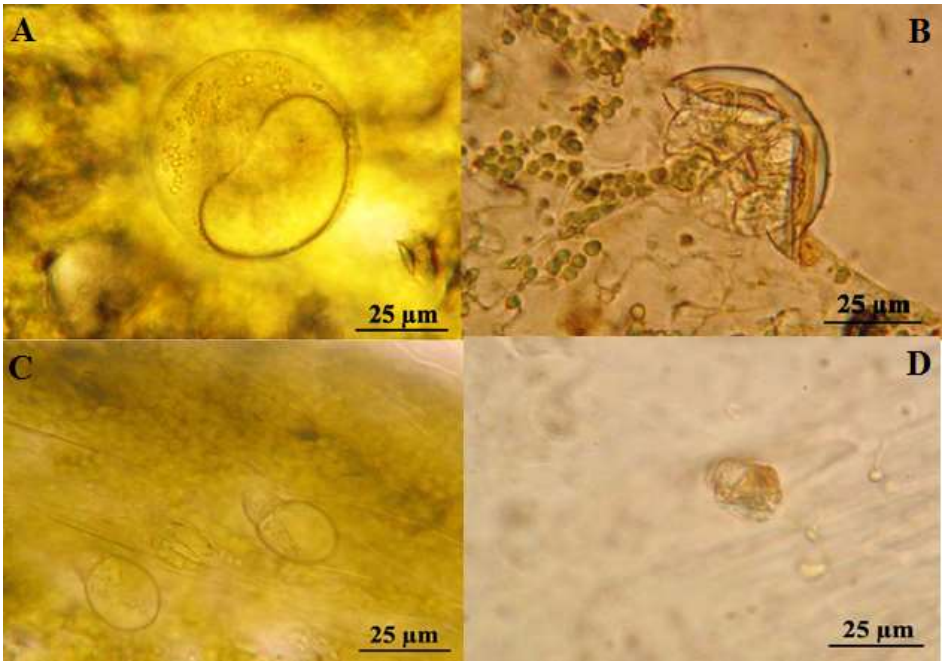


Fig. 3.5. *Microscopic analysis of* C. grandiflora*. glandular trichome. A and C before the experiment. B after affecting with CFD and D after affecting with FAD*

The CFD and FAD procedures studied in this work involve contacting a bed of intact herbaceous material with artificial finger and pressure with CO_2 or N_2O, thus disrupting the glandular trichomes in which the essential oils are encapsulated. As a result, the extraction of volatile compounds with CFD and FAD is often preceded by a mechanical treatment of the matrix in order to rupture these glandular trichomes. Glandular trichome before and after this fast chrushing and pressure showed evidence of disruption due to a bursting action from within the interior of the glands (Fig. 3.5). Two different extraction methods (CFD and FAD) produce distinguishable physical changes in the plant material. Fig. 3.5 compares scanning optical micrographs of *C. grandiflora*. The structures of the untreated plant material can be compared with those of the material treated by CFD or FAD. The untreated *C. grandilfora* leaf shows glandular trichomes which contain

volatile oils; amongst glandular trichomes, two types are recognized – unicellular and multicellular glandular trichomes. The capitate trichomes were situated on the adaxial and abaxial leaf surface. They consisted of a basal cell, a short mono or bicellular stalk and a rather large one or two celled secretory head. After CFD and FAD, cuticle and cell walls have been affected and it can be observed damages of the oil-containing glands. Glandular trichomes (Fig. 3.5 B and D) seem to be destroyed. The changes observed for CFD and FAD extractions showing clearly that the cells are broken and damaged during crushing and decompression treatment, but the mechanical strain induced by the rapid decompression in the surface tension of the glandular wall, causing it to crumble or rupture more readily, than pressure with CO_2 or N_2O.

3.3 ANTIOXIDANT ACTIVITY OF PLANT HYDRODISTILLATION RESIDUES

Valorization of plant residues after essential oil distillation is an important task. Dry herbal products are mainly composed of nondigestable carbohydrates and their derivatives incorporated into the cell walls, which in their native form are of a little value for food and/or nutraceutical applications. However, non-volatile secondary metabolites may be of interest for such applications and therefore one of the main tasks of this study was to assess antioxidant potential and the amount of polyphenolics in *C. grandiflora, M. odorata* and *T. farfara* hydrodistillation residue, which actually may be considered as some kind of "herbal soup"consisting of solid and liquid fractions. It should be said that in some cases deodorized plant extracts produced from the essential oil free material were reported as possessing higher antioxidant power than the extracts isolated from the whole material [86, 108]. The composition of hydrodistillation fractions will depend on the solubility of plant constituents in boiling water, which is a high polarity solvent. The results of RSC of extracts in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ reactions as well as their reducing power in FRAP assay are listed in chapter 3.3.1, 3.3.2 and 3.3.3. The RSC measurements in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays are easy, rapid and sensitive methods and therefore most frequently applied for the preliminary assessment of antioxidant potential of various natural substances. Although the basic principles are similar, the $ABTS^{\bullet+}$ scavenging assay is preferable for its ability to evaluate RSC of both lipophilic and hydrophilic antioxidants.

3.3.1 Antioxidant properties of deodorized residues extracts of C. grandiflora in electron/hydrogen transfer based assays

The model reaction systems containing stable $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and reducing power in FRAP were used to assess radical scavenging capacity (RSC) of deodorized *C. grandiflora* extracts. These methods are simple and widely used for the fast screening of plant antioxidant properties and they provide quite reliable preliminary information on the presence of antioxidatively active constituents in the extracts.

The concentration necessary to decrease the initial concentration of $DPPH^{\bullet}$ by 50% (IC_{50}) was chosen to compare RSA of different extracts (Fig. 3.6). A low IC_{50} is the result of high radical scavenging effect.

IC_{50} values of *C. grandiflora* extracts (solid residue) in $DPPH^{\bullet}$ assay varied from 1.10 (AE) to 0.23 (ME) mg/mL (Fig. 3.6), while TEAC values in $ABTS^{\bullet+}$ reaction were from 0.11 (AE) to 0.66% (EE) (Fig. 3.7). It is evident that lower polarity acetone (AE) extract was several times weaker radical scavenger than polar alcoholic (ME, EE) extract. It is in agreement with many previously published results which showed that polar solvents extract more antioxidants from botanicals than lower polarity solvents [86, 108, 185]. Methanol and ethanol as organic solvents are quite similar, however, the EE extract was stronger radical scavenger than ME in $ABTS^{\bullet+}$ reaction. Moreover, ethanol is a food grade solvent and from this point of view is preferable to the toxic methanol. It is also interesting noting that in $DPPH^{\bullet}$ reaction the RSC of alcoholic extracts obtained from the solid residue were quite similar to the RSC of water extracts, 0.23 (ME), 0.22 (EE), 0.22 (FD) and 0.18 (SD) mg/mL, respectively. Meaning that, some antioxidativey active compounds do not dissolve in boiling water and still remain in the solid residue. FRAP assay is a versatile method and can be readily applied to both aqueous, alcohol and acetone extracts of different plants. In this assay, the antioxidant activity is determined on the basis of the ability to reduce ferric (III) iron to ferrous (II) iron and the results are expressed in mg ferrous iron equivalents per mL of sample.

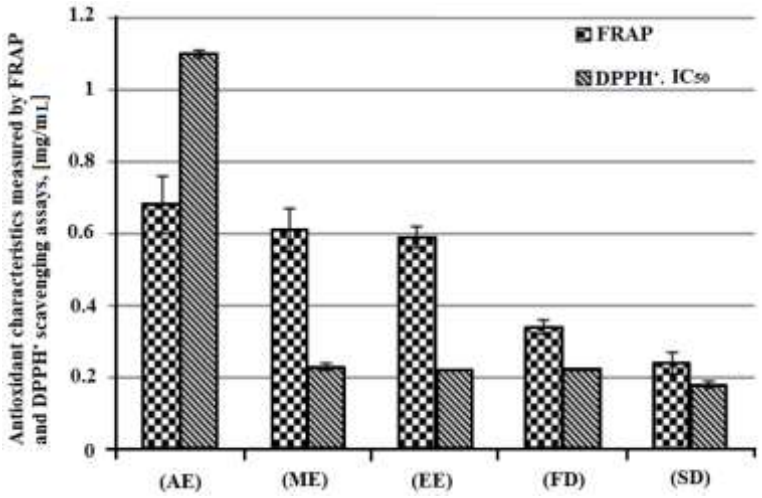


Fig. 3.6. *Ferric reducing/antioxidant power (FRAP) and DPPH radical scavenging assay of* C. grandiflora

In FRAP assay a ferric salt (Fe III) is used as an antioxidant and its redox potential (0.70 V) is comparable to that of $ABTS^{\bullet+}$ (0.68 V), therefore there is not much difference between TEAC assay and FRAP assay [186]. However, the results obtained by these two assays for C. *grandiflora* extracts were rather different, which shows that the assay type plays a most important role in antioxidant activity measurements. The ferric reducing power of different extracts was in the range of 0.24–0.68 mg Fe (II)/mL (Fig. 3.6) and the most remarkable difference was observed for the AE, which was several times weaker antioxidant in TEAC assay (Fig. 3.7), while in FRAP assay AE was the strongest reducing agent. ME and AE produced from the solid hydrodistillation residue of plant were remarkably stronger antioxidants than dried water extracts. The observed differences between $DPPH^{\bullet}/ABTS^{\bullet+}$ and FRAP methods can be explained by different solvent polarities and pH in the reaction media [187].

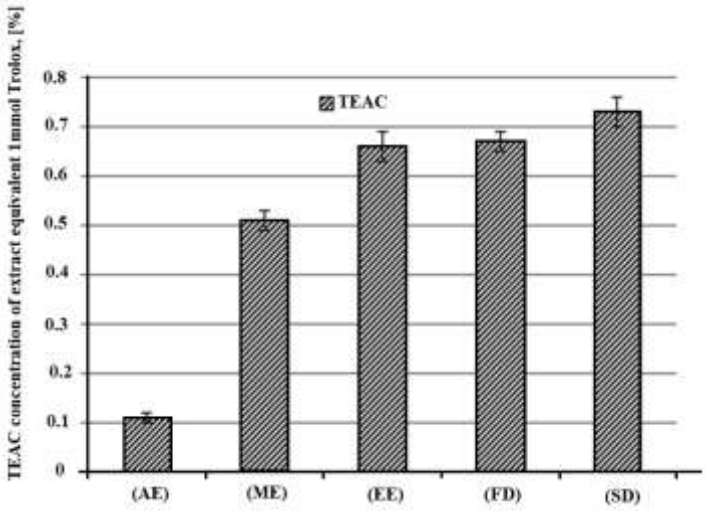


Fig. 3.7. *TEAC values in ABTS radical cation decolourisation assay of* C. grandiflora

3.3.2 *Antioxidant properties of deodorized residues of* M. odorata *from two different geographical origins in electron/hydrogen transfer based assays*

The soil, climate and altitude may be affected on the antioxidant activity; therefore the aim of this study was to determine the dependence of antioxidants properties and plant origin.

Antioxidant analysis shows that all extract plant residues samples were antioxidatively active; however, their RSC varied in a wide range (Fig. 3.8). The RSC of French (FR) origin extracts was from 0.12±0.00 to 0.83±0.08 mg/mL in DPPH$^{\bullet}$ test and from 0.02±0.00 to 0.79±0.07 mg/mL in FRAP reaction system, while that of Lithuanian (LT) plant extracts varied from 0.11±0.00 to 0.98±0.08 mg/mL in DPPH$^{\bullet}$ test and from 0.14±0.01 to 0.99±0.02 mg/mL in FRAP reaction system (Fig. 3.8).

In DPPH$^{\bullet}$ test, *M. odorata* AE and ME extracts from FR grown plants shows little better antioxydant activity than those of the same botanical source but from different geographical origin (LT), while FRAP test show that LT ME, EE and WE extracts have better antioxidant activity compared to those from FR country. Based on the result obtained from DPPH$^{\bullet}$ analysis, it is important to notice that there is not significant similitude between the same plant from different climate and country (LT and FR), but only important

difference is obtained by using different solvents extraction. On the other hand, the results obtained from the FRAP analyses, shows that the plants located in LT has more antioxidant activity than located in the Midi-Pyrénées zone (FR), exept AE extract.

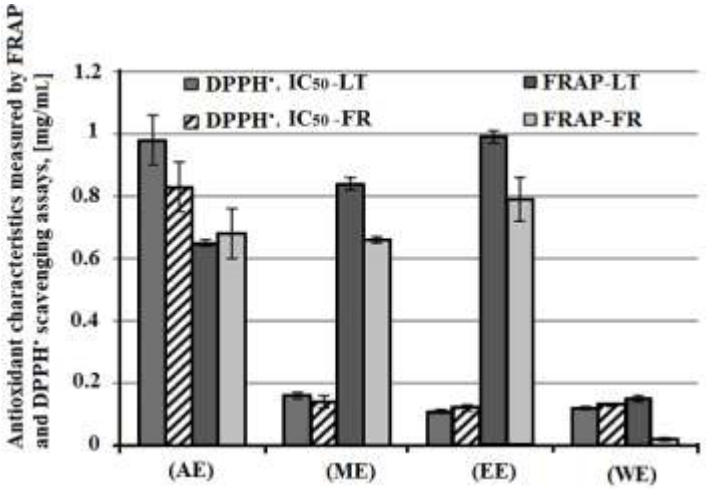


Fig. 3.8. *Ferric reducing/antioxidant power (FRAP) and DPPH radical scavenging assay of* M. odorata *from Lithuania and France*

The previous data demonstrated that measurement of RSC in model system is a reliable method to assess antioxidant properties of plant extracts. However, it is known that antioxidants act very differently with various free radicals. In order to obtain more information about radical scavenging properties of *M. odorata* extracts, we introduced the second model system ($ABTS^{\bullet+}$) to test RSC. ABTS radical cation decolourisation assay, showed similar results compared to those obtained in $DPPH^{\bullet}$ reaction. EE and ME extracts were stronger radical scavengers than AE extract in the $ABTS^{\bullet+}$ assay. The concentrations of extracts were equivalent to the activity of 1mmol of Trolox solution in $ABTS^{\bullet+}$ reaction are given in Fig. 3.9.

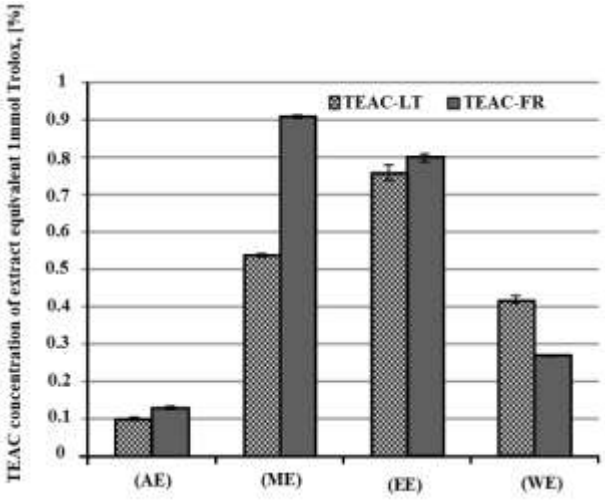


Fig. 3.9. *TEAC values in ABTS radical cation decolourisation assay of* M. odorata *from Lithuania and France*

The strongest antioxidant activity was demonstrated by EE (from 0.76 to 0.8%) and ME (from 0.54 to 0.91%) extracts. All extracts were remarkably stronger antioxidants than AE extracts in $DPPH^{\bullet}$, FRAP and $ABTS^{\bullet+}$ reaction systems. Comparing different origins, the RSC in $ABTS^{\bullet+}$ reaction systems we can see the FR origin extracts were stronger, than the extracts from LT plants materialexcept WE extract. But in $DPPH^{\bullet}$ assay, the RSC of EE and WE extracts was slightly stronger than that of ME, while AEs were approximately 6-9 times weaker radical scavengers than polar extracts (Fig. 3.8). So far as polar phenolic constituents are dominant antioxidants in the majority medicinal and aromatic plants, this result is in agreement with many previously performed studies [188]. Preliminary screening of antioxidant potential indicate that non-volatile fraction of *M. odorata* contain relatively high amount of phenolic compounds which might be of interest for the isolation of valuable food and or nutraceutical ingredients. It also showed that phytochemical composition of plant's secondary metabolites depends on several factors, such as cultivation area, climatic conditions, vegetation phase, genetic modifications and others; therefore, evaluation of plant properties from different geographical regions is an interesting task, which was in the focus of numerous studies [188-189].

3.3.3 *Antioxidant activity in different anatomic parts of* T. farfara *from two different geographical origins*

Although some pure compounds such as a number of phenolics, mucopolysaccharides and water-soluble polysaccharides have been isolated from the flower buds of *T. farfara* [151], no flavonoids have been reported. So far, there are no studies performed on the *T. farfara* residues after plant hydrodistillation. However, these deodorized residues may contain a panel of bioactive molecules, previously not determined and therefore not used. Recent developments in green chemistry and speculation on plant material for industrial uses led to exploit plant residues after extraction and transformation process. Latest studies have proved that some species may contain a lot of bioactive molecules such as antioxidants [188]. There is no information on antioxidant activities in *T. farfara* deodorized residues and different anatomical parts of this plant. Therefore, it was interesting to characterize the antioxidant activities of *T. farfara* deodorized residues obtained after flowers-stems (FS) hydrodistillation and extracts of leaves (L) and roots (R) for inexpensive exploitation. The antioxidant activity in different anatomical parts, FS, L and R of *T. farfara* plants of two different origins were analyzed in our work.

The IC_{50} values for DPPH radical scavenging activity of *T. farfara* anatomical parts were the lowest for FR R AE extract (0.88 mg/mL), and LT R AE extract (0.54 mg/mL), the highest antioxidant activity were in FR L AE extract (0.05 mg/g) (Fig.3.10).

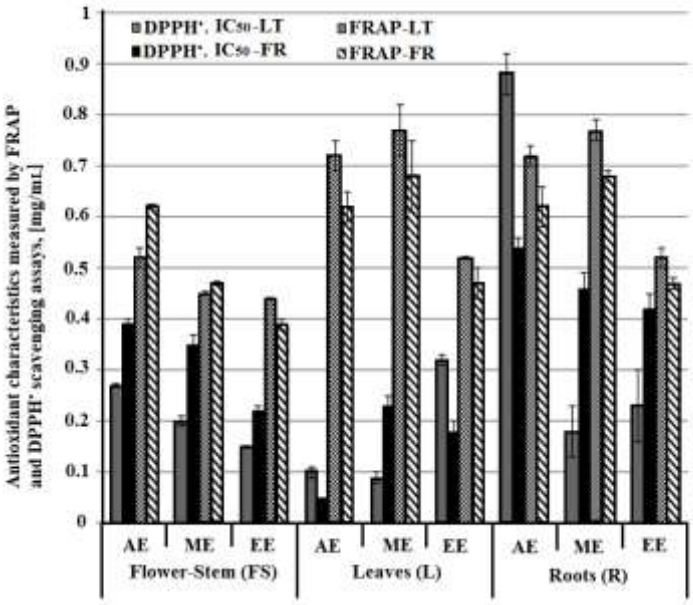


Fig. 3.10. *Ferric reducing/antioxidant power (FRAP) and* DPPH *radical scavenging assay of different anatomical parts of* T. farfara *from Lithuania and France*.

IC_{50} values of *T. farfara* FS, L and R extracts in $DPPH^{\bullet}$ assay varied from 0.09 (L ME) to 0.88 (R AE) mg/mL in Lithuanian extracts and from 0.05 (L AE) to 0.54 (R AE) mg/mL in FR extracts. As expected, low polarity AE extracts FS and R were weaker radical scavengers than polar alcoholic extracts in $DPPH^{\bullet}$ reaction that is in agreement with many previous reports [86, 108, 185]. In contrast, another antioxidant activity screening method, applicable for both lipophilic and hydrophilic antioxidants $ABTS^{\bullet+}$ decolourisation assay, exhibited rather inverse range compared to those obtained in $DPPH^{\bullet}$ reaction (Fig. 3.11). For TEAC values in $ABTS^{\bullet+}$ reaction AE (FS) and roots (R) extracts were the most effective ABTS radical scavengers, than FS ME, R ME and FS EE, but R EE extract showed higher activity, than R AE. The main difference is that $DPPH^{\bullet}$ can be only dissolved in organic solvents (e.g., methanol), while $ABTS^{\bullet+}$ is soluble in both aqueous and organic media. Therefore, the $ABTS^{\bullet+}$ test can be performed in hydrophilic and lipophilic systems [165].

The ferric reducing power (FRAP) assay of the different parts of the plant extracts was in the range of 0.18-0.77 mg Fe (II)/mL in LT extracts and 0.15-0.68 mg Fe (II)/mL in FRh extracts. Antioxidant activity of FS extracts

measured by FRAP reaction showed the same relationships as did $ABTS^{\bullet+}$. In FRAP assay a ferric salt (Fe III) is used as an antioxidant and its redox potential is comparable to that of $ABTS^{\bullet+}$, therefore the results of TEAC and FRAP assays showed similar trends as previously reported [186]. FS AE extracts were remarkably stronger antioxidants than other (FS ME and FS EE) extracts in $ABTS^{\bullet+}$ and FRAP reaction, but for leaves extracts relationships between $ABTS^{\bullet+}$ and FRAP did not find, LT L EE showed higher activity in $ABTS^{\bullet+}$ and lowest in FRAP reaction, the observed difference can by explained by different pH in the reaction media (pH_{FRAP}=3.6 and pH_{ABTS}=7.4).

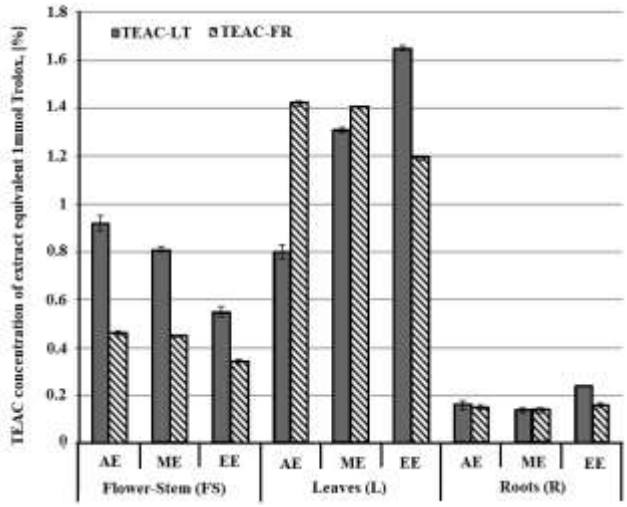


Fig. 3.11. *TEAC values in ABTS radical cation decolourisation assay of different anatomical parts of* T. farfara *from Lithuania and France*

Comparing antioxidant activity indicators we observed that the *T. farfara* extracts isolated from LT and FR origin were different. The differences in climatic conditions (the amount of rainfall during plant development was nearly three times less abundant in Lithuania (340 mm) than in France (971 mm), the average temperature was also lower in Lithuania and it might have the impact on these differences; however, other factors, such as soil, sunlight may also have the impact on plant properties and composition. Determination of the effects of these factors on *T. farfara* properties would require special experimental design which was beyond the scope of this study.

3.4 CONTENT OF TOTAL PHENOLIC COMPOUNDS (TPC), FLAVONOIDS AND FLAVONOLS

Phenolic components are very important plant constituents with radical scavenging ability due to their hydroxyl groups. It has been established that phenolic compounds are the major plant constituents with antioxidant activity that results from their redox properties. Phenolic compounds are a class of antioxidant agents which can adsorb and neutralize the free radicals [190]. Application of Folin-Ciocalteu reagent gained popularity and is commonly known as the total phenols assay, although the standard reagent used in this method measures a sample‘s reducing capacity [186]. The content of phenolic compounds was determined from regression equation of a calibration curve ($y=8.3166x + 0.2207$, $R^2=0.9985$) and expressed in gallic acid equivalents (GAE) in *C. grandiflora, M. odorata* and *T. farfara* extracts.

Table 3.8. *Total amount of plant phenolic compounds, flavonoids and flavonols*

Plant	Part of the plant	Extraction solvent	Total phenolic compounds (TPC), mg GAE/g		Total flavonoids mg/g plant extract (in RE)		Total flavonols mg/g plant extract (in RE)	
			LT	FR	LT	FR	LT	FR
C. grandiflora	Leaves (L)	Acetone (AE)	-	28.01±0.12	-	1.80±0.03	-	1.18±0.01
		Methanol (ME)	-	64.80±0.22	-	4.30±0.09	-	0.88±0.02
		Ethanol (EE)	-	82.80±1.02	-	2.44±0.17	-	0.75±0.01
		Water (FD)	-	58.90±0.72	-	0.79±0.05	-	0.03±0.00
		Water (SD)	-	72.78±1.11	-	1.24±0.05	-	0.10±0.01
M. odorata	Leaves (L)	Acetone (AE)	10.78 ± 0.02^{a}	39.19 ± 0.32^{b}	1.87 ± 0.08^{a}	2.07 ± 0.04^{b}	0.27 ± 0.02^{a}	0.87 ± 0.09^{b}
		Methanol (ME)	62.32 ± 0.08^{a}	85.25 ± 1.32^{b}	1.63 ± 0.02^{a}	9.61 ± 0.78^{b}	1.36 ± 0.01^{b}	0.61 ± 0.04^{a}
		Ethanol (EE)	80.60 ± 0.97^{b}	68.97 ± 0.30^{a}	4.00 ± 0.31^{a}	4.15 ± 0.71^{a}	0.98 ± 0.02^{b}	0.75 ± 0.08^{a}
		Water (WE)	35.55 ± 0.06^{a}	55.03 ± 0.04^{b}	0.36 ± 0.02^{b}	0.23 ± 0.06^{a}	0.34 ± 0.01^{b}	0.08 ± 0.00^{a}
T. farfara	Flower and Stem (FS)	Acetone (AE)	94.79 ± 0.35^{a}	123.16 ± 0.12^{b}	9.20 ± 0.17^{a}	9.18 ± 0.03^{a}	0.70 ± 0.01^{b}	0.49 ± 0.01^{a}
		Methanol (ME)	94.55 ± 0.22^{b}	66.22 ± 0.30^{a}	5.95 ± 0.03^{a}	5.12 ± 0.09^{a}	0.53 ± 0.02^{b}	0.27 ± 0.02^{a}
		Ethanol (EE)	66.45 ± 0.15^{b}	48.79 ± 0.22^{a}	3.25 ± 0.09^{b}	2.20 ± 0.07^{a}	0.39 ± 0.01^{b}	0.19 ± 0.01^{a}
	Leaves (L)	Acetone (AE)	102.00 ± 0.02^{a}	151.36 ± 1.87^{b}	7.91 ± 0.30^{a}	8.21 ± 0.06^{a}	0.96 ± 0.03^{b}	0.86 ± 0.02^{a}
		Methanol (ME)	144.33 ± 1.20^{a}	204.57 ± 1.54^{b}	12.16 ± 0.15^{b}	11.02 ± 0.30^{a}	1.73 ± 0.02^{b}	0.82 ± 0.03^{a}
		Ethanol (EE)	157.25 ± 0.08^{b}	131.88 ± 0.99^{a}	10.96 ± 0.15^{b}	7.81 ± 0.30^{a}	1.98 ± 0.05^{b}	0.42 ± 0.02^{a}
	Roots (R)	Acetone (AE)	6.06 ± 0.01^{a}	13.58 ± 0.01^{b}	0.47 ± 0.02^{b}	0.29 ± 0.02^{a}	0.25 ± 0.01^{b}	0.01 ± 0.00^{a}
		Methanol (ME)	41.08 ± 0.78^{a}	41.64 ± 0.03^{a}	0.35 ± 0.01^{b}	0.22 ± 0.00^{a}	0.17 ± 0.01^{b}	0.14 ± 0.00^{a}
		Ethanol (EE)	38.87 ± 0.65^{a}	41.30 ± 0.06^{b}	0.47 ± 0.03^{b}	0.22 ± 0.02^{a}	0.19 ± 0.02^{b}	0.12 ± 0.00^{a}

Superscript letters (a–b) indicate if the values for the extracts isolated with the same solvent from Lithuanian and French origin plants are statistically different ($P \leq 0.05$); Values are mean ± SD of triplicate measurements.

In *C. grandiflora* extracts it was from 28.0±0.12 in AE to 82.8±1.32 mg GAE/g in EE (Table 3.8). The concentration of TPC was lower in FD samples than in SD. Most likely, the reason for this finding is that the yield of FD (8.20±0.25% (w/w)) was higher than SD (5.15±0.10% (w/w)) and phenolic compounds in FD were more diluted. It is in agreement with previously obtained results for black currant buds [188]. It can be observed that the content of phenolics in the extracts correlates with their antiradical activity; strong correlation was observed between total phenolics and $DPPH^{\bullet}$ (R^2=0.8204) and $ABTS^{\bullet+}$ scavenging capacity (R^2=0.8023), confirming that phenolic compounds are important contributors to the antioxidant properties of these extracts. However, the correlation between TPC and FRAP was very weak (R^2=0.157), which suggests that in FRAP assay nonphenolic compounds may play more important role.

Consequently, *M. odorata* extracts isolated from the essential oil distilation by-products, particularly by such polar solvents as ethanol, methanol and water might be promising sources of natural antioxidants. The content of TPC extracted from *M. odorata* was solvent dependent and varied in the range of 10.78±0.02 to 85.25±1.02 mg GAE/g (Table 3.8). The content of polyphenols isolated by different solvents decreased in the following order: ME > EE > WE > AE for FR *M. odorata* and EE > ME > WE > AE for LT *M. odorata*. EE and ME are quite similar protonic solvents; however both of them were tested because EE is more acceptable for the isolation of food grade ingredients. The differences in the TPC content in EE and ME between LT and FR origin plants were not remarkable, however, the biggest sum of TPC in FR *M. odorata* (ME+WE+AE=179.47 mg GAE/g) was higher than in LT *M. odorata* (EE+WE+AE=126.93 mg GAE/g). It is interesting to note that lower polarity phenolics extracted with acetone were almost 4 times more abundant in FR *M. odorata* than in LT *M. odorata*.

The *M. odorata* water extracts have a strong hydroxyl radical inhibitory activity relative to the other investigated extracts, whereas its phenol content is lower, according to Table 3.8. This finding showed that the content of total phenolics in herbs is not a reliable indicator of their antioxidant activity. The structures of the individual constituents need to be elucidated and assessed in order to obtain more precise results and information. To support of this argument, we compared the TPC with RSC. Thus, the correlation between the RSC and TPC of the ten samples was studied using a linear regression analysis. The correlation coefficient between TPC and FRAP values (R^2=0.283), $DPPH^{\bullet}$ scavenging activity (R^2=0.3078), and $ABTS^{\bullet+}$ assay (R^2=0.7006). Usually the TPC are in a strong correlation with RSC, however in our study of *M. odorata* this correlation was weak, less than 0.8. For instance, the content of TPC in WE and AE of FR *M. odorata* was remarkably higher that in LT *M. odorata*, while the differences in the RSC between the extracts isolated from the two different origin plants with the same solvents were negligible (Fig. 3.8). EE and ME contained high amount of TPC and showed very good RSC, while the content of TPC in WE, which also possessed good RSC was remarkably lower than in EE and ME extracts.

The correlation between phenols content and these two $DPPH^{\bullet}$, FRAP assays was found to be very weak, less than 0.5. These low correlation values between total phenols and the antioxidative activity suggest that the major antioxidant compounds in studied extracts might be nonphenolics. TPC in different anatomical parts, L, FS and R were determined of *T. farfara* from two origins. Various solvents (ethanol, methanol and acetone) were used to achieve extraction of active substances with diversity in their polarity. The highest concentration of TPC in FS extracts of *T. farfara* were in AE followed by ME and EE except for ME isolated from *T. farfara* from LT; it contained similar amount of TPC to AE. It is

interesting noting that AE of FR origin plants contained higher amount of TPC than AE of LT origin *T. farfara*, while the content of TPC in ME and EE was higher in LT origin plants. However, the sums of TPC in AE+ME and AE+EE were similar (189.4 mg GAE/g) or very close (161.2 and 171.0 mg GAE/g) for both origin plants.

The concentrations of TPC in L and R extracts were inverse than in FS extracts. The highest concentration of TPC in the LT *T. farfara* was L EE extract, but for the FR origin was L ME extract. The ME extracts of R have higher concentration of TPC, than EE and AE extracts. However, the sums (AE+ME+EE) of TPC in different anatomical part of *T. farfara* were very different, for LT and FR FS were (255.9 and 238.2 mg GAE/g), but for L the TPC were two time highest (403.6 and 487.8 mg GAE/g) respectively. Therefore, high amount of TPC in L extracts showed very good RSC, while the content of TPC in FS and R extracts, possessed lower RSC.

So far as acetone and alcohol were used as consecutive extraction solvents it may be suggested that individual phenolic compounds are present in the plants of two origins at different proportions. Results of the present study showed that acetone does not provide exhaustive extraction of phenolic compounds; polar solvent should be applied as a second solvent. According to our study, the high content of these phenolic compounds in AE FS extract of *T. farfara* could explain their high $ABTS^{\bullet+}$ scavenging capacity and FRAP. This reducing capacity of the extracts may serve as an indicator of potential antioxidant activities through the action of breaking the free radical chain by donating hydrogen atom [191].

It can be observed that the content of phenolics in the FS, L and R extracts correlates with their antiradical activity; strong correlation was observed between total phenolics and FRAP in FS and R extracts (R^2=0.8341 and R^2=0.7935 respectively), also we found correlation between TPC and $DPPH^{\bullet}$ for R extract (R^2=0.6969) and TPC with $ABTS^{\bullet+}$ assay in L extracts (R^2=0.553), confirming that phenolic compounds are important contributors to the antioxidant properties of these extracts. However, the correlations between TPC, $DPPH^{\bullet}$, $ABTS^{\bullet+}$ in FS extracts and $DPPH^{\bullet}$, FRAP in L extracts were very weak, less than 0.5.

Flavonoids are important group of phenolic compounds; therefore, their concentration was assessed separately, by exploring the ability of flavonoids to form yellow colour chelate type internal complexes with Al^{3+}, which may be measured spectrophotometrically.

The content of flavonoids, in rutin equivalents (RE), of *C. grandiflora* varied from 0.79 (in FD extract) to 4.30 mg (rutin)/g (in ME extract). The concentration of flavonoids was higher in alcoholic extracts than in AE. Relatively low amounts of flavonoids were determined in aqueous extracts of *C. grandiflora*, which contained high amount of TPC and the differences between FD and SD again may be explained by the differences in extract yield. There was no correlation between the amount of flavonoids and RSC.

The content of flavonols was also estimated spectrophotometrically and it was found that the concentration of this class of compounds varied from 0.03 to 1.18 mg RE/g of plant extract (regression equation of a calibration curve y=6.0727x + 0.0504, R^2=0.9929). The highest amounts were measured in the AE extract, while in water extracts this class of compounds was present in trace concentrations. Flavonols are known as important compounds in terms of radical scavenging properties. It may be observed that the extract with higher content of flavonols showed lower IC_{50} values in $DPPH^{\bullet}$ assay.

The total flavonoids and the flavonol subclass of the *M. odorata* plant extracts were also measured. EE extracts had the highest flavonoid contents 4.00±0.31 and 4.15±0.71 mg RE/g dry weight plant extract, respectively, whereas WE extracts although demonstrating high RSC contained the lowest contents of flavonoids, phenolics and flavonols. It is noteworthy that, apart from the phenolic compounds, there may be other secondary metabolites in plants that could be responsible for antioxidant activities. The *M. odorata* water extracts have a strong hydroxyl radical inhibitory activity relative to the other investigated extracts, whereas its phenol content is lower, according to Table 3.8. This finding showed that the content of total phenolics in herbs is not always a reliable indicator of their antioxidant activity

The amounts flavonoids and flavonols extracted from different anatomical part and origins of *T. farfara* were analyzed. The highest amounts flavonoids and flavonols were found in LT origin plants for all their anatomical parts than in FR origin. It is important to note that the climatic conditions in the countries were very different; mean temperature and rainfall amount during the month of collection were higher in Midi-Pyrenées-France (10.5°C and 97.1 mm) than in Kaunas-Lithuania (6.9°C and 34.0 mm). These factors may be among the reasons of the differences in chemical composition.

High concentrations of flavonoids were determined in FS and L extracts, than R extract. The highest flavonoid content was found in the AE extract from the FS (9.20 and 9.18 mg RE/g) and in the ME extracts from the L (12.16 and 11.02 mg RE/g).

However, the content of total flavonoids and flavonols measured by using spectrophotometric method [192] cannot be directly used as a reliable indicator of the total antioxidant capacity. The structures of individual constituents need to be elucidated and assessed in order to obtain more comprehensive information.

3.5 ANTIOXIDANT ACTIVITY OF EXTRACTS IN SUNFLOWER (*HELIANTHUS ANNUUS*) OIL

Numerous plant extracts have been studied in various lipid systems. The results of these studies showed that there is further need for measurements of plant composition and properties; in many cases data obtained are still insufficient and sometimes contradictory, depending on the origin of primary plant material, extraction solvent, lipid substrate and method chosen for stability tests. Recently, many antioxidants were isolated from aromatic and medicinal plants [193-196]. The primary antioxidant activity analysis data obtained in sunflower oil are presented in this chapter. Sunflower oil is largely used in Europe. It is rather unstable because of the presence of a substantial amount of α-linolenic acid (60-65%) and can be used for the assessment of antioxidant properties of plant extracts. The antioxidant activity of various plant extracts in sunflower oil has been studied previously. Rosemary, sage, nutmeg, clove and allspice extracts showed their capacities to stabilize sunflower oil [97, 197-198]. Edible oils containing high percentage of polyunsaturated fatty acids are very sensitive to auto and photooxidation. So far as autooxidation proceeds via radical formation chain reaction, the compounds able to transfer electron and/or hydrogen may retard the process of lipid oxidation. Various methods exists to assess the effects of antioxidants on lipid oxidation; however, the Oxipres method, which was used to evaluate the antioxidant activity (AA) of extracts in our study, is a very convenient procedure as it is performed without using any chemicals. The change of oxygen pressure in the reaction vessel at the end of the induction period indicating rapid formation of hydroperoxides can be quite precisely measured. This is measured from the cross-section point of tangents of the first part and the subsequent part of

the curve recording the pressure changes. For edible oils analysis, the optimum conditions are: 5.0 g samples; initial pressure 0.5 MPa; temperature 100°C [198].

Antioxidant activity of *C. grandiflora* essential oil was reported previously and PF at the concentration of 0.2% was 1.63 [177]. In case of adding plant extracts at 0.05, 0.1 and 0.2% concentrations to sunflower oil, the pressure drop was delayed for some period of time, indicating the antioxidative effect of the added substance (Table 3.9). The effect of 0.2% EE extract on the stability of sunflower oil during accelerated oxidation was twice lower as compared with 0.02% butylated hydroxytoluene (BHT). Different concentrations of each extract were tested to receive more information about antioxidant properties of *C. grandiflora* extracts in sunflower oil. In general, all extracts showed oil stabilizing effect, which increased with increasing concentration of the extract in the oil. The effectiveness of the 0.2% concentration of plant extracts decreases in the following order: EE > ME > SD > AE > FD. However, the antioxidant activity of *C. grandiflora* EE was most effective and considerably exceeded the effect of other used herbal (*M. odorata* and *T. farfara*) extracts. However, all *C. grandiflora* and *T. farfara* extracts possessed slightly higher positive effect on such characteristics as IP, PF and AA as compared with *M. odorata.* For instance, 0.05% addition of AE, ME, EE and WE of LT and FR origin *M. odorata*, prolonged induction period by 2.5%, 0.5%, 1% 9.5% and 3.5%, 0%, 3.5%, 14.5% respectively as compared with the blank samples (Table 3.9). The changes of IP in sunflower oil after addition of *M. odorata* were not considerable; e. g. IP of the sample with 0.05% of *M. dodrata* ME was almost equal to IP of the sample without additives. It should be noted that so far as plant extracts are very complex mixtures consisting of various compounds the concentration of pure compound, BHT in oil was 2.5 times lower (0.02%) than the concentration of the extracts (0.05%), but synthetic (BHT) antioxidant was stronger, than plant extracts.

Table 3.9. *Antioxidant activity (AA) of the extracts of* C. grandiflora, M. odorata and T. farfara *as compared with the effect of BHT and their effect on the stability of sunflower oil, expressed in protection factor values (PF)*

			Plant origin					
Plant	**Solvent**	**Conc**	Lithuania	France	Lithuania	France	Lithuania	France
		%	**IP**		**PF**		**AA**	
C. grandiflora	AE	0.20	-	2.68±0.01	-	1.34±0.00	-	0.35±0.00
		0.10	-	2.72±0.02	-	1.36±0.01	-	0.33±0.00
		0.05	-	2.48±0.00	-	1.24±0.00	-	0.23±0.00
	ME	0.20	-	2.81±0.01	-	1.41±0.01	-	0.39±0.00
		0.10	-	2.71±0.03	-	1.36±0.02	-	0.34±0.01
		0.05	-	2.58±0.02	-	1.29±0.01	-	0.28±0.00
	EE	0.20	-	2.98±0.01	-	1.49±0.00	-	0.47±0.00
		0.10	-	2.93±0.03	-	1.47±0.02	-	0.45±0.01
		0.05	-	2.78±0.00	-	1.39±0.00	-	0.38±0.00
	FD	0.20	-	2.21±0.01	-	1.31±0.00	-	0.29±0.00
		0.10	-	2.02±0.01	-	1.27±0.00	-	0.26±0.00
		0.05	-	2.01±0.00	-	1.15±0.00	-	0.14±0.00
	SD	0.20	-	2.78±0.03	-	1.39±0.02	-	0.38±0.01
		0.10	-	2.51±0.02	-	1.26±0.01	-	0.25±0.00
		0.05	-	2.37±0.01	-	1.19±0.00	-	0.18±0.00
M. odorata	AE	0.20	2.17±0.03	2.19±0.01	1.09±0.02	1.10±0.01	0.08±0.02	0.09±0.01
		0.10	2.09±0.02	2.11±0.01	1.05±0.01	1.06±0.01	0.04±0.01	0.05±0.01
		0.05	2.05±0.02	2.07±0.01	1.03±0.01	1.04±0.01	0.02±0.01	0.03±0.01
	ME	0.20	2.19±0.01	2.10±0.01	1.10±0.00	1.05±0.01	0.09±0.00	0.05±0.01
		0.10	2.07±0.00	2.02±0.01	1.04±0.00	1.01±0.01	0.03±0.00	0.01±0.01
		0.05	2.01±0.01	2.00±0.01	1.01±0.01	1.00±0.00	0.00±0.00	0.00±0.01
	EE	0.20	2.30±0.00	2.30±0.01	1.15±0.01	1.15±0.00	0.14±0.01	0.14±0.01
		0.10	2.10±0.01	2.20±0.01	1.05±0.01	1.10±0.01	0.05±0.01	0.10±0.01
		0.05	2.02±0.01	2.07±0.01	1.01±0.01	1.04±0.01	0.01±0.01	0.03±0.01
	WE	0.20	2.36±0.04	2.42±0.01	1.18±0.01	1.21±0.01	0.17±0.01	0.20±0.01
		0.10	2.21±0.01	2.34±0.01	1.11±0.01	1.17±0.01	0.10±0.01	0.16±0.01
		0.05	2.19±0.01	2.29±0.01	1.10±0.01	1.15±0.01	0.09±0.01	0.14±0.01

(*Table 3.9 continue*)

Plant	Solvent	Conc %	Plant origin: Lithuania IP	France IP	Lithuania PF	France PF	Lithuania AA	France AA
T. farfara-FS	AE	0.20	2.72±0.02	2.54±0.01	1.30±0.01	1.22±0.00	0.32±0.01	0.23±0.00
		0.10	2.61±0.03	2.50±0.01	1.25±0.02	1.20±0.00	0.26±0.02	0.21±0.00
		0.05	2.56±0.02	2.34±0.03	1.22±0.01	1.12±0.02	0.24±0.01	0.13±0.02
	ME	0.20	2.57±0.01	2.67±0.01	1.23±0.01	1.28±0.01	0.24±0.00	0.29±0.00
		0.10	2.49±0.01	2.51±0.01	1.19±0.01	1.20±0.01	0.20±0.00	0.21±0.00
		0.05	2.46±0.01	2.38±0.01	1.18±0.01	1.14±0.01	0.19±0.00	0.15±0.00
	EE	0.20	2.59±0.01	2.75±0.01	1.24±0.01	1.32±0.01	0.25±0.00	0.33±0.00
		0.10	2.55±0.01	2.64±0.01	1.22±0.01	1.26±0.01	0.23±0.00	0.28±0.00
		0.05	2.48±0.00	2.53±0.01	1.19±0.00	1.21±0.00	0.20±0.00	0.22±0.00
T. farfara- L	AE	0.20	2.80±0.03	2.82±0.01	1.40±0.03	1.41±0.01	0.39±0.03	0.40±0.01
		0.10	2.62±0.01	2.64±0.01	1.31±0.01	1.32±0.01	0.30±0.01	0.31±0.01
		0.05	2.58±0.01	2.59±0.00	1.29±0.01	1.30±0.00	0.28±0.01	0.29±0.00
	ME	0.20	2.32±0.01	2.29±0.01	1.16±0.01	1.15±0.01	0.15±0.01	0.14±0.01
		0.10	2.30±0.01	2.21±0.01	1.15±0.01	1.11±0.01	0.14±0.01	0.10±0.01
		0.05	2.28±0.02	2.18±0.02	1.14±0.02	1.09±0.02	0.14±0.02	0.09±0.02
	EE	0.20	2.46±0.01	2.39±0.01	1.23±0.01	1.20±0.01	0.22±0.01	0.19±0.01
		0.10	2.40±0.01	2.36±0.01	1.20±0.01	1.18±0.01	0.19±0.01	0.17±0.01
		0.05	2.30±0.01	2.28±0.01	1.15±0.01	1.14±0.01	0.14±0.01	0.14±0.01
T. farfara -R	AE	0.20	2.62±0.00	2.53±0.01	1.31±0.00	1.27±0.01	0.30±0.00	0.26±0.01
		0.10	2.39±0.01	2.48±0.01	1.20±0.01	1.24±0.01	0.19±0.01	0.23±0.01
		0.05	2.26±0.02	2.30±0.01	1.13±0.02	1.15±0.01	0.13±0.02	0.14±0.01
	ME	0.20	2.41±0.01	2.45±0.01	1.21±0.01	1.23±0.01	0.20±0.01	0.22±0.01
		0.10	2.38±0.01	2.40±0.00	1.19±0.01	1.20±0.00	0.18±0.01	0.19±0.00
		0.05	2.31±0.01	2.33±0.01	1.12±0.01	1.17±0.01	0.15±0.01	0.16±0.01
	EE	0.20	2.58±0.03	2.60±0.01	1.29±0.03	1.30±0.01	0.28±0.03	0.29±0.01
		0.10	2.50±0.01	2.51±0.01	1.25±0.01	1.26±0.01	0.24±0.01	0.25±0.01
		0.05	2.42±0.01	2.46±0.01	1.21±0.01	1.23±0.01	0.20±0.01	0.22±0.01
BHT			0.02±0.00		4.07±0.00	2.04±0.00	1.00±0.00	
Sunflower oil			0.00±0.00		2.00±0.00	1.00±0.00	-	

Also from these data it may be observed that there are no significant differences between the same plants of two different origins, but the important difference are obtained in case of using different extraction solvents: WEs of *M. odorata* possessed stronger antioxidant activity, than AE, ME and EE. For LT *T. farfara* we observed that the highest IP was in AE extract from FS and L but for R was EE extract. In FR origin the EE extracts of FS and R showed the most effective effect, but for L AE was stronger. BHT was active antioxidant allowing stabilizing lipid for two times longer than *T. farfara* extracts. Comparing the influence of the same solvent and plant origin in Oxipres test it was found the same regularities between *M. odorata* and *T. farfara*, there were no significant differences between origins (LT and FR), the only important difference is obtained by the use of different solvents for extraction.

Summarizing the results obtained by oxipres method it can be stated that almost all herbal extracts obtained some AA by stabilizing sunflower oil during storage. In terms of effectiveness the extracts at 0.2 % concentration can be put into the following succession: *C. grandiflora* > *T. farfara* > *M. odorata*. It has also should be emphasized that no positive correlation was found between the TPC and AA. For instance, *M. odorata* extracts which were rich in the TPC possessed the lowest AA as compared with the roots of *T. farfara* (low amount of TPC and medium AA) and *C. grandiflora* (medium TPC and highest AA). It should be noted that different solvents were used for the isolation of phenolic compounds (ethanol or methanol) and (acetone). However, we suppose that the solvent in this case was an important factor, so far as the procedure of the extraction in Soxhlet apparatus is sufficiently efficient to isolate herb phenolics with ethanol or methanol, probably due to their higher

compatibility with the medium or the more polar nature of the contained analytes. The absence of the correlation between the TPC and AA of the extracts can be better explained by the well-known fact that the AA of individual phenolic compounds can vary in a very wide range.

3.6 ASSESSMENT OF RADICAL SCAVENGERS (RSC) BY ON-LINE HPLC-DPPH• METHOD

3.6.1 Radical scavengers in C. grandiflora *extracts*

The on-line RP-HPLC-DPPH• method can be used for a rapid assessment of radical scavenging properties of separated in chromatographic column compounds which are present in complex mixtures such as plant extracts [199-200]. The HPLC/UV/DPPH• chromatograms of EE and FD extracts (Fig. 3.12) indicate that several separated compounds are able to scavenge radicals. This study was aimed at the identification of individual components in the extracts; the most abundant compound in EE was identified as rosmarinic acid, which is well known natural antioxidant.

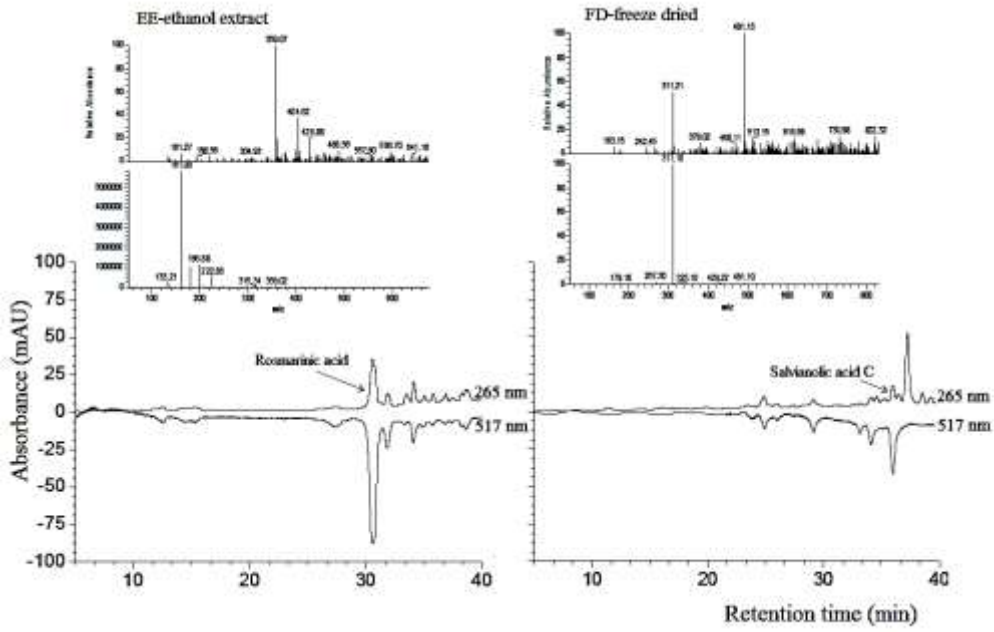


Fig. 3.12. *HPLC–DPPH• chromatograms of* C. grandiflora *extracts and m/z of identified compounds*

In LC-MS spectra rosmarinic acid (RT=30.6 min.) in a positive ionization mode gave m/z at 359 and characteristic fragmentation at m/z 161, which possessed a very high RSC (49.8% of total area of the peaks).

It was described by other authors that rosmarinic acid is one of the most common caffeic acid esters occurring in the Lamiaceae family plants. The production of this compound has been investigated both in numerous Lamiaceous plants and in tissue cultures of several species. Zgorka *et al*. described the possible role of rosmarinic acid as the chemotaxonomic marker of the Lamiaceae family [201]. Rosmarinic acid is known as an antiviral, antibacterial, antioxidant, anti-inflammatory and immunostimulating agent [202].

High amount of the radical scavenger eluted from the *C. grandiflora* FD at approximately 35.9 min: it was identified as salvianolic acid C. In LC-MS spectra it gave m/z 491 and characteristic fragmentation at m/z 311, and possessed a very high radical scavenging activity (30.4% of total area of the peaks).

Salvianolic acid was previously found in *Salvia miltiorrhiza root*; this polyphenolic acid protects against dopamine-induced apoptosis in SH-SY5Y cells and may be effective in treating neurodegenerative diseases associated with oxidative stress [203].

To the best of our knowledge rosmarinic acid and salvianolic acid C were not previously reported in *C. grandiflora*. Thus, these results showed that the residue of WEs from *C. grandiflora* possess strong antioxidant activity and therefore might be promissing for industrial applications, e.g. in foods and the cosmetics, as a means of protecting against negative effects of excessive free radicals.

3.6.2 Phytochemical composition and RSC of M. odorata *constituents*

The assessment of antioxidant properties of *M. odorata* by TPC and RSC-DPPH$^{\bullet}$ assays suggests that there might be remarkable differences in the extract composition between the plants of FR and LT origin, particularly in case of WE and AE. It is known that the antioxidant activity of individual phenolic compounds can differ significantly depending on their molecular structure; therefore the content of TPC in plant extracts is not very informative indicator of their RSC. The structures of isolated constituents need to be elucidated and assessed in order to obtain more precise results. An on-line HPLC–DPPH$^{\bullet}$ method is a convenient option for the preliminary screening of RSC of the compounds present in the extracts. Therefore, further studies were performed to evaluate RSC of chromatographically separated constituents. Kaempferol-7-O-glucoside and cynarin were identified in *M. odorata* and they were the major antioxidatively active compounds in the analyzed extracts, however the former compound was more abundant in all LT *M. odorata* extracts while the latter one was dominant in WE of FR *M. odorata*. To the best of our knowledge these compounds were not previously reported in *M. odorata*. Peak areas of kaempferol-7-O-glucoside and cynarin in EE and WE were in the range of 639.7-6038.2 and 0-849.1, while their input in the total RSC was 34.5-97.1 and 0-45.4%, respectively (Table 3.10). Kaempferol-7-*O*-glucoside was previously found in *Smilax china*; this flavonoid induced G2/M phase arrest and apoptosis on HeLa cells in a p53-independent manner [204]. Cynarin is an ester formed from quinic acid and two units of caffeic acid and was reported as biologically active chemical constituent of artichoke 58 years ago [205]; diuretic, choleretic and hepatoprotective activities of artichokes leaves extract were often related to the cynarin content [204-205]. In addition, cynarin is responsible for the sensation of sweetness that occurs after eating artichokes; it appears to suppress bitter tongue taste receptors while enhancing the sweet ones; consequently it may also be responsible for the sweet taste of *M. odorata*. Most recently it was reported that cynarin, which was abundant in sunflower (*Helianthus annuus*) sprouts possessed both antiglycative and antioxidant activities and was able to intervene against glycoxidation [206].

The chromatographic profiles of WE (Fig. 3.13) obtained by the on-line HPLC–DPPH$^{\bullet}$ methods clearly show that FR *M. odorata* contain more radical scavenging constituents than LT *M. odorata*. Strong antioxidant cynarin was present in FR *M. odorata* in remarkably higher amounts than in LT *M. odorata*, however, the content of kaempferol-7-O-glucoside was more abundant in LTMO (Fig. 3.13, Table 3.10). Total peak area of radical scavenging peaks in W of LT *M. odorata* and FR *M. odorata* was 2814.6 and 1801.4, respectively, and

these values are in agreement with RSC (IC_{50}) of these extracts, which were 0.12 and 0.13 mg/l, respectively.

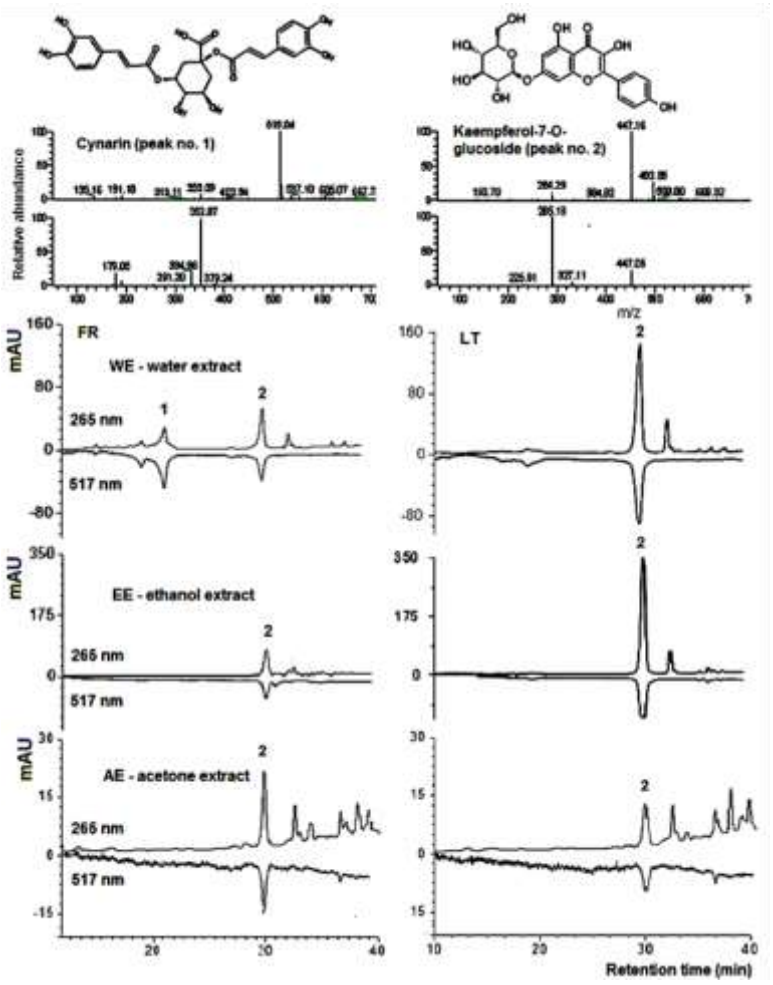


Fig. 3.13. *HPLC–DPPH$^{\bullet}$ chromatograms of* Myrrhis odorata *extracts of French (FR) and Lithuanian (LT) origin and m/z and the structures of identified compounds*

However, in case of EE direct comparison of on line and batch evaluation of RSC is not relevant. The IC_{50} of EE of FR *M. odorata* was only slightly lower than that of LT *M. odorata*, while peak area of the main EE constituent kaempferol-7-O-glucoside was more than 4 times bigger in the LT. Possibly EE of FR contains other radical scavengers which were not detected at the applied HPLC analysis conditions. As it may be observed in the chromatograms the extracts contain more antioxidatively active compounds, however, their input in the total RSC was less remarkable and constituted from 0.8% (WE of LT *M. odorata*) to 20.5% (EE of FR *M. odorata*) of the total RSC (Table 3.10). Mass spectra and UV data was not sufficient to identify these compounds. Also the content of detected constituents in AE was remarkably lower than in EE and WE.

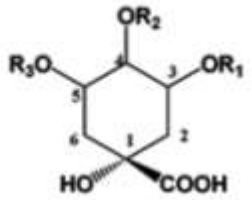
Table 3.10. *Composition and radical scavenging capacity of* Myrrhis odorata *constituents evaluated by the on-line HPLC-UV-DPPH$^{\bullet}$ method (ni=not identified)*

Plant origin			Ethanol extract EE		Water extract WE		Acetone extract AE	
	Active compound	RT, min	Peak area, mV×s	% of total RSC	Peak area, mV×s	% of total RSC	Peak area, mV×s	% of total RSC
FR	ni	8.5	-	-	51.4	3.4	-	-
	ni	13.2	-	-	201.2	10.8	-	-
	Cynarin	15.5	-	-	849.1	45.4	-	-
	ni	26.7	-	-	26.4	1.4	-	-
	K-7-O-glc	29.7	1436.6	77.2	639.7	34.5	375.1	90.4
	ni	30.7	229.9	12.4	-	-	-	-
	ni	32.0	58.0	3.1	33.6	1.8	-	-
	ni	34.2	67.6	3.6	-	-	-	-
	ni	35.0	26.5	1.4	-	-	-	-
	ni	36.5	-	-	-	-	32.6	7.9
LT	ni	15.5	-	-	231.2	7.8	-	
	K-7-O-glc	29.8	6038.2	97.1	2547.8	86.4	214.4	77.2
	ni	35.5	55.5	0.9	22.3	0.5	-	-
	ni	36.4	115.9	1.9	-	-	43.4	15.6
	ni	37.9	-	-	13.3	0.3	-	-

3.6.3 Evaluation of active compounds from T. farfara

Flavonoid glycosides, quercetin 3-O-β-L-arabinopyranoside and quercetin 3-O-β-D-glucopyranoside [152] as well as quinic, chlorogenic and dicaffeoylquinic acids were reported previously in *T. farfara* flower bud extract from China [153]. The on-line HPLC-DPPH$^{\bullet}$ method was used for the detection of radical scavenging components. The *T. farfara* extract of the flowers-stems had clear differences from the extracts obtained from other parts of the plant. The R AE exhibited smaller peaks than those seen in the extracts of the L and FS (Fig. 3.14). The analysis revealed that FS, L and R extracts contain 5 similar active components: quinic acid, dicaffeoylquinic acid, quercetin-pentoside and kaempferol-glucoside, while other detected compounds were different. The ESI-MS of the AE, ME and EE of *T. farfara* from two origins were qualitatively different (Table3.11). Compounds 3, 4 and 5 showed $[M-H]^-$ signal at m/z 515 with fragmentation at m/z 353 and 179, from which a molecular formula of $C_{25}H_{24}O_{12}$ was assigned. The ion at m/z 353 indicated a chlorogenic acid fragment derived from the loss of a caffeoyl group. The ion at m/z 179 indicated fragments of caffeic acid moieties. These MS data indicated that the compounds 3-5 were dicaffeoylquinic acid isomers. As chromatographic separation was performed, it wasn't possible to determine which isomers of dicaffeoylquinic acid were present, however all isomers are common components of *T. farfara* and possess antioxidant activity [117]. Usually, the location of the caffeoyl groups in dicaffeoylquinic are at C-3'; C-4', C-3'; C-5' and C-1'; C-5' (fig.3.14, A).

A)



Caffeoyl (R=OH)

Compound	R_1	R_2	R_3
3-Caffeoylquinic acid (3-CQA)	Caffeoyl	H	H
4-Caffeoylquinic acid (4-CQA)	H	Caffeoyl	H
5-Caffeoylquinic acid (5-CQA)	H	H	Caffeoyl
5-Feruloylquinic acid (5-FQA)	H	H	Feruloyl
3,4-Dicaffeoylquinic acid (3,4-diCQA)	Caffeoyl	Caffeoyl	H
3,5-Dicaffeoylquinic acid (3,5-diCQA)	Caffeoyl	H	Caffeoyl
4,5-Dicaffeoylquinic acid (4,5-diCQA)	H	Caffeoyl	Caffeoyl

B)

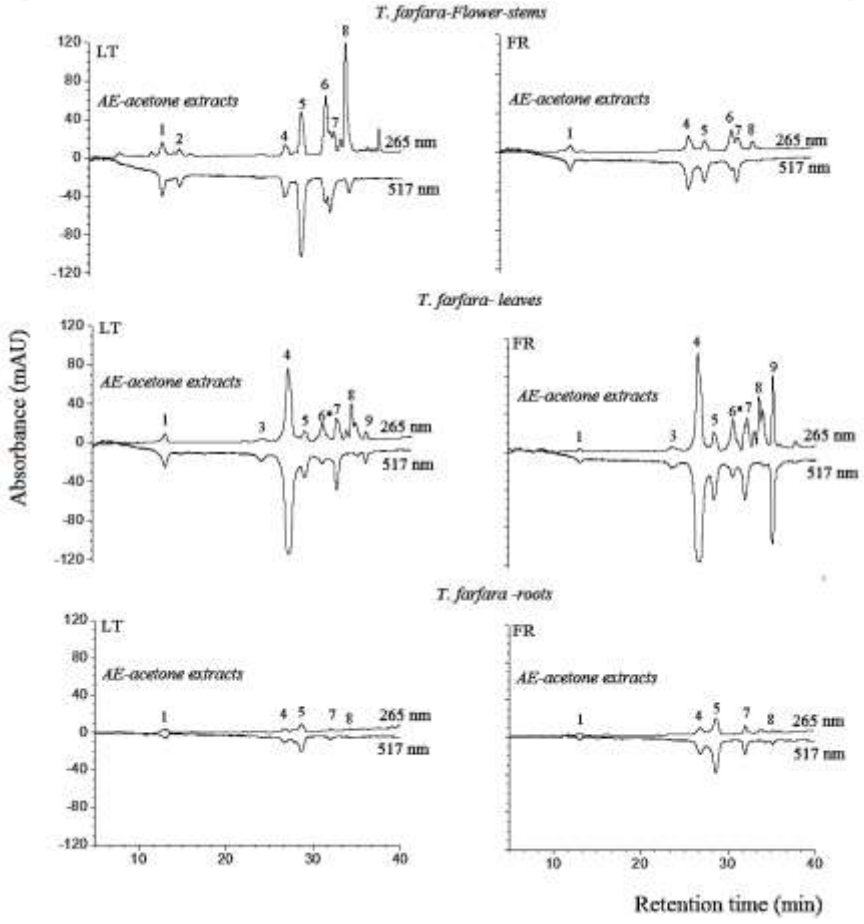


Fig. 3.14. **A)** *Structure of dicaffeoylquinic acid derivatives.* **B)** *HPLC–DPPH chromatograms of acetone (AE) extracts of T. farfara from Lithuanian (LT) and French origin (FR)*

The ESI-MS of the LT *T. farfara* FS extracts presented ions corresponding to: 1-(m/z 191)-quinic acid, 2-(m/z 354)-chlorogenic acid, 3-5 (m/z 515)-dicaffeoylquinic acid, 6-(m/z 609)-quercetin-3-rutinoside (rutin), 7-(m/z 434)-quercetin-pentoside and 8-(m/z 447)-kaempferol-glucoside. FS extract of FR origin plants was qualitatively different: in this

extract chlorogenic acid was absent. Quinic acid, chlorogenic acid, rutinoside and dicaffeoylquinic acid isomers were previously isolated from the flower buds of *T. farfara* [153] while, to the best of our knowledge, quercetin-pentoside and kaempferol-glucoside were not previously reported in this plant.

Table 3.11. *Composition and radical scavenging capacity of* Tussilago farfara *constituents evaluated by the on-line HPLC-UV-DPPH$^{\bullet}$ method*

Plant origin	Part of the plant			Acetone extract		Methanol extract		Ethanol extract	
		Active compound	RT, min	Peak area, mV×s	% of total RSC	Peak area, mV×s	% of total RSC	Peak area, mV×s	% of total RSC
FR	*T. farfara-Flower-stems*	Quinic acid	13.1	365.3	8.4	7.2	0.4	718.8	18.3
		CGA	-	-	-	-	-	-	-
		diCQA	26.8	150.9	16.9	863.3	46.3	871.4	22.2
		diCQA	28.6	1226.2	13.8	645.3	44.9	513.6	13.1
		Rutin	31.3	539.8	9.8	-	-	261.1	6.6
		Q-pent	31.9	1159.3	13.1	58.0	6.3	800.5	21.6
		K-3-O-glc	34.2	862.9	5.8	12.4	0.7	154.5	3.9
LT		Quinic acid	12.9	458.3	8.3	387.9	9.4	1054.8	15.9
		CGA	15.0	185.3	3.6	208.0	6.3	126.8	2.6
		diCQA	26.7	915.6	10.0	699.3	12.6	834.1	11.7
		diCQA	28.5	3385.3	39.6	917.7	23.4	1657.7	24.9
		Rutin	31.1	756.6	12.5	411.7	11.5	942.9	18.9
		Q-pent	31.9	1464.6	17.1	987.1	15.7	1065.4	16.0
		K-3-O-glc	34.2	87.3	1.0	141.7	2.8	141.5	4.2
FR	*T. farfara- leaves*	Quinic acid	13.0	95.6	0.5	195.1	1.0	304.4	1.8
		diCQA	23.5	196.2	1.0	84.3	0.5	322.4	1.9
		diCQA	26.6	7203.1	36.5	5292.3	28.3	8564.1	50.6
		diCQA	28.3	1909.7	5.1	889.5	4.8	1359.4	8.0
		Q-3-O-glc	30.8	997.1	5.1	947.1	5.1	974.3	5.8
		Q-pent	31.8	2003.4	10.2	2455.8	13.1	2681.2	15.8
		K-3-O-glc	34.1	432.4	2.2	224.8	1.2	348.9	2.1
		triCQA	35.1	3467.7	17.6	3940.2	21.1	2206.9	13.0
LT		Quinic acid	12.9	318.4	2.3	112.3	0.5	221.9	1.8
		diCQA	23.4	234.5	1.7	248.2	1.0	212.7	1.7
		diCQA	26.5	6726.8	47.7	9519.9	37.7	3149.7	25.1
		diCQA	28.3	1298.5	9.2	1676.6	6.6	1113.7	8.9
		Q-3-O-glc	30.9	644.4	4.6	1819.7	7.2	691.9	5.5
		Q-pent	31.9	1620.3	11.5	3703.2	14.7	1896.9	15.1
		K-3-O-glc	34.2	182.4	1.3	601.2	2.4	729.7	5.8
		triCQA	35.1	287.1	2.0	1293.8	5.1	1113.7	8.9
FR	*T. farfara -Roots*	Quinic acid	13.0	131.6	2.0	126.2	11.0	332.5	20.0
		diCQA	26.8	625.9	9.4	288.7	25.2	317.8	19.1
		diCQA	28.6	1676.3	25.1	196.4	17.1	237.8	14.3
		Q-pent	31.9	695.7	10.4	228.9	20.0	355.5	21.4
		K-3-O-glc	34.2	218.1	3.3	91.5	8.0	87.2	5.2
LT		Quinic acid	12.9	102.1	10.8	187.9	10.1	162.3	2.4
		diCQA	26.8	101.4	10.6	371.2	20.0	474.6	7.0
		diCQA	28.6	516.6	54.1	399.6	21.5	1711.6	25.4
		Q-pent	31.9	107.9	11.3	544.8	29.3	1036.1	15.4
		K-3-O-glc	34.1	8.7	0.9	158.3	8.5	578.6	8.6

CGA-chlorogenic acid, diCQA-dicaffeoylquinic acid, Q-3-O-glc-quercetin- glucoside, Q-pent-quercetin-pentoside, K-3-O-glc- kaempferol-glucoside.

The MS data of the *T. farfara* L extracts presented ions corresponding to *m/z* 463-quercetin-glucoside (6*) and m/z 678- tricaffeoylquinic acid (9); however, these ions were not found in the MS of flowers-stems and roots extracts. On the other hand quercetin-3-rutinoside was present only in FS extracts.

RSC of *T. farfara* extracts isolated from LT origin plants showed slightly better antioxidant activity than those of FR origin; however, based on DPPH$^{\bullet}$ assay (Fig. 3.10), no significant differences in IC_{50} values were observed between the same plant extract from different countries. Chromatograms profiles of extracts in HPLC–DPPH$^{\bullet}$ assay (Fig. 3.14) were not similar between the two origin plants. *T. farfara* FS extracts isolated from LT origin plants showed more big peaks in negative chromatogram comparing to FR origin plants. Dicaffeoylquinic acids and quercetin-pentoside were major active compounds with total RSC (39.6%), (10%), (17.1%) and (16.9%), (13.8%), (13.1%) respectively (Table 3.11) in LT and FR AE deodorized FS extracts.

Major active compounds from LT AE L extracts was dicaffeoylquinic acids and quercetin-pentoside, but in FR origin was dicaffeoylquinic acid, quercetin-pentoside and tricaffeoylquinic acid and these compounds have RSC (47.7%), (9.2%), (11.5%) and (36.5%), (10.4%), (17.6%) respectively.

As it has already been noted, phytochemical composition of plant's secondary metabolites depends on several factors, such as cultivation area, climatic conditions, vegetation phase, genetic modifications, part of plants and others; therefore evaluation of plant properties from different geographical regions was in the focus of numerous studies [147, 207].

3.7 THE POSSIBILITIES OF TECHNOLOGICAL IMPLEMENTATION

So far as the yields of volatile compounds which might be isolated from the selected aromatic plants are relatively low, it would be more economical to focus future efforts on the processing of by-products into high-added value nonvolatile substances, such as antioxidants. The performed studies suggest that solid and liquid residues remaining after essential oil isolation from the whole plant material could have potential uses in food industry and some other applications. In addition to reducing the waste associated with by-products production, could provide additional economic value to the aromatic plant material. Phenolic compounds from by-products may be extracted and spray dried producing powder with high content of phenolic compounds and strong antioxidant capacity; the product could be used as a natural additive or healthy food ingredient in foods and nutraceuticals.

All experiments were performed with small amounts of plant material on laboratory scale. Therefore it would be very important to upscale the production quantities. First step in implementation of the results would be testing of technology in pilot plant equipment and afterwards it may be upscaled to the industrial level. Possible technological scheme for the valorization of aromatic plant by-products processing is presented in Fig. 3.15.

The procedures used in this work on a laboratory scale may be implemented by using existing commercial equipment, possibly by adding to the existing lines some new apparatus fitting selected plant material properties. As an example an existing technological scheme developed by the company Dig-Maz [208] with some modifications (Fig. 3.15) may be applied; it represents the full technology scheme for plant material processing: the production line is divided into six stages, which are briefly outlined further in this section (detailed description of the technological scheme is given in appendix).

First stage is conducted for checking of the primary raw materials for various impurities and grinding. This stage is recommended by Dig-Maz using their equipment i.e., the raw materials

are filled into the feeding funnel (AT 06) of the conveyor belt FB-4 (segregation line). The conveyor belt (FB 4) is transferring the raw material through the metal detector (GLS) to the cutting mill (GSL 180/120). After the cutting mill the crushed material particles are filled in the feeding tank. The feeding tank is placed on a balance and filled as long until the adjusted weight is obtained. A lifting-tipping device (filling device) is loading the extraction plant (hydrodistillation).

Second stage is a hydrodistillation process, resulting in one of the products - essential oil and two-type of by-products (liquid and solid) - liquid residue containing water soluble plant constituents and solid residue, containing water insoluble plant substances. This stage was not included into the existing technological scheme developed by the company Dig-Maz; therefore some modifications were introduced by adding a hydrodistillation apparatus with condenser and spray dryer, which will be used in third stage. Hydrodistillation is a method of extraction, which is sometimes used instead of steam distillation. This process of extraction is one of the most frequently used traditional methods of extraction. In this process the plants are soaked in the water; then the water container is heated untill the steam comes out together with volatile oil and afterwards is cool for condensation. The oil is collected from the top of the hydrosol.

The remaining by-products in the third stage can be processed according to our proposed technology: the liquid residue is filtered, thus solid particles can be separated, and sprayed in spray dryer to obtain a second product – powder containing active water soluble components such as glycosidic polyphenol compounds, phenolic acids and others. Spray drying is a very fast method. The short drying time, and consequently fast stabilization of material at moderate temperatures, means spray drying is also suitable for heat-sensitive substances. As a technique, spray drying consists of four basic stages: atomization, drying, particle formation, and their recovery. A liquid stock is atomized into droplets by means of a nozzle or rotary atomizer. Nozzles use pressure or compressed gas to atomize the feed while rotary atomizers employ an atomizer wheel rotating at high speed. Hot process gas (air or nitrogen) is brought into contact with the atomized liquid guided by a gas disperser, and evaporation begins. The balance between temperatures, of rate and droplet size controls the drying process. As the liquid rapidly evaporates from the droplet surface, a solid particle forms and falls to the bottom of the drying chamber. The powder is recovered from the exhaust gas using a cyclone or a bag later. The whole process generally takes no more than a few seconds. In addition, agglomeration may be used as a final stage, which performed by rewetting spray dried particles by mixing them with a mist of water and vapor and finally by gentle impacting to form the right size of light and stable agglomerates.

The remaining solid residue after hydrodistillation is dried at a temperature of 30-40°C (fourth stage) and then is transported by conveyor belt to the extraction; this stage was also added to the Dig-Maz technological scheme.

Extraction of solid residue is carried out using selected solvent; later the resulting extract is concentrated and the solvent is evaporated, thus obtaining a third product - the extract containing water insoluble substances. In this stage commercial DIG - MAZ Extraction system (DIG-MAZ 500) may be used. The DIG-MAZ extraction system is designed as a multifunctional system which combines important methods of extraction, such as percolation, digestion, maceration as well as distillation in one compact plant. The extraction process can be performed under pressure up to 10 bar and under vacuum. DIG-MAZ extraction plants' special counter-current feature, causing the solvent to stream through the herbs bottom up, makes them a unique extraction system. The process starts with the filling the extractor with

hackled solids. The lid opens the whole cross-section of the extraction vessel at the touch of a button whereby an optimum filling is ensured. Batch-extraction of fine-grained solids proceeds by leaching with solvents, such as water and ethanol. Thus, the extract filtration is executed by bottom and lid sieves then, the solids are squeezed using the sieve bottom, which is employed as movable press piston and controlled via hydraulics. The good extraction is then dehumidified reducing the solvent loss and extraction vessel is tipped over on a horizontal axis and completely evacuated with the help of the hydraulically shifted sieve bottom. Finally, in the Vacuum evaporation, the solid-content of the extract is increased by vacuum evaporation at short holding time. The solvent is condensed, accumulated and can be re-circulated into the extraction process [208]. A solid residue after extraction (mainly lignocelullosic substances) may be further processed, for instance by transporting it to the pressing line for producing biodegradable cups for plants or briquettes for fuel or to composting line (these processes were not studied in the present work; however such technologies recently have been developed).

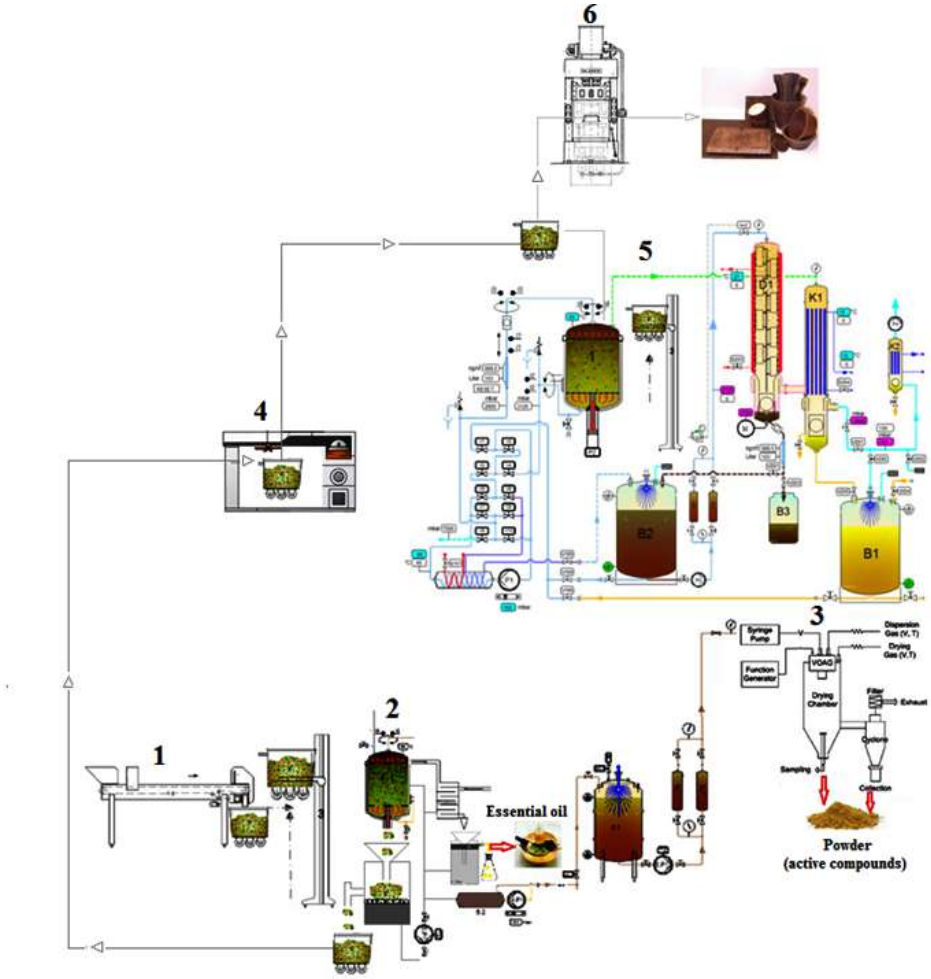


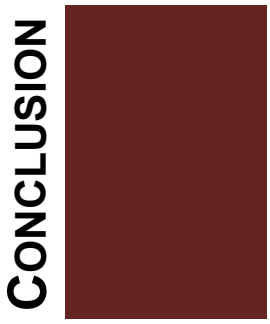
Fig. 3.15. *Technological scheme for the implementation of agrorefinery concept for the efficient processing of aromatic and medicinal plant material into different products [205]*

Industrial implementation of the technology would require additional optimization of process parameters, such as temperature, the ratio of solvent and plant material, particle size, process time and others. In fact, main processes could give three main products – essential oil,

water soluble fraction and water insoluble fraction, which may be isolated with organic solvents. These fractions may be further processed in to finer and more specific products. For instance, rectification column may be used as an additional apparatus for fractionation of volatile oil into different fractions possessing special odour and antimicrobial properties, while nonvolatile fractions may be further fractionated by chromatographic methods into the targeted compound's groups possessing strong antioxidant and other biological activities.

In our study it was shown that phytochemical composition of the selected aromatic and medicinal plants was highly dependent on their origin of cultivation and vegetation phase. Therefore, another important recommendation for the industrial implementation is associated with plant material selection and cultivation as well as various harvesting issues. These issues should be carefully considered by selecting cultivation area, agrotechnological aspects, plant chemo- and biotype peculiarities. These issues are important for the standardization of the products, which may be obtained by using agrorefinery concept. They should be focused on the crop yield and the concentrations of the most valuable constituents in the plant material harvested for processing. It should be considered that phytochemical composition of plant's secondary metabolites depends on several factors, such as cultivation area, climatic conditions, vegetation phase, genetic modifications and others. Therefore, comprehensive assessment of all these factors could help identifying genetic potential, which could assist in improvements in breeding programs and diversification of its industrial uses. Also, additional future work should include further investigation of the bioactivity; bioavailability and toxicology of phytochemicals need to be carefully assessed by the *in vitro* and *in vivo* assays.

Undoubtedly, functional foods as representing an important, innovative and rapidly growing part of the overall food market may be considered as one of the main end user of the developed plant products. Other areas of application include cosmetics, pharmaceuticals, and fine chemicals for other uses. So far as the majority of such products are intended for human consumption, future work should also be focused on investigating their design, sensory properties, consumer acceptability and also careful assessment of all potential risks which might arise from the natural compounds recovered from by-products. Furthermore, investigations on stability and interactions of phytochemicals with other food ingredients during processing and storage need to be performed.



4 CONCLUSION

1. Based on main essential oil components, *M. odorata* of French and Lithuanian origin may be preliminary assigned to the same *E*-anethole, methyleugenol and *E*-nerolidol chemotype. However, the percentage of estragol was more than 4 times higher in essential oil of French origin plant materialwhereas the percentage of β-caryophyllene, germacrene-D, spathulenol and limonene was significantly higher in the Lithuanian origin oil. The climatic conditions from two studied areas countries are different; mean temperature and rainfall amount during the month of collection were higher in France than in Lithuania; due to these differences the former oil had stronger anise and licorice-type flavor notes while the latter one possessed stronger fresh, sweet, woody, spice, clove dry, earthy, herbal, and fruity notes.

2. The main constituents of *T. farfara* volatile fraction of French and Lithuanian origin plants were 1-nonene (34.1±0.0%) and *n*-tricosane (21.7±0.1%), respectively. Chemical composition of *T. farfara* volatiles in flowers and stems were compared for the first time; microscopy analysis revealed that stems contain secretory hairs (glandular trichoma), the organs for essential oil supply suggesting that all the aerial parts of *T. farfara* might be useful for essential oil production.

3. Two new analytical methods for the analysis of volatile compounds, namely Crushing Finger Device (CFD) and Flash Aroma Dispenser (FAD) were developed and tested as alternatives to the traditional extraction methods; it was shown that these methods may avoid some disadvantages of traditionally used analysis techniques, such as the thermal degradation of volatile compounds or large amounts of raw materials.

4. All extracts isolated from plant hydrodistillation residues were antioxidatively active. *M. odorata* and *C. grandiflora* alcoholic and water extracts were remarkably stronger antioxidants than acetone (AE) extracts in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ reaction systems. Radical scavenging capacity (RSC) of extracts obtained from the solid residue was quite similar to the RSC of water extracts, showing that some antioxidatively active compounds do not dissolve in boiling water and still remain in the solid residue. Some differences were determined in the antioxidant activity indicators of *T. farfara* and *M. odorata* extracts isolated from Lithuanian and French origin plants proving that such factors as cultivation area, climatic conditions, vegetation phase, genotype and others may be important in the accumulation of active constituents in the plants.

5. Plants extract additives retarded sunflower oil oxidation; *C. grandiflora* extract added at 0.2 % had the strongest effect followed by *T. farfara* and *M. odorata*. However, synthetic butylated hydroxytoluene (BHT) antioxidant in oil was 10 times lower (0.02%) than the concentration of the extracts (0.2%), but synthetic (BHT) antioxidant was stronger, than plant extracts. It has also should be emphasized that no positive correlation was found between the total amount of phenolic compounds (TPC) and antioxidant activity (AA); for instance, *M. odorata* extract possessing high TPC value was weaker antioxidant that *T. farfara* root extract containing lower TPC.

6. Several antioxidants were identified in the studied plants by using modern chromatographic and spectrometric analysis techniques such as on-line HPLC-$DPPH^{\bullet}$ method. Rosmarinic acid was the major antioxidant in *C. grandiflora* ethanol extract, while salvianolic acid C was major active compound in freeze-dried extract. Kaempferol-7-O-glucoside and cynarin were identified as the major antioxidatively active compounds in *M. odorata* extracts; these compounds were not previously reported in *M. odorata* and

C. grandiflora. Eight antioxidant compounds were identified in *T. farfara* two of them (quercetin-pentoside and kaempferol-glucoside) previously not reported in this plant.

7. *C. grandiflora, M. odorata* and *T. farfara* examined in the study contain valuable functional constituents - antioxidants and volatile aroma compounds. The results obtained in this study may be considered as a first step in valorizing the processing of these raw materials in order to obtain high-added value substances, present both in volatile and non-volatile fractions, which can found wider practical application in different areas, particularly in formulation and production of food additives, functional food components and healthy supplements. Agrorefinery approach applied in this study may substantially increase the feasibility of practical implementation in terms of agro-technological, economical, processing and application issues.

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APPENDIX 1

TECHNICAL SPECIFICATION

Table A1.1 Technical specification

Item	Description	Remarks	Apparatus
1	Raw material acceptance • Feeding funnel • Metal detector • Conveyor belt • Cutting mill • Screw-conveyor	**Raw material acceptance RAS 55** **Feeding funnel AT 06** **Dimensions:** length: 800 mm width:1,000 mm height 600 mm 1,000 mm **Metal detector GLS** Tunnel-metal detectors with rectangular outlet opening are detecting all magnetic and nonmagnetic metal pollutions **Conveyor belt FB-4** (Segregation line) **Dimensions:** length: 4,000 mm width:1,000 mm height:1,000 mm total height:1,600 mm power:0.5 kW **Cutting mill GSL 180/120** The rotor geometry with V-diagonal cut guarantees highquality grist with a low dust content. **Dimensions:** length: 450 mm width:1,035 mm height:750 mm power:3 kW **Screw-conveyor ST-4** evacuation of the crushed raw material from the cutting mill **Raw material container FW 250** endless, leak-proof welded bead edge wall thickness: 2mm substruction stiffened to 5mm by a double bottom. **Extractor** **Filling device RSE 55** stainless steel profile with sledge and fork for the reception of the raw material container. Gear motor attached consoles. The raw materials are filled into the feeding funnel of the conveyor belt (segregation line). The conveyor belt is transferring the raw material through the metal detector to the cutting mill. Before the cutting mill pollutions or foreign matter can be sorted out. After the cutting mill the crushed material particles are filled in the feeding tank. The feeding tank is placed on a balance and filled as long until the adjusted weight is obtained. A lifting-tipping device (filling device) is loading the extraction plant.	 **GLS - Metal detector**  **Conveyor belt FB-4**  **GSL 180/120 - Cutting mill**  **Screw-conveyor**  **Raw material container**  **Filling device**

2	Hydrodistillation system • Extractor • Water tank • Cooling water out/into • Hot water out • Vacuum/gas inlet • Condenser • Heat control • Separator	Hydrodistillation is one of the simplest, oldest and primitive process known to man for obtaining essential oils from plants. Mostly used by small scale producers of essential oils In water / hydrodistillation the plant material is almost entirely covered with water as suspension in the still which is placed on a furnace Water is made to boil and essential oil is carried over to the condenser along with the steam. Useful for distillation of powders of spices and comminuted herbs etc.	 **Extractor-ALAMBIC PRO 2**
3	Spray drying • Air intel • Spray cylinder • Spray nozzle • Aspiration filter • Cyclone chamber • Collecting vessel • Pump	Spray drying is a very fast method of drying due to the very large surface area created by the atomization of the liquid feed and high heat transfer coefficients generated. The short drying time, and consequently fast stabilization of feed material at moderate temperatures, means spray drying is also suitable for heat-sensitive materials. .	 **GALAXIE Spray Dryer Model 2520**
4	Oven • Temperature controller and adjustable • Motor control push buttons • Low pressure air circulation system	Successful drying depends on heat, air dryness and air circulation.	
5	Extraction system • Extractor • Valve block-control • Hydraulic system • Heat exchanger • Evaporation plant • Extract tank • Filter • Extract concentrator • Solvent vessel	Filling the extractor with hackled solids. The lid opens the whole cross-section of the extraction vessel at the touch of a button whereby an optimum filling is ensured. Batch-extraction of fine-grained solids by leaching with solvents, such as water and ethanol. Free choice of solvent flow direction in the extractor: inflow from the bottom or from top, flow direction also changeable according to pressure. Both, extract and solvent can be re-circulated. Extract filtration: Executed by bottom and lid sieves, if necessary additionally by an external filter. Squeezing the solids: Executed by a solid-squeezer that is integrated into the extract vessel: the sieve bottom is employed as movable press piston and controlled via hydraulics. The extraction good is dehumidified and the solvent loss reduced. Evacuation of the extractor: The extraction vessel is tipped over on a horizontal axis and completely evacuated with the help of the hydraulically shifted sieve bottom – and	 **DIG-MAZ 500 - Extractor**  **Extract concentrator**

		all this in one single working step. The handling of the leached residuals is thereby substantially simplified. Vacuum evaporation: The solid-content of the extract is increased by vacuum evaporation at short holding time. The solvent is condensed, accumulated and can be re-circulated into the extraction process.	
6	Hydraulic press • Hydraulic pump • Vane pump • Pump with axial pistons for the working-phase. • Calibration valve • Double acting cylinder	Most hydraulic presses are driven by one or more cylinders that use fluid pressure.	

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Publications et participation à des congrès

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Antioxidant properties and essential oil composition of *Calamintha grandiflora* L.

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ABSTRACT

Essential oil was isolated from the leaves of *Calamintha grandiflora* L. by hydrodistillation and analysed by gas chromatography–mass spectrometry (GC–MS). The most abundant compounds in *C. grandiflora* essential oil were isomenthone, neo-isomenthol, pulegone and isomenthol, constituting 34.07%, 7.65%, 19.83% and 19.54%, respectively. The residues obtained after hydrodistillation were separated into the solid and liquid fractions, the former one was extracted with acetone, methanol and ethanol, while the latter one was sprayed or freeze dried. Antioxidant potential of extracts was evaluated by $DPPH^{\cdot}$ (batch and HPLC-on-line) and $ABTS^{\cdot+}$ radical scavenging, ferric ion reduction (FRAP) assays and by the effect on oil peroxidation in Oxipres apparatus. The content of total phenolic compounds, flavonoids and flavonols was determined spectrophotometrically. Rosmarinic and salvianolic C acids were identified as the main antioxidants in *C. grandiflora*.

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1. Introduction

Aromatic and medicinal plants have been used for food flavouring, preservation, nearly all-medicinal therapies and other applications since prehistoric time. Nowadays, such plants remain an important source for natural aromas and some other ingredients for foods, nutraceuticals and cosmetics, while over 80% of developing world population still rely on herbal remedies for primary healthcare. Search of new natural bioactive compounds for nutraceuticals, pharmaceuticals, cosmetics and other products has led to the increase in bio-prospecting, i.e. collection, investigation and utilisation of diverse biological resources. It is important that the plants are renewable resources of biologically active compounds, while the biodiversity of chemical profiles of plants offers a vast list of phytochemicals which has to be properly assessed. Therefore, the studies of plant chemical composition and discovery of new components are of great interest for scientists and society (Augustyniak et al., 2010). Many medicinal and aromatic plants contain large amounts of antioxidants, such as polyphenols, which may play an important role in adsorbing and neutralising free radicals, quenching singlet and triplet oxygen, or decomposing hazardous peroxides. Consumption of plant phytochemicals possessing antioxidant potential have been associated with lower occurrence of several human diseases and lower mortality rates (Anderson et al., 2001).

Herbs are often defined as any part of a plant that is used in the diet for its aromatic properties; however, they were also reported as a source of various bioactive phytochemicals, particularly possessing high antioxidant power (Kähkönen et al., 1999; Velioglu, Mazza, Gao, & Oomah, 1998). Volatile fractions of plants, traditionally called essential oils, were also studied for their antioxidative properties (Botsoglou, Grigoropoulou, Botsoglou, Govaris, & Papageorgiou, 2003; Lagouri & Boskou, 1995; Ruberto & Baratta, 2000) and were proposed as potential substitutes for synthetic antioxidants (Mimica-Dukić, Bozin, Soković, Mihajlović, & Matavulj, 2003).

C. grandiflora is an aromatic plant of the Lamiaceae family possessing a strong and penetrating mint odour, which is actually used in fresh state as a condiment in French "*Nouvelle Cuisine*". Volatile compounds of this herb from southern European regions were investigated; however, the publications on *C. grandilfora* properties and chemical composition are very scarce. Pulegone was reported as the major compound in *C. grandiflora* from Greece and France, whereas isopinocamphone was the dominating constituent in the plants collected in Turkey (Baser & Özek, 1993; Carnat, Chossegros, & Lamaison, 1991; Souleles & Argyriadou, 1990). The reports on antioxidative activity of *C. grandiflora* were not found in the available literature sources. Agricultural and industrial residues and/or by-products obtained during processing of plant raw materials, e.g. during their distillation for essential oil recovery (the wastes may constitute up to 99.5% of the raw material) may represent potential natural sources for antioxidants and other valuable substances. However, currently such residues are under exploited. Considering this fact the aims of this study were evaluation of essential oil composition of *C. grandiflora* from Midi-Pyrenées region of south-west of France and especially assessment of hydrodistillation by-products as a possible source of antioxidants.

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2. Materials and methods

2.1. Plant material and reagents

The leaves of large flowered calamint (*Calamintha grandiflora* L. Moench.) were collected in Midi-Pyrenées (south-west of France) in May, 2009 at flowering vegetation phase, which is considered as a preferable time in terms of phytochemical composition and functional properties of herbs.

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH$^{\cdot}$, 95%), anhydrous sodium carbonate, gallic acid, Trolox 97%, 2,6-*di*-*tert*-butylhydroxytoluene (BHT), rutin were from Sigma–Aldrich Chemie (Steinheim, Germany); 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diamonium salt (ABTS), standard Folin–Ciocalteu's phenol reagent, KCl, NaCl, Na_2HPO_4, and $K_2S_2O_8$ were from Merck (Darmstadt, Germany); KH_2PO_4 was from Jansen Chimica (Beerse, Belgium); acetone was from Standard (Lublin, Poland); methanol, 98% acetic acid were from Lachema (Brno, Czech Republic) and ethanol was from Stumbras (Kaunas, Lithuania). 2,4,6-tripyridyl-*s*-triazine (TPTZ) were from Fluka Chemicals (Steinheim, Switzerland), aluminium trichloride hydrate, sodium acetate from Reachim (Riga, Latvia).

2.2. Preparation of essential oil and extracts

Fifty g of air dried leaves were mixed with 500 ml of distilled water and the essential oil was isolated by hydrodistillation in a Clevenger type apparatus over 3 h. The obtained oil was separated from the water and dried over anhydrous sodium sulphate. Isolation of essential oils was performed in duplicate and the samples were stored in a freezer prior to further analysis.

The residues of hydrodistillation were separated into the solid and liquid fractions. The solid residue was dried at 30 °C and extracted with acetone, methanol and ethanol, while the liquid fraction (water extract) was divided into two parts. One was spray-dried and the other one freeze-dried. The yields of the extracts were 5.15 ± 0.10–8.20 ± 0.25% for a liquid fraction (water extracts) and 11.30 ± 0.10%, 14.61 ± 0.35% and 15.80 ± 0.30% for acetone, methanol and ethanol extracts of solid fractions, respectively. The extractions were performed in triplicate.

2.3. Gas chromatography and mass spectrometry (GC–MS)

The essential oil was analysed on a Varian 3900 GC-FID and Agilent 5973 GC–MS using a fused-silica capillary column Agilent DB5 (30 m length, 0.25 mm id., 0.25 μm film thickness). GC oven temperature was programmed from 30 °C (5 min hold) to 250 °C (10 min hold) at 5 °C/min. Diluted in *n*-pentane samples (1 μl, 1/100 v/v) were manually injected at 250 °C in the split mode at a ratio of 1:20. Injection and detection temperature was 250 °C. Identification of volatiles was based on retention indexes calculated by using n-hydrocarbons and mass spectra by computerised matching of essential oil compounds with NIST 2008 and WILEY libraries.

2.4. DPPH$^{\cdot}$ radical scavenging assay

Radical scavenging capacity (RSC) of plant extracts against stable 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH$^{\cdot}$, Sigma–Aldrich Chemie, Steinheim, Germany) was determined spectrophotometrically by a slightly modified method of Brand-Williams and coworkers (Brand-Williams, Cuvelier, & Berset, 1995). The solution of DPPH$^{\cdot}$ in methanol (6.5×10^{-5} M) was prepared daily before measurements on a UV/visible light spectrophotometer Spectronic Genesys 8 (Thermo Spectronic, Rochester, NY) at 515 nm. Extract solutions were prepared by dissolving dry *C. grandiflora* extract in methanol. Two ml of this solution were mixed with 50 μl of different concentration (3, 2.5, 2, 1.5 and 1 mg/ml) extract solution in 1 cm path length disposable microcuvette. The samples were kept in the dark for 30 min at room temperature and then the decrease in absorbance was measured. The absorbance of a blank sample containing the same amount of methanol and DPPH$^{\cdot}$ solution was prepared and measured daily. The experiment was carried out in triplicate. The RSC was calculated by the following formula: $I = [(A_B - A_A)/A_B] \times 100$, where I is DPPH$^{\cdot}$ inhibition, %; A_B is the absorbance of a blank sample ($t = 0$ min); A_A is the absorbance of extract solution ($t = 30$ min). The amount of extract required to decrease the initial DPPH$^{\cdot}$ concentration in the reaction by 50% is referred as an effective concentration, IC_{50}.

2.5. ABTS$^{\cdot+}$ radical cation decolourisation assay

The RSC of extracts was also measured by ABTS$^{\cdot+}$ radical cation assay (Re et al., 1999). A stock solution of ABTS (2 mM) was prepared by dissolving in 50 ml of phosphate buffered saline (PBS) obtained by dissolving 8.18 g NaCl, 0.27 g KH_2PO_4, 1.42 g Na_2HPO_4 and 0.15 g KCl in 1 l of ultra pure water. If the pH was lower than 7.4, it was adjusted with NaOH. Ultra pure water was used to prepare 70 mM solution of $K_2S_2O_8$. ABTS$^{\cdot+}$ radical cation was produced by reacting 50 ml of ABTS stock solution with 200 μl of $K_2S_2O_8$ solution and allowing the mixture to stand in the dark at room temperature for 15–16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the assessment of extracts, the ABTS$^{\cdot+}$ solution was diluted with PBS to obtain the absorbance of 0.800 ± 0.030 at 734 nm. Three millilitres of ABTS$^{\cdot+}$ solution were mixed with 30 μl ethanol solution of leaf extract in 1 cm path length microcuvette. The absorbance was read at ambient temperature after 1, 4, 6 and 10 min. PBS solution was used as a blank sample. All determinations were performed in triplicate. The percentage decrease of the absorbance at 734 nm was calculated by formula: $I = [(A_B - A_A)/A_B] \times 100$, where I is ABTS$^{\cdot+}$ inhibition, %; A_B is the absorption of blank sample ($t = 0$ min); A_A is the absorption of extract solution ($t = 10$ min). The RSC was expressed as a percentage of inhibition of Trolox (6-hydroxy-2.5.7.8-tetramethylchroman-2-carboxylic acid) mM equivalent per 1000 g using prepared calibration curve. The extent of quenching of ABTS$^{\cdot+}$ radical by extract was compared with standard amounts of Trolox. The concentrations of Trolox standard used for calibration curve were 0.001, 0.01, 0.1, 0.5, 1, 1.5 mM/l.

2.6. Ferric reducing/antioxidant power (FRAP) assay

The ability of plant extracts to reduce ferric ion to the ferrous one, (FRAP assay) is another indicator frequently used for assessing antioxidant power (Benzie & Strain, 1996). Ferrous ion (Fe^{2+}) produced in this assay forms a blue complex (Fe^{2+}/TPTZ) absorbing at 593 nm. Briefly, the reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \times 6H_2O$ at 10:1:1 (v/v/v). Firstly, 300 μl of freshly prepared FRAP reagent was heated to 37 °C and an absorbance (A_0) of a blank reagent was read at 593 nm in a Biotek EL808 microplate reader (Vermont, USA). Then 10 μl of 0.1% extract solution in water and 30 μl H_2O were added (final dilution of samples in the reaction mixture was 1:34) and the absorbance (A) was recorded every 1 min during the whole monitoring period, which lasted up to 30 min. The change in the absorbance (ΔA_{593} nm) between the final reading and A_0 was calculated for each sample and related to the ΔA_{593} nm of a Fe^{2+} reference solution which was measured simultaneously.

2.7. Analysis of total phenolic compounds (TPC)

The content of TPC in extracts was determined with Folin–Ciocalteu reagent (Folin & Ciocalteu, 1927). A calibration curve was prepared by using 1 ml reference gallic acid solutions in ethanol (0.025, 0.075, 0.100, 0.175 and 0.350 mg/ml), which were mixed with 5 ml of a standard Folin–Ciocalteu reagent and diluted with distilled water (1:10) and 4 ml of 7.5% sodium carbonate solution in distilled water. The absorption was read after 30 min at 765 nm. The concentration of TPC was expressed in mg of gallic acid equivalents (GAE) per g of plant extract. The TPC was calculated by the following formula: $c = c \times V/m$, where c is the concentration of gallic acid, determined form the calibration curve (mg/ml); V is the volume of plant extract (100 ml); m is the weight of pure plant extract (g).

2.8. Determination of flavonoids

The percentage of flavonoids was measured using rutin as a reference (State Pharmacopoeia of USSR., 1989). One millilitre of plant extract solution in 95% ethanol (10 g/l) and 1 ml of aluminium trichloride solution in 95% ethanol (20 g/l) were pipetted into a 25 ml volumetric flask and made up to volume with 95% ethanol. The absorbance was read at 415 nm after 40 min at 20 °C. Blank samples were prepared from the mixture of 1 ml of plant extract and one drop of diluted acetic acid. The absorbance of a reference solution, which was prepared by using 1 ml of rutin solution instead of plant extract, was read simultaneously. Rutin solution was prepared from 0.05 g of dried at 130–150 °C for 3 h rutin, which was diluted in 100 ml of 95% ethanol. All determinations were performed in triplicate. The percentage of flavonoids in plant extracts was calculated by formula: $X = (A \times m_0 \times 100 \times 10)/(A_0 \times m \times 100)$, where A is the absorbance of extract; A_0 is the absorbance of rutin; m is the weight of pure extract (g); m_0 is the weight of rutin (g).

2.9. Determination of flavonols

The content of flavonols was determined by the previously reported method (Yermakov, Arasimov, & Yarosh, 1987). Series of reference rutin solutions containing 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 mg/ml of rutin were prepared. Two millilitres of such reference were mixed with 2 ml of aluminium trichloride solution (20 g/l) in 95% ethanol and 6 ml of sodium acetate solution in ethanol (50 g/l) were added. The absorbance was read at 440 nm after 2.5 h at 20 °C and the calibration curve on the dependence of the absorbency on the concentration of rutin was drawn. Plant extract samples were prepared under the same conditions by using 2 ml of extract (10 g/l) in 95% ethanol instead of rutin. All determinations were performed in triplicate. The percentage of flavonols was calculated by the formula: $X = (C \times V \times 100)/(m \times 100)$, where C is the concentration of rutin, determined form the calibration curve (mg/ml); V is the volume of plant extract (ml); m is the weight of pure plant extract (g).

2.10. Measurement of the effect of extracts on oil oxidation in Oxipres apparatus

The samples were prepared by mixing sunflower oil with 0.05%, 0.1% and 0.2% of extracts. Five grams of oil were placed in a reactor tube and thermostated at 110 °C under oxygen atmosphere (5 bars) in Oxipres apparatus (Mikrolab, Aarhus, Denmark). Pressure changes which occur due to the absorption of oxygen consumed for oil oxidation were recorded. The protection factor (PF) value of sunflower oil in case of using plants extracts and their antioxidant activity (AA) were calculated by the following formulas: $PF = IP_X/IP_K$; $AA = (IP_X - IP_K)/(IP_{BHT} - IP_K)$, where IP_X is induction period of sample with additive (h); IP_K is induction period of sample without additive (h); IP_{BHT} is induction period of sample with synthetic antioxidant BHT (h).

2.11. On-line HPLC–DPPH assay

The extracts were analysed on a HPLC/UV system supplemented with $DPPH^{\cdot}$ radical scavenging detector. In this system two chromatograms were recorded simultaneously, one of which representing UV absorbance of effluent at 265 nm prior to the reaction, while a mirror chromatogram was obtained recording absorbance at 517 nm after reaction of effluent with $DPPH^{\cdot}$ solution in the reaction coil. The mobile phase was supplied to the column by a model 9012 HPLC gradient pump (Varian, USA) at flow rate 0.75 ml/min. The samples of 20 μl were injected into the HPLC system by means of Cheminert C1 injector (Valco Instruments, USA). Reverse-phase LiChroSpher RP-18e 5 μm 12.5 × 0.4 cm column and 0.5 × 0.4 cm precolumn (Merck, Germany) were used for separation. The $DPPH^{\cdot}$ reagent was prepared by dissolving 0.01 M $DPPH^{\cdot}$ in 0.1 M sodium citrate buffer (pH 7.6), methanol and acetonitrile (50:25:25 v/v). It was continuously supplied into a reaction coil (3 m of 0.25 mm id fused-silica capillary) by a model 2200 HPLC pump (Bischoff, Germany) at a flow rate of 0.75 ml/min. Signals were acquired at 265 and 517 nm wavelengths by means of Linear 206 PHD and Linear UVIS 200 UV–VIS detectors, respectively. Solution A (bidistilled water with 0.05% TFA) and B (methanol with 0.05% TFA) were used as the mobile phase components for gradient elution. Extracts were separated using the following gradient: 10% of B at 0 min, 25% of B at 5 min, 40% of B at 25 min, 95% of B at 40 min, 95% of B at 43 min and 10% of B at 44 min. Clarity chromatography software (DataApex, Czech Republic) was used for data acquisition.

2.12. HPLC and LC–MS analysis

LC system consisted of a Thermo-Fisher Spectra System (TFSP, San Jose, CA) P1000XR pump, a TFSP 6000LP Photodiode Array Detector and a TFSP AS 3000 autosampler. Separation of compounds was performed on a Varian Pursuit XRs 5 C18 column (250 mm × 4.6 mm ID, 5 μm) using a linear gradient of 0–30 min., 3–97% of acetonitrile (A) to double distilled water with 0.1% TFA (v/v) (B), 30–35 min., 20–80% of A to B, 35–45 min., 3–97% of A to B. Flow rate was 1 ml/min and 10 μl of the sample was injected.

Mass spectra were acquired using a Thermo-Fisher LCQ mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an atmospheric pressure chemical ionisation source (APCI) using both positive and negative ion mode. The APCI source operating parameters were as follows: the capillary and APCI vaporizer temperatures were set to 250 and 450 °C, respectively, and the spray was stabilised with nitrogen sheath and auxiliary gas (80 and 25 arbitrary units, respectively). Discharge current was 5 μA and capillary voltage was +15 and −15 V in the positive and negative ion mode, respectively. The mass spectra were acquired in the data dependent mode with wideband activation (i.e. the most intense ion obtained for each scan in the full mass spectrum is further submitted to 38% collision energy for MS/MS).

2.13. Statistical analysis

The values are expressed as a means of 3 replicates with standard deviations (SD). Correlation coefficients (R) to determine the relationship between two variables, different RSC tests, RSC tests and content of TPC, flavonoids and flavonols were calculated using MS Excel software (CORREL statistical function).

3. Results and discussion

3.1. Essential oil composition

The yield of *C. grandiflora* essential oil was 0.25 ± 0.07%. It was reported previously that the yield of oil obtained by steam distillation from dried leaves of *C. grandiflora* was approximately 0.35% (El Kalamouni, Dobravalskytė, Raynaud, Venskutonis, & Talou, 2010).

In total, 28 compounds were identified in *C. grandiflora* essential oil (Table 1). Oxygenated monoterpenes (>87%) constituted the major fraction, followed by sesquiterpenes (8.7%). The percentage of monoterpene hydrocarbons was only slightly higher than 1.3%. In general, the chemotype of analysed *C. grandiflora* essential oil may be characterised by the oxygenated *p*-menthane skeleton monoterpenes, namely menthone, isomenthone, isomenthol, neo-isomenthol and pulegone. Isomenthone (34.07% of oil content) was the major constituent in *C. grandiflora* oil. The same structures were most abundant in Greek and previously studied French origin plants, however their percentages were different; pulegone in Greek *C. grandiflora* constituted 35.2%, menthone 20.2% and isomenthone 15.2% (Souleles & Argyriadou, 1990), while in the oil from France, pulegone, isomenthone, neo-isomenthone and menthol were found at the concentrations of 27.6%, 24.7%, 23.7% and 10.8%, respectively (Carnat et al., 1991). A completely different *C. grandiflora* chemotype was reported in Kütahya Domaniç-Daritepe region of Turkey; isopinocamphone was the major compound constituting 52.6% in the total oil (Baser & Özek, 1993). It is not surprising because chemical polymorphism is characteristic for many Lamiaceae family species. For instance, 5 chemotypes were identified for another Lamiaceae family species *Thymus pulegioides* previously; some of these chemotypes produced completely different terpene compounds, such as geraniol/geranial/neral, thymol and linalool (Ložienė & Venskutonis, 2005). In addition, the composition of essential oils varies very much depending on annual climatic changes; therefore even within the same chemotype quite remarkable variations in essential oil composition are possible.

3.2. Antioxidant properties of extracts in electron/hydrogen transfer based assays

Valorisation of plant residues after essential oil distillation is an important task. Dry herbal products are mainly composed of non-digestable carbohydrates and their derivatives incorporated into the cell walls, which in their native form are of a little value for food and/or nutraceutical applications. However, non-volatile secondary metabolites may be of interest for such applications and therefore one of the main tasks of this study was to assess antioxidant potential and the amount of polyphenolics in *C. grandiflora* hydrodistillation residue, which actually may be considered as some kind of 'herbal soup' consisting of solid and liquid fractions. It should be said that in some cases deodorised plant extracts produced from the essential oil free material were reported as possessing higher antioxidant power than the extracts isolated from the whole material (Bandonienė, Venskutonis, Gruzdienė, & Murkovic, 2002; Povilaitytė, Venskutonis, & R., 2000). The composition of hydrodistillation fractions will depend on the solubility of plant constituents in boiling water, which is a high polarity solvent. The results of RSC of extracts in $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ reactions as well as their reducing power in FRAP assay are listed in Table 2.

The RSC measurements in $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ assays are easy, rapid and sensitive methods and therefore most frequently applied for the preliminary assessment of antioxidant potential of various natural substances. Although the basic principles are similar, the $ABTS^{\cdot+}$ scavenging assay is preferable for its ability to evaluate RSC of both lipophilic and hydrophilic antioxidants. IC_{50} values of *C. grandiflora* extracts in $DPPH^{\cdot}$ assay varied from 1.10 (AE) to 0.23 (ME) mg/ml, while TEAC values in $ABTS^{\cdot+}$ reaction were from 0.11% (AE) to 0.73% (SD). It is evident that lower polarity acetone

Table 1
Composition of *C. grandiflora* essential oil (GC area%).

No	Compounds	RI[a]	RI[th]	Peak area%	Aroma characterisation (Burdock, 2005)
1	α-Pinene	934	939	0.03	Pine, turpentine-like
2	Sabinene	973	975	0.19	
3	β-Pinene	980	979	0.21	Turpentine with dry, woody, or resinous aroma
4	Myrcene	986	991	0.09	Pleasant, sweet, balsamic, plastic odour
5	3-Octanol	993	991	0.25	Sweet, oily, nutty, warm, herbaceous odour
6	Limonene	1029	1029	0.71	Pleasant, lemon-like
7	cis-β-Ocimene	1033	1037	0.08	
8	Terpinolene	1070	1089	0.03	Sweet, pine odour with somewhat sweet, citrus flavour
9	Linalool	1099	1091	0.04	Floral
10	p-Menth-8-en-3-ol	1150	1150	0.24	
11	Menthone	1158	1153	4.40	Mint-like
12	Isomenthone	1169	1162	34.07	Peppermint
13	Isomenthol	1181	1183	7.65	Minty
14	Neo-isomenthol	1193	1187	19.83	Minty
15	Pulegone	1243	1237	19.54	Pleasant, somewhat similar to peppermint and camphor
16	Piperitone	1255	1253	1.28	Camphor-like odour, sharp, minty flavour
17	Isomenthyl acetate	1304	1305	0.03	
18	Copaene	1362	1377	0.04	
19	β-Bourbonene	1369	1388	0.13	
20	β-Caryophyllene	1397	1409	1.99	Terpene odour, midway between that of cloves and turpentine
21	Santalene	1443	1447	0.37	
22	Germacrene-D	1477	1485	3.55	
23	Bicyclogermacrene	1495	1500	1.78	
24	α-Farnesene	1501	1506	0.11	
25	γ-Cadinene	1518	1514	0.12	Pungent
26	Isoeugenyl acetate	1578	1568	0.93	Weak, rose-carnation, somewhat spicy, clove-like odour and initially burning, then sweet taste
27	Caryophyllene oxide	1584	1583	0.43	
28	α-Cadinol	1655	1654	0.20	
			Total	98.32	

th- relative retention indices (literature values (Adams, 2007)).
[a] Retention indices relative to C8–C22 alkanes on a nonpolar DB-5 column.

Table 2
Antioxidant characteristics of *C. grandiflora* extracts measured by FRAP, $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ scavenging assays.

Extract type	Yield, % (w/w)	FRAP, mg/ml	$DPPH^{\cdot}$, IC_{50} mg/ml	$ABTS^{\cdot+}$ scavenging effect (%)	TEAC concentration of extract (%) equivalent 1 mmol Trolox
Acetone (AE)	11.30 ± 0.10	0.68 ± 0.08	1.10 ± 0.01	5.90 ± 0.03	0.11 ± 0.01
Methanol (ME)	14.61 ± 0.35	0.61 ± 0.06	0.23 ± 0.01	18.67 ± 1.07	0.51 ± 0.02
Ethanol (EE)	15.80 ± 0.30	0.59 ± 0.03	0.22 ± 0.00	23.56 ± 1.10	0.66 ± 0.03
Freeze dried (FD)	8.20 ± 0.25	0.34 ± 0.02	0.22 ± 0.00	23.79 ± 0.05	0.67 ± 0.02
Spray dried (SD)	5.15 ± 0.10	0.24 ± 0.03	0.18 ± 0.01	25.76 ± 2.74	0.73 ± 0.03

Table 3
Total amount of plant phenolic compounds, flavonoids and flavonols in *C. grandiflora* extracts.

Plant extracts	Total phenolic compounds, mg/g plant extract (in GAE)	Total flavonoids, mg/g plant extract (in RE)	Total flavonols, mg/g plant extract (in RE)
Acetone (AE)	28.01 ± 0.12	1.80 ± 0.03	1.18 ± 0.01
Methanol (ME)	64.80 ± 0.22	4.30 ± 0.09	0.88 ± 0.02
Ethanol (EE)	82.80 ± 1.02	2.44 ± 0.17	0.75 ± 0.01
Freeze dried (FD)	58.90 ± 0.72	0.79 ± 0.05	0.03 ± 0.00
Spray dried (SD)	72.78 ± 1.11	1.24 ± 0.05	0.10 ± 0.01

extract was several times weaker radical scavenger than polar alcoholic extract. It is in agreement with many previously published results which showed that polar solvents extract more antioxidants from botanicals than lower polarity solvents (Bandonienė et al., 2002; Dapkevicius et al., 2002; Povilaitytė & Venskutonis, 2000). Methanol and ethanol as organic solvents are quite similar, however, the ethanol extract was stronger radical scavenger than methanol in $ABTS^{\cdot+}$ reaction. Moreover, ethanol is a food grade solvent and from this point of view is preferable to the toxic methanol. It is also interesting noting that the RSC of alcoholic extracts obtained from the solid residue were quite similar to the RSC of water extracts. Meaning that some antioxidatively active compounds do not dissolve in boiling water and still remain in the solid residue.

FRAP assay is a versatile method and can be readily applied to both aqueous, alcohol and acetone extracts of different plants. In this assay, the antioxidant activity is determined on the basis of the ability to reduce ferric (III) iron to ferrous (II) iron and the results are expressed in mg ferrous iron equivalents per ml of sample. In FRAP assay a ferric salt (Fe III) is used as an antioxidant and its redox potential (0.70 V) is comparable to that of $ABTS^{\cdot+}$ (0.68 V), therefore there is not much difference between TEAC assay and FRAP assay (Huang, Ou, & Prior, 2005). However, the results obtained by these two assays for *C. grandiflora* extracts were rather different. The ferric reducing power of different extracts was in the range of 0.24–0.68 mg Fe (II)/ml (Table 2) and the most remarkable difference was observed for the AE, which was several times weaker antioxidant in TEAC assay, while in FRAP assay AE was the strongest reducing agent. ME and AE produced from the solid hydrodistillation residue of plant were remarkably stronger antioxidants than dried water extracts. The observed differences between $DPPH^{\cdot}$/$ABTS^{\cdot+}$ and FRAP methods can be explained by different solvent polarities and pH in the reaction media (Koleva, Van Beek, Linssen, Groot, & Evstatieva, 2002).

Application of Folin–Ciocalteu reagent gained popularity and is commonly known as the total phenols assay, although the standard reagent used in this method measures a sample's reducing capacity (Huang et al., 2005). The content of phenolic compounds was determined from regression equation of a calibration curve ($y = 8.3166x + 0.2207$, $R^2 = 0.9985$) and expressed in gallic acid equivalents (GAE); in *C. grandiflora* extracts it was from 28.0 mg/g in AE to 82.8 mg/g in EE (Table 3). The concentration of TPC was lower in freeze dried samples than in spray dried. Most likely, the reason for this finding is that the yield of FD was higher than SD and phenolic compounds in FD were more diluted. It is in agreement with previously obtained results for blackcurrant buds (Dvaranauskaitė et al., 2008). It can be observed that the content of phenolics in the extracts correlates with their antiradical activity; strong correlation was observed between total phenolics and $DPPH^{\cdot}$ ($R^2 = 0.8204$) and $ABTS^{\cdot+}$ scavenging capacity ($R^2 = 0.8023$),

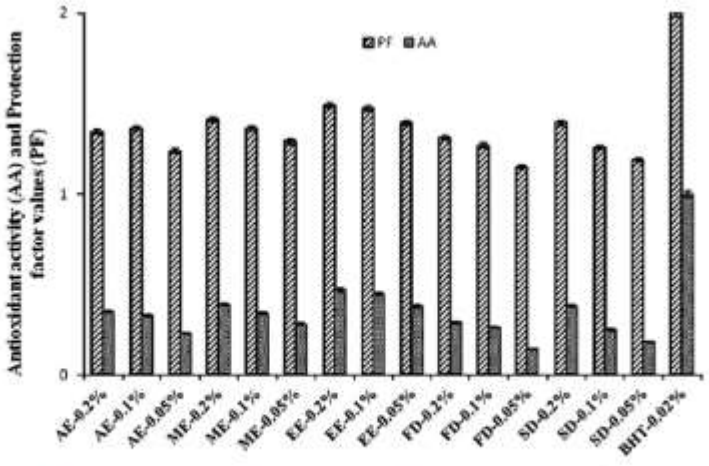


Fig. 1. Antioxidant activity (AA) and protection factors (PF) of *C. grandiflora* extracts in of sunflower oil.

confirming that phenolic compounds are important contributors to the antioxidant properties of these extracts. However, the correlation between TPC and FRAP was very weak (R^2 = 0.157), which suggests that in FRAP assay nonphenolic compounds may play more important role.

Flavonoids are important group of phenolic compounds; therefore, their concentration was assessed separately, by exploring the ability of flavonoids to form yellow colour chelate type internal complexes with Al^{3+}, which may be measured spectrophotometrically. The content of flavonoids, in rutin equivalents, varied from 0.79 (FD) to 4.30 mg/g (ME) Table 3. The concentration of flavonoids was higher in alcoholic extracts than in AE. Relatively low amounts of flavonoids were determined in aqueous extracts of *C. grandiflora*, which contained high amount of total phenolics and the differences between FD and SD again may be explained by the differences in extract yield. There was no correlation between the amount of flavonoids, total amount of phenolics and radical scavenging.

The content of flavonols was also estimated spectrophotometrically and it was found that the concentration of this class of compounds, expressed in rutin equivalents varied from 0.03 to 1.18 mg/g of plant extract (regression equation of a calibration curve $y = 6.0727x + 0.0504$, $R^2 = 0.9929$). The highest amounts were measured in the AE extract, while in water extracts this class of

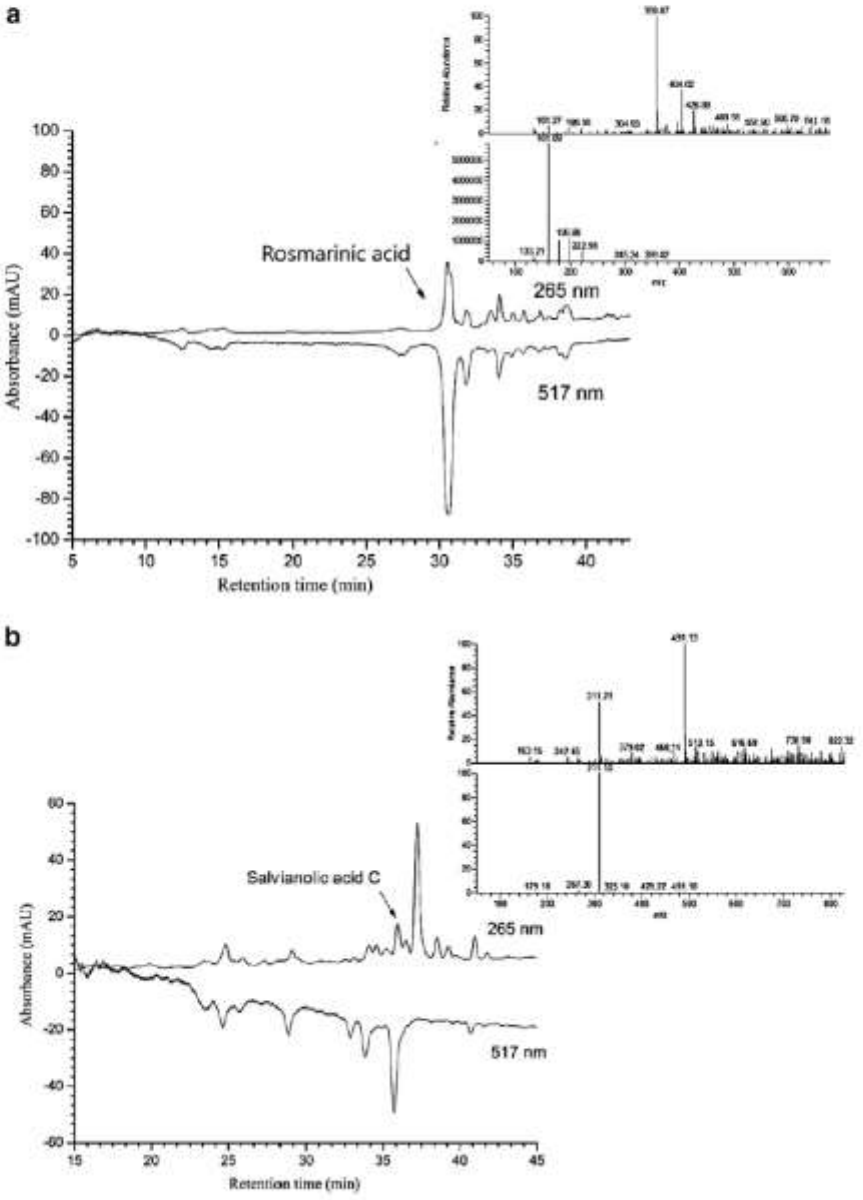


Fig. 2. On-line HPLC-DPPH· scavenging assay profile *C. grandiflora* extracts: (A) EE extract, (B) FD extract.

compounds was present in trace concentrations. Flavonols are known as important compounds in terms of radical scavenging properties. It may be observed that the extract with higher content of flavonols showed lower IC_{50} values in DPPH· assay. However, in case of *C. grandiflora* the content of total flavonoids and flavonols measured by using spectrophotometric method can not be considered as a reliable indicator of the total antioxidant capacity. The structures of individual constituents need to be elucidated and assessed in order to obtain more comprehensive information.

3.3. Antioxidant activity of extracts in sunflower oil

Edible oils containing a high percentage of polyunsaturated fatty acids are very sensitive to auto- and photo-oxidation. So far as auto-oxidation proceeds via radical formation chain reaction, the compounds able to transfer electron and/or hydrogen may retard the process of lipid oxidation. Various methods exist to assess the effects of antioxidants on lipid oxidation, however, the Oxipres method, which was used to evaluate the antioxidant activity (AA) of extracts in our study, is a very convenient procedure as it is performed without using any chemicals. The change of oxygen pressure in the reaction vessel at the end of the induction period indicating rapid formation of hydroperoxides can be quite precisely measured.

Antioxidant activity of *C. grandiflora* essential oil was reported previously and PF at the concentration of 0.2% was 1.63 (El Kalamouni et al., 2010). In case of adding plant extracts at 0.05%, 0.1% and 0.2% concentrations to sunflower oil, the pressure drop was delayed for some period of time, indicating the antioxidative effect of the added substances (Fig. 1). However, the effect of 0.2% EE extract on the stability of sunflower oil during accelerated oxidation was two times lower as compared with 0.02% synthetic antioxidant butylated hydroxytoluene (BHT). Different concentrations of each extract were tested to receive more information about antioxidant properties of *C. grandiflora* extracts in sunflower oil. In general, all extracts showed oil stabilising effects, which increased with increasing concentration of the extract in the oil. The effectiveness of the 0.2% concentration of plant extracts decreases in the following order: EE > ME > SD > AE > FD.

3.4. Assessment of radical scavengers by the on-line HPLC-DPPH· method

The on-line RP-HPLC-DPPH· method can be used for a rapid assessment of radical scavenging properties of separated in chromatographic column compounds, which are present in complex mixtures such as plant extracts (Koleva, Niederländer, & van Beek, 2000; Wu, Huang, Tung, & Chang, 2007). The HPLC/UV/DPPH· chromatograms of EE and FD extracts (Fig. 2) indicate that several separated compounds are able to scavenge radicals. Although this study was not aimed at the comprehensive identification of individual components in the extracts, the most abundant compound in EE was identified as rosmarinic acid, which is a well known natural antioxidant. In LC–MS spectra rosmarinic acid (RT = 30.6), which possessed a very high RSC (49.8% of total area of the peaks) in a positive ionisation mode gave m/z at 359 and characteristic fragmentation at m/z 161.

High amount of radical scavenger eluted from the *C. grandiflora* FD water extract at approximately 35.9 min. Radical scavengers may also be observed in the chromatogram of FD extract, one of the strongest radical scavengers was identified as salvianolic acid C, which possessed a very high RSC (30.4% of total area of the peaks). In LC–MS spectra its peak produced m/z 491 and characteristic fragmentation at m/z 311.

To the best of our knowledge rosmarinic acid and salvianolic acid C were not previously reported in *C. grandiflora*. Thus, these results showed that the residue of water extracts from *C. grandiflora* possesses antioxidant activity and this finding might be considered for applications in food, cosmetic and other relevant products as a way to protect them against oxidative deterioration.

4. Conclusions

Summarising all data obtained, it can be concluded that *C. grandiflora* examined in our study contain valuable biologically active components, such as antioxidants and volatile aroma compounds, which could be found wider practical application in different areas, particularly in formulation and production of food additives, functional food components and healthy supplements. Due to moderate antioxidant activities, development of plant products for practical uses should be focused on a complex exploitation of all valuable properties characteristic to the plants. Such approach would help increasing the feasibility of practical implementation in terms of agro-technological, economical, processing and application issues.

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Antioxidant Properties and Composition of Deodorized Extracts of *Tussilago farfara* L.

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Abstract: Residues obtained after isolating essential oil very often constitute more than 99% of the total raw material. Such residues are poorly exploited although they may represent a potential sustainable source for valuable natural products. This study investigated antioxidant properties and the composition of bioactive compounds (total phenolics, flavonoids, flavonols) present in the deodorized extracts of *Tussilago farfara* flowers and stems collected in Lithuania and South of France, which were isolated with acetone, methanol or ethanol. Online HPLC/UV/DPPH scavenging assay showed that among 8 identified by HPLC/MS compounds, dicaffeoylquinic acids and quercetin pentoside were the major radical scavengers in the *T. farfara* extracts.

Keywords: *Tussilago farfara* L.; Deodorized extracts; Antioxidants; Radical-scavenging capacity.

1. Introduction

There is an increasing demand for natural bioactive compounds which may be used in foods, nutraceuticals, pharmaceuticals, cosmetics and other products; therefore comprehensive bio-prospecting, i.e. collection, investigation and utilisation of diverse biological resources remain an important scientific and practical task. Plant kingdom is a tremendous renewable resource of biologically active compounds, whereas biodiversity of plant chemical profiles offers a vast list of phytochemicals which has to be properly assessed. Therefore, the studies of plant chemical composition and discovery of new components are of great interest for scientists and society [1]. The processing of plant origin raw materials results in the production of by-products that may be rich sources of natural products, including antioxidatively active phenolic compounds [2]. The availability of phenolic compounds from agricultural and industrial residues, their extraction and antioxidant activity were reviewed more than 10 years ago [3]. Phenolic compounds with antioxidant activity were reported in various agricultural by-products, such as rice [4], buckwheat [5] and almond hulls [6].

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The residues obtained during essential oil distillation may constitute up to 99.5% of the raw material and therefore represent a potential source for antioxidants and other valuable substances, however currently such residues are under exploited. The content of phenolic and other valuable compounds in plants depends on several factors, such as cultivation zone, climatic conditions, vegetation phase, genetic pecularities. Therefore, the composition and the quality of plant material grown in various sites and geographical zones may be remarkably different [7]. *Tussilago farfara* (Asteraceae), commonly called coltsfoot has been traditionally used as a medicinal herb to treat lung ailments, such as asthma as well as various coughs by way of smoking. *T. farfara* leaves and flowers possess expectorant activity and are used for chronic dry cough and various pulmonary diseases [8]. Volatile constituents of *T. farfara* were studied previously, while to the best of our knowledge the residues remaining after their distillation from the plant were not evaluated until now [9-10]. The extracts of *T. farfara* were shown to exhibit various activities, such as antioxidant and antimicrobial as well as inhibitory effects on NO synthesis in LPS-activated macrophage and diacylglycerol acyltransferase activity [11-12]. Some phenolics, mucopolysaccharides and water-soluble polysaccharides were isolated from the flower buds of *T. farfara* [13], however the presence of flavonoids has not been reported until now.

It should be noted that besides beneficial bioactive compounds present in the herb, it was found to contain toxic pyrrolizidine alkaloids (PAs), mainly senkirkine, which was recognised by The International Agency for Research on Cancer (IARC) as possessing "sufficient or limited evidence" for the carcinogenicity [14]. As a result, in order to minimize the amount of toxic PAs ingested, the German health authorities limited the daily intake of toxic PAs to 1 μg [15]. Most recently Committee on Herbal Medicinal Products recommended to keep exposure to PAs as low as practically achievable, as recommended by IPCS 1988, EFSA 2007, BfR 589 2007 [16]. To achieve this requirement some free of the toxic compounds *T. farfara* clones were selected and introduced [17].

Deodorized plant residues may contain a number of bioactive compounds, which was shown by the studies of some other species [18]. So far as there is no information on the properties of *T. farfara* by-products, the main aim of this study was to evaluate the antioxidant potential of *T. farfara* deodorized residues obtained after hydrodistillation of flowers and stems collected from two different geographical origins.

2. Materials and Methods

2.1. Plant material

The flowers and stems of coltsfoot (*Tussilago farfara* L.) were collected in Midi-Pyrenées (south-west of France 43° 36′ 16.2″ N/ 1° 26′ 38.4″ E) and in Kaunas Botanical Garden of Vytautas Magnus University, Lithuania (54° 52′ 14″ N/ 23° 54′ 40″ E), during April, 2010 at flowering phase. Climatic conditions in both locations were favourable for the plant development; however the mean temperature and the amount of rainfalls were higher in Midi-Pyrenées (10.5°C and 971 mm) than in Kaunas (6.9°C and 340 mm). The plants were dried at ambient temperature in a ventilated room.

2.2. Preparation of extracts

The volatile compounds were removed by hydrodistillation and the remaining solid residue was dried at 30 °C and extracted with acetone, methanol or ethanol; the extracts obtained are further referred as AE, ME and EE, respectively. The yield of the extracts varied from 10.3 to 12.8% (w/w). The extractions were performed in triplicate.

2.3. DPPH˙ radical scavenging assay

Radical scavenging capacity (RSC) of *T. farfara* extracts against stable 2.2-diphenyl-2-picrylhydrazyl hydrate (DPPH˙, Sigma-Aldrich Chemie, Steinheim, Germany) was determined on a UV/Vis spectrophotometer Spectronic Genesys 8 (Rochester, USA) at 515 nm by a slightly modified method of Brand-Williams and co-workers [19]. Extract solutions were prepared by dissolving dry *T.*

farfara extract in methanol. The solution of DPPH$^{\bullet}$ in methanol (6.5×10^{-5} M) was prepared daily before measurements. Two mL of this solution were mixed with 50 µL of extract solution in a 1 cm path length microcuvette and after 30 min the decrease in absorbance was read. Blank sample with the same amount of methanol and DPPH$^{\bullet}$ was prepared and measured daily. The RSC was calculated by the following formula: I=[(AB-AA)/AB]×100, where I is DPPH$^{\bullet}$ inhibition, %; AB is the absorbance of a blank sample (t=0 min); AA is the absorbance of extract solution (t=30 min). The amount of extract required to decrease the initial DPPH$^{\bullet}$ concentration in the reaction by 50% is referred as an effective concentration, IC_{50}. The measurements were carried out in triplicate for each solvent.

2.4. ABTS$^{\bullet+}$ radical cation decolourisation assay

The RSC of extracts was also measured by ABTS$^{\bullet+}$ radical cation assay [20]. Stock solution of ABTS (2 mM) was prepared by dissolving in 50 mL of phosphate buffered saline (PBS) obtained by dissolving 8.18 g NaCl, 0.27 g KH_2PO_4, 1.42 g Na_2HPO_4 and 0.15 g KCl in 1 L of ultra-pure water. If the pH was lower than 7.4, it was adjusted with NaOH. Ultra-pure water was used to prepare 70 mM solution of $K_2S_2O_8$. ABTS$^{\bullet+}$ radical cation was produced by reacting 50 mL of ABTS stock solution with 200 µL of $K_2S_2O_8$ solution and allowing the mixture to stand in the dark at room temperature for 15–16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the assessment of extracts, ABTS$^{\bullet+}$ solution was diluted with PBS to obtain the absorbance of 0.800±0.030 at 734 nm. Three mL of ABTS$^{\bullet+}$ solution were mixed with 30 µL ethanol solution of *T. farfara* extract in 1 cm path length microcuvette. The absorbance was read at ambient temperature after 1, 4, 6 and 10 min. PBS solution was used as a blank sample. All determinations were performed in triplicate. The percentage decrease of the absorbance at 734 nm was calculated by the formulae: I=[(AB-AA)/AB] × 100, where I is ABTS$^{\bullet+}$ inhibition, %; AB is the absorption of blank sample (t=0 min); AA is the absorption of extract solution (t=10 min). The RSC was expressed as a percentage of inhibition of Trolox (6-hydroxy-2.5.7.8-tetramethylchroman-2-carboxylic acid) mM equivalent per 1000 g using prepared calibration curve. The extent of quenching of ABTS$^{\bullet+}$ radical by extracts was compared with standard amounts of Trolox. The concentrations of Trolox standard used for calibration curve were 0.001, 0.01, 0.1, 0.5, 1, 1.5 mM/L.

2.5. Ferric reducing antioxidant power (FRAP)

The ability of plant extracts to reduce ferric ion (FRAP assay) is another indicator frequently used for assessing antioxidant power [21]. Ferrous ion (Fe^{2+}) produced in this assay forms a blue complex (Fe^{2+}/TPTZ) absorbing at 593 nm. Briefly, the reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3\times6H_2O$ at 10:1:1 (v/v/v). Firstly, 300 µL of freshly prepared FRAP reagent was heated to 37 °C and an absorbance (A_0) of a blank reagent was read at 593 nm in a Biotek EL808 microplate reader (Vermont, USA). Then 10 µL of 0.1% extract solution in water and 30 µL H_2O were added (final dilution of samples in the reaction mixture was 1:34) and the absorbance (A) was recorded every 1 min during the whole monitoring period which lasted up to 30 min. The change in the absorbance (ΔA593 nm) between the final reading and A_0 was calculated for each sample and related to the ΔA593 nm of a Fe^{2+} reference solution which was measured simultaneously.

2.6. Determination of total phenolic compounds (TPC)

The content of TPC in extracts was determined with Folin–Ciocalteu reagent [22]. Calibration curve was prepared by using 1 mL reference gallic acid solutions in ethanol (0.025, 0.075, 0.100, 0.175 and 0.350 mg/mL), which were mixed with 5 mL of a standard Folin-Ciocalteu reagent and diluted with distilled water (1:10) and 4 mL of 7.5% sodium carbonate solution in distilled water. The absorption was read after 30 min at 765 nm. The concentration of TPC was expressed in mg of gallic acid equivalents (GAE) per g of plant extract.

2.7. Determination of flavonoids

The percentage of flavonoids was measured using rutin as a reference [23]. One mL of plant extract solution in 95% ethanol (10 g/L) and 1 mL of aluminum trichloride solution in 95% ethanol (20 g/L) were pipetted into a 25 mL volumetric flask and made up with 95% ethanol. The absorbance was read at 415 nm after 40 min at 20 °C. Blank samples were prepared from the mixture of 1 mL of plant extract and 1 drop of diluted acetic acid. The absorbance of a reference solution, which was prepared by using 1 mL of rutin solution instead of plant extract, was measured simultaneously. Rutin solution was prepared from 0.05 g of dried at 130-150 °C for 3 h rutin, which was diluted in 100 mL of 95% ethanol. All determinations were performed in triplicate. The percentage of flavonoids in plant extracts was calculated by formula: $X=(A\times m_o\times 100\times 10)/(A_o\times m\times 100)$, where A is the absorbance of extract; A_o is the absorbance of rutin; m is the weight of pure extract (g); m_o is the weight of rutin (g).

2.8. Determination of flavonols

The content of flavonols was determined by the previously reported method [24]. Series of reference rutin solutions containing 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 mg/mL of rutin were prepared. Two mL of reference were mixed with 2 mL of aluminum trichloride solution (20 g/L) in 95% ethanol and 6 mL of sodium acetate solution in ethanol (50 g/L) were added. The absorbance was read at 440 nm after 2.5 h at 20 °C and the calibration curve on the dependence of the absorbency on the concentration of rutin was drawn. Plant extract samples were prepared under the same conditions by using 2 mL of extract (10 g/L) in 95% ethanol instead of rutin. All determinations were performed in triplicate. The percentage of flavonols was calculated by the formula: $X=(C\times V\times 100)/(m\times 100)$, where C is the concentration of rutin, determined form the calibration curve (mg/mL); V is the volume of plant extract (mL); m is the weight of pure plant extract (g).

2.9. On-line radical scavenging assay (HPLC/UV/DPPH$^{\bullet}$)

The extracts were analyzed on a HPLC/UV system supplemented with DPPH$^{\bullet}$ radical scavenging detector. Two chromatograms were recorded simultaneously, one of which representing UV absorbance of effluent at 265 nm prior to the reaction, while a mirror chromatogram was obtained at 517 nm after reaction of the effluent with DPPH$^{\bullet}$ solution in the reaction coil. The mobile phase was supplied to the column by a model 9012 HPLC gradient pump (Varian, USA) at a flow rate 0.75 mL/min. The samples (20 µL) were injected into the HPLC system by means of Cheminert C1 injector (Valco Instruments, USA). Reverse-phase LiChroSpher RP-18e 5 µm 12.5×0.4 cm column and 0.5×0.4 cm precolumn (Merck, Germany) were used for separation. The DPPH$^{\bullet}$ reagent was prepared by dissolving 0.01 M DPPH$^{\bullet}$ in 0.1 M sodium citrate buffer (pH=7.6), methanol and acetonitrile (50:25:25 v/v). It was continuously supplied into a reaction coil (3 m of 0.25 mm id fused-silica capillary) by a model 2200 HPLC pump (Bischoff, Germany) at a flow rate of 0.75 mL/min. The signals were acquired at 265 and 517 nm wavelengths by means of Linear 206 PHD and Linear UVIS 200 UV-VIS detectors, respectively. Solution A (bidistilled water with 0.05% TFA) and B (methanol with 0.05% TFA) were used as a mobile phase components for gradient elution. The extracts were separated using the following gradient: 10% of B at 0 min, 25% of B at 5 min, 40% of B at 25 min. 95% of B at 40 min. 95 % of B at 43 min and 10% of B at 44 min. Clarity chromatography software (DataApex. Czech Republic) was used for data acquisition.

2.10. LC-MS analysis

LC system consisted of a Thermo-Fisher Spectra System (TFSP, San Jose, CA) P1000XR pump, a TFSP 6000LP Photodiode Array Detector and a TFSP AS 3000 autosampler. Separation of compounds was performed on a Varian Pursuit XRs 5 C18 column (250 mm×4.6 mm ID, 5 µm) using a linear gradient of 0–30 min., 3–97% of acetonitrile (A) to bidistilled water with 0.1% TFA (v/v) (B), 30–35 min., 20–80% of A to B, 35-45 min., 3-97% of A to B. Flow rate was 1 mL/min and 10 µL of the sample was injected.

Mass spectra were acquired using a Thermo-Fisher LCQ mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an atmospheric pressure chemical ionization source (APCI) using both positive and negative ion mode. The APCI source operating parameters were as follows: the capillary and APCI vaporizer temperatures were set to 250 °C and 450 °C, respectively, and the spray was stabilized with nitrogen sheath and auxiliary gas (80 and 25 arbitrary units, respectively). Discharge current was 5 μA and capillary voltage was +15 V and −15 V in the positive and negative ion mode, respectively. The mass spectra were acquired in the data dependent mode with wideband activation (i.e. the most intense ion obtained for each scan in the full mass spectrum is further submitted to 38% collision energy for MS/MS).

2.11. Statistical analysis

For antioxidant activity measurements, mean values and standard deviations were calculated from at least three replicates using MS Excel 2003. Statistical analysis was performed by using one-way analysis of the variance (ANOVA), followed by the Duncans' post hoc test to compare the means showing significant variation ($p<0.05$). All the data were subjected to variance analysis using the GLM procedure of SAS [25]. Analyses were performed using MSStat (ANALYT MTC, Muehlheim, Germany, MS Statistical Software Version 3.02u); looking for differences in the plant origins.

3. Results and Discussion

3.1. Antioxidant properties of extracts in electron/hydrogen transfer based assays

$DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging and FRAP assays were used for assessing antioxidant activity of *T. farfara* extracts (Table 1). These methods are simple and widely used for the fast screening of plant antioxidant properties and they provide quite reliable preliminary information on the presence of antioxidatively active constituents in the extracts. In general, the reaction is based on the ability of radicals to accept either an electron or hydrogen atom. The extracts were isolated consecutively by using increasing polarity solvents, acetone, methanol or ethanol. Ethanol and methanol are quite similar protonic solvents; however both of them were tested because ethanol is more acceptable for the isolation of food and pharmaceutical grade ingredients. The results demonstrated that extract RSC was dependent on the solvent polarity, the type of free radical used in the reaction and the origin of plant material. The IC_{50} values of *T. farfara* extracts in $DPPH^{\bullet}$ assay were from 0.15 (EE) to 0.27 (AE) mg/mL for the extracts isolated from the plants grown in Lithuania and 0.22 (EE) to 0.39 (AE) mg/mL for the plants grown in France, showing that EE possessed stronger RSC than AE and ME in this assay. In this case the extracts isolated with polar solvents were stronger $DPPH^{\bullet}$ radical scavengers, which is in agreement with previously published results [26-28]. However, EE extracts were less effective in scavenging $ABTS^{\bullet+}$ than AE and ME. Although the principle of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays is similar, the latter is applicable both for lipophilic and hydrophilic antioxidants; in addition $DPPH^{\bullet}$ can be dissolved only in organic solvents (e.g., methanol), while $ABTS^{\bullet+}$ is soluble in both aqueous and organic media. Therefore, $ABTS^{\bullet+}$ assay can be performed in hydrophilic and lipophilic systems [20].

FRAP assay is a versatile method and can be readily applied to aqueous, alcohol and acetone extracts of different plants. In this assay, the antioxidant activity is determined on the basis of the ability to reduce ferric (III) iron to ferrous (II) iron and the results are expressed in mg ferrous iron equivalents per mL of sample. In FRAP assay, similarly to $ABTS^{\bullet+}$, acetone extracts were significantly stronger antioxidants than ME and EE. The ferric reducing antioxidant power of different extracts was in the range of 0.44–0.52 mg Fe (II)/mL for Lithuanian origin plants and 0.39-0.62 mg Fe (II)/mL for French origin plants. In FRAP assay a ferric salt (Fe III) is used as an antioxidant and its redox potential (0.70 V) is comparable to that of $ABTS^{\bullet+}$ (0.68 V), therefore the results of TEAC and FRAP assays showed similar trends [29].

Table 1. Antioxidant characteristics, the content of total phenolic compounds, flavonoids and flavonols in the extracts isolated from *T. farfara*

Characteristics	Lithuania			France		
	Acetone (AE)	Methanol (ME)	Ethanol (EE)	Acetone (AE)	Methanol (ME)	Ethanol (EE)
$DPPH^{\bullet}$ scavenging (IC_{50}), mg/mL	0.27 ± 0.00^{a}	0.20 ± 0.01^{a}	0.15 ± 0.00^{a}	0.39 ± 0.01^{b}	0.35 ± 0.02^{b}	0.22 ± 0.01^{b}
$ABTS^{\bullet+}$ scavenging, %	31.7 ± 0.60^{b}	28.3 ± 0.20^{b}	19.6 ± 0.30^{b}	17.2 ± 0.25^{a}	16.0 ± 0.10^{a}	13.1 ± 0.15^{a}
TEAC, %, equivalent 1mmol Trolox	0.92 ± 0.03^{b}	0.81 ± 0.01^{b}	0.55 ± 0.02^{b}	0.46 ± 0.01^{a}	0.45 ± 0.00^{a}	0.34 ± 0.01^{a}
FRAP, mg/mL	0.52 ± 0.00^{a}	0.45 ± 0.00^{a}	0.44 ± 0.00^{b}	0.62 ± 0.02^{b}	0.47 ± 0.01^{a}	0.39 ± 0.01^{a}
Total phenolic compounds, mg GAE/g	94.8 ± 0.35^{a}	94.6 ± 0.22^{b}	66.4 ± 0.15^{b}	123.2 ± 0.12^{b}	66.2 ± 0.30^{a}	48.8 ± 0.22^{a}
Total flavonoids, mg RE /g	9.20 ± 0.17^{a}	5.95 ± 0.03^{b}	3.25 ± 0.09^{b}	9.18 ± 0.03^{a}	5.12 ± 0.09^{a}	2.20 ± 0.07^{a}
Total flavonols, mg RE /g	0.70 ± 0.01^{b}	0.53 ± 0.02^{b}	0.39 ± 0.01^{b}	0.49 ± 0.01^{a}	0.27 ± 0.02^{a}	0.19 ± 0.01^{a}

Superscript letters indicate if the values for the extracts isolated with the same solvent from Lithuanian and French origin plants are statistically different

Comparing antioxidant activity indicators it may be observed that the extracts isolated from Lithuanian origin *T. farfara* in most cases possessed higher RSC than French origin plants, although in FRAP assay these differences were negligible. The differences in climatic conditions (the amount of rainfall during plant development was nearly three times less abundant in Lithuania than in France, the average temperature was also lower in Lithuania) might have the impact on these differences, however, other factors, such as plant chemotype, soil, sunlight may also have the impact on plant properties and composition. Determination of the effects of these factors on *T. farfara* properties would require special experimental design which was beyond the scope of this study.

3.2. Content of total phenolic compounds (TPC), flavonoids and flavonols

Phenolic compounds are very important plant antioxidants due to the presence of hydroxyl groups in their structure. It was established that phenolic compounds are the major constituents with antioxidant activity in many plants which are able to adsorb and neutralize the free radicals [30]. Among them flavonoids is the most abundant group of natural constituents found in various plants, which exhibit antioxidant activity through radical scavenging or chelating mechanisms [31]. The content of polyphenols, flavonoids and flavonols in the extracts isolated from two origin plants by three different solvents is presented in Table 1. The highest concentration of all three compound groups was in AE followed by ME and EE except for ME isolated from *T. farfara* from Lithuania; it contained similar amount of TPC to AE. It is interesting noting that AE of French origin plants contained higher amount of TPC than AE of Lithuanian origin *T. farfara*, while the content of TPC in ME and EE was higher in Lithuanian origin plants. However, the sums of TPC in AE+ME and AE+EE were similar (189.4 mg/g) or very close (161.2 and 171.0 mg/g) for both origin plants. So far as acetone and alcohol were used as consecutive extraction solvents it may be suggested that individual phenolic compounds are present in the plants of two origins at different proportions. The content of flavonoids, expressed in rutin equivalents was from 2.20 to 9.48 mg/g. In general, these results are in agreement with previously published data [26-27]. It is obvious that acetone does not provide exhaustive extraction of phenolic compounds, flavonoids and flavonols from the solid plant residue obtained after hydrodistillation; polar solvent should be applied as a second solvent.

The RSC and reducing capacity of plant extracts may serve as an indicator of potential antioxidant activities through the action of breaking the free radical chain by donating hydrogen atom [32]. Usually the content of TPC measured in the extracts isolated from whole plant material is in a good correlation with RSC of such extracts. However, for *T. farfara* extracts isolated from the

hydrodistillation residue such correlations were more complex. Strong correlation (R^2) was observed between TPC, total flavonoids and FRAP; 0.8341 and 0.7964, respectively, suggesting that phenolic compounds might be important contributors to the antioxidant properties of these extracts. However, the correlation between TPC, total flavonoids and RSC was very weak, less than 0.5: in DPPH$^{\bullet}$ assay 0.269 and 0.3803, respectively; in ABTS$^{\bullet+}$ assay 0.1905 and 0.3036, respectivelly. The correlation coefficient between total flavonols and FRAP (0.3368) or DPPH$^{\bullet}$ (0.005) was found to be very weak, but strong correlation was observed between total flavonols and RSC in ABTS$^{\bullet+}$ assay (0.7949).

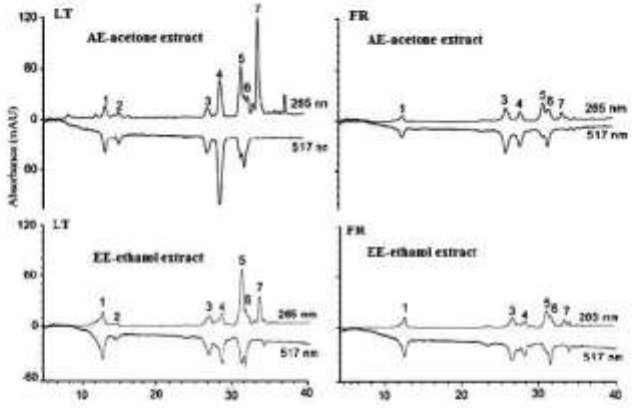


Figure 1. HPLC–UV–DPPH chromatograms of acetone (AE) and ethanol (EE) extracts of *T. farfara* from Lithuanian (LT) and French origin (FR).

3.3. Assessment of radical scavengers by the on-line HPLC-DPPH$^{\bullet}$ method

Flavonoid glycosides quercetin-3-O-β-L-arabinopyranoside and quercetin-3-O-β-D-glucopyranoside [33] as well as quinic, chlorogenic and dicaffeoylquinic acids were reported previously in *T. farfara* flower bud extract from China [11]. The on-line HPLC-DPPH$^{\bullet}$ method was used for the detection of radical scavenging components. The ESI-MS of the AE, ME and ET of *T. farfara* extracts from two origins (Figure 1) were qualitatively different. Compounds 3 and 4 showed [M-H]- signal at *m/z* 515 with fragmentation at *m/z* 353 and 179, from which a molecular formula $C_{25}H_{24}O_{12}$ was assigned. The ion at *m/z* 353 indicated a chlorogenic acid fragment derived from the loss of a caffeoyl group. The ion at *m/z* 179 indicated fragments of caffeic acid moieties. These MS data indicated that the compounds 3 and 4 were dicaffeoylquinic acid isomers. HPLC/MS data was not sufficient to determine exact isomers of dicaffeoylquinic acid; it is known that both of them are common components of *T. farfara* and exhibit antioxidant activity [11]. Usually, the location of the caffeoyl groups in dicaffeoylquinic are at C-3'; C-4' or C-3'; C-5'.

The ESI-MS of the *T. farfara* extracts from Lithuania gave ions corresponding to: 1-(*m/z* 191)-quinic acid, 2-(*m/z* 354)-chlorogenic acid, 3-4 (*m/z* 515)-dicaffeoylquinic acid, 5-(*m/z* 609)-quercetin-3-rutinoside (rutin), 6-(*m/z* 434)-quercetin-pentoside and 7-(*m/z* 447)-kaempferol-glucoside. In the extracts of French origin chlorogenic acid was not detected. Quinic, chlorogenic and dicaffeoylquinic acids and rutinoside were previously isolated from the flower buds of *T. farfara* [11], whereas, to the best of our knowledge, quercetin-pentoside and kaempferol-glucoside were not previously reported in this plant. In general, the RSC of *T. farfara* extracts from the plants grown in Lithuania was slightly higher than those of French origin (Table 1). The profiles of chromatograms of *T. farfara* extracts from different growing locations obtained by HPLC–DPPH$^{\bullet}$ method were also different (Figure 1): the number of negative peaks in the extracts of Lithuanian *T. farfara* origin was higher. Dicaffeoylquinic acids and quercetin-pentoside were major active compounds with total RSA 39.6, 10 and 17.1% (AE from Lithuania) and 16.9, 13.8 and 13.1% (AE from France), respectively (Table 2). Phytochemical composition of plant secondary metabolites depends on several factors, such as cultivation area, climatic conditions, vegetation phase, genetic peculiarities and others; therefore evaluation of plant properties from different geographical regions was in the focus of numerous studies [30-31].

Table 2. Composition and radical scavenging capacity of *Tussilago farfara* constituents evaluated by the on-line HPLC-UV-DPPH$^{\bullet}$ method

Plant origin	Active compound	Acetone extract			Methanol extract		Ethanol extract	
		RT, min	Peak area, mV×s	% of total RSC	Peak area, mV×s	% of total RSC	Peak area, mV×s	% of total RSC
FR	Quinic acid	13.1	365.3	8.4	7.2	0.4	718.8	18.3
	CGA	-	-	-	-	-	-	-
	diCQA	26.8	150.9	16.9	863.3	46.3	871.4	22.2
	diCQA	28.6	1226.2	13.8	645.3	44.9	513.6	13.1
	Rutin	31.3	539.8	9.8	-	-	261.1	6.6
	Q-pent	31.9	1159.3	13.1	58.0	6.3	800.5	21.6
	K-3-O-glc	34.2	862.9	5.8	12.4	0.7	154.5	3.9
LT	Quinic acid	12.9	458.3	8.3	387.9	9.4	1054.8	15.9
	CGA	15.0	185.3	3.6	208.0	6.3	126.8	2.6
	diCQA	26.7	915.6	10.0	699.3	12.6	834.1	11.7
	diCQA	28.5	3385.3	39.6	917.7	23.4	1657.7	24.9
	Rutin	31.1	756.6	12.5	411.7	11.5	942.9	18.9
	Q-pent	31.9	1464.6	17.1	987.1	15.7	1065.4	16.0
	K-3-O-glc	34.2	87.3	1.0	141.7	2.8	141.5	4.2

CGA: chlorogenic acid, diCQA: dicaffeoylquinic acid, Q-pent: quercetin-pentoside, K-3-O-glc: kaempferol-glucoside.

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NATIVE VS EXTRACTED ESSENTIAL OIL: FROM CHEMICAL COMPOSITION TO BIOLOGICAL ACTIVITIES

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1 INTRODUCTION

Aromatic, medicinal and spicy plants are an immense and sustainable source of natural compounds with various beneficial properties. Such materials have been used since ancient times for various applications, particularly healing of diseases, flavouring of foods and formulation of fragrances. Some of these plants are now grown commercially and produce a variety of ingredients. However, some medicinal and aromatic herbs for some reason have not found wide application and sometimes are referred to as 'forgotten plants'. Taking into account the increasing demand for natural ingredients that might be used as food additives, components of functional foods and nutraceuticals as well as for other applications, it is reasonable to revisit the 'forgotten plants' to assess their applicability and benefits using modern scientific methods. The Midi-Pyrenées region of southwest France has a rich, but sometimes forgotten wild plant heritage, including a remarkable diversity of aromatic and medicinal plants. Recently, the MIPAROM Trek initiative has been conducted to collect and analyse various regional and 'forgotten' aromatic plants, including such species as *Tanacetum balsamita, Myrrhis odorata, Calamintha grandiflora* and *Monarda didyma*. The first phase in the assessment of these plants includes analysis of their essential oils, antioxidative properties and antimicrobial activity. The essential oil composition and properties of plants grown in various regions of the world and selected for this investigation have been studied previously. For instance, it was reported that the essential oil of *T. Balsamita* grown in Iran contained carvone and β-thujone as the main constituents; the results of the antimicrobial activity of the essential oil, according to the disc diffusion method and minimal inhibition concentration (MIC) values, indicated that the oil exhibited moderate to high antimicrobial activity against eight Gram-positive and Gram-negative bacteria (*Bacillus subtilis, B. pumulis, Enterococcus faecalis, Staphylococcus aureus, S. epidermidis, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) and three fungi (*Candida albicans, Saccharomyces cerevisiae* and *Aspergillus niger*).[1] In another study, the essential oil of *T. balsamita* subsp. *Balsamita* from Turkey was found to contain *trans*-chrysanthenol, chrysanthenyl acetate, linalool oxide, camphor and 1,8-cineole as the major components. The antimicrobial activities of the oil and two antibiotics against Gram-positive and Gram-negative bacteria and two yeasts were investigated *in vitro*, and the results showed the oils to possess moderate

activities against all test microorganisms.[2] This example clearly shows that a remarkable diversity in the chemical composition may be characteristic of the same plant species, particularly of those grown in different parts of the world. The chemical composition and antimicrobial activities of essential oil isolated from *M. odorata* were investigated against seven bacterial and six fungal species. Activity against bacteria and *C. albicans* was investigated using the bioautographic test on TLC plates, while all the other fungi were tested using the microdilution test. *M. odorata* oil showed the lowest effect compared to commercial products.[3] The composition of *C. grandiflora* essential oil had been previously studied.[4,5] However, no reports were found in the available literature sources on the antimicrobial and antioxidant properties of this species. Oxidative and microbial deterioration are the main processes damaging huge amounts of foods. Lipid-containing foods can be stored for a limited time period because of their insufficient resistance to oxidation. Moreover, in dry foods, which are more stable from the microbiological point of view, oxygen can easily enter the lipid phase, and the stability of such foods in many cases is substantially lower than in the presence of water. Therefore, the stability of dry foods should be carefully tested and, if necessary, the lipid fraction should be protected by adding antioxidants. During storage of oils, fats and other fat-containing foods, lipid oxidation is still a major cause of food quality deterioration in spite of wide use of several antioxidants. The most widely used synthetic antioxidants, *tert*-butylhydroxyanisole (BHA) and *tert*-butylhydroxytoluene (BHT), are quite volatile compounds and easily decompose at high temperatures.[6] There are some serious questions concerning the safety and toxicity of some synthetic antioxidants related to their metabolism and possible absorption and accumulation in body organs and tissues.[7] Therefore, development of preparations based on the antioxidative properties of natural compounds is highly desirable. The antioxidant extracts isolated from various herbs were reported to be effective in retarding the development of rancidity in oils and fatty foods; however, the antioxidant activity of these extracts depends on the concentration and chemical structure of active antioxidant components in the raw materials. Since ancient times, aromatic plants have been widely used as flavourings. During the last few years they have also become a source of natural antioxidants and antibacterial agents.[8] Essential oils are considered among the most important antimicrobial agents present in plants and may also possess antioxidant and anti-inflammatory activities. The increasing antibiotic resistance of some pathogens that are associated with diseases is another major concern. Therefore, there has been increasing interest in developing new, effective and non-toxic antimicrobial compounds. Several authors have proposed essential oils as natural preservative agents.[9]

In the present study, a preliminary screening of antioxidant and antimicrobial activities of Midi-Pyrenées aromatic plants grown in France, namely *Tanacetum balsamita, Calamintha grandiflora, Myrrhis odorata* and *Monarda didyma*, was carried out. Essential oils from these plants were added to sunflower oil, and oxidative deterioration was measured by the Oxipress method. The antimicrobial activities of the essentials oils were evaluated against nine pathogenic bacteria present in various foods.

We have designed an apparatus,[10] to mimic the crushing of leaves between your fingers as done by farmers or flavorists to sensorially evaluate the odor of aromatic plants. This so-called Artificial Crushing Finger (ACF) device was based on a modified artificial mouth and allows us to perform a rapid evaluation of the essential oil with only a couple of leaves using a controlled Dynamic Headspace (DHS) concentration of the volatiles emitted by the crushed fresh leaves. In the present paper we report the comparison of the chromatographic profiles of essential oil of leaves of our model plant, *Calamintha grandiflora*,[11] with the ones obtained with leaves processed by ACF-DHS.

2. MATERIALS AND METHODS

2.1 Volatile Analysis

The ACF was swept with nitrogen (60 mL/min). The crushing of leaves was set up using a motorized Teflon piston having transverse and rotative movements. Emitted volatiles were then concentrated on a Tenax trap located at the top of the device during 10 minutes; three replicates were performed.

2.1.1 Static Headspace (ACF/SPME)

Two entire fresh leaves of *Calamintha Grandiflora* were placed in the glass reactor of the ACF. Volatiles emitted after crushing were absorbed during 10 min using a SPME syringe equipped with a Carboxen/PDMS/DVB fiber. Three replicates were performed.

2.1.2 Gas Chromatographic Analysis

Volatile analysis (SPME) was performed on a Varian 3900 gas chromatograph equipped with FID and an Agilent 5973 Network GC-MS. (DHS/ACF, EO) analysis was performed with a Perkin Elmer Clarus 500 GC-MS-FID equipped with a TurboMatrix TD thermodesorber. A fused-silica DB5 column (0.25 mm x 30 m, 0.25 µm film thickness) was used throughout this study. Temperature program used was: 30°C for 5min, then 5°C/min up to 200°C and 10 min isotherm at 200°C. Identification of volatiles was based on comparison of their retention times with those of authentic samples (calculated RI based on n-hydrocarbons series kit) and on computerized matching with a commercial mass spectra data bank (NIST 2005 and Wiley)

2.2 Plant Materials

The leaves of costmary, also known as alecost, balsam herb, Bible leaf (*Tanacetum balsamita* L.), cicely or sweet cicely (*Myrrhis odorata* L. Scop.), large flowered calamint (*Calamintha grandiflora* L. Moench.) and bergamot, also known as scarlet beebalm, scarlet monarda, oswego tea, or crimson beebalm (*Monarda didyma* L.) were used in the study. The plants were collected in Midi-Pyrenées (south west of France) in April–May 2008 at the vegetation phase which is considered preferable in terms of the functional properties of herbs. Essential oils were isolated from ground plants by hydrodistillation in a Clevenger type apparatus.

2.3 Antioxidant Test

The antioxidant activity of the essentials oils was determined in sunflower oil, using an Oxipress apparatus (Mikrolab Aarhus, Denmark). In the course of oxidation, oxygen consumption is evaluated by measuring changes in its pressure in a hermetically sealed 200 ml vessel containing oxygen and a sample. Five grams of sunflower oil (pure, with essential oils and with the synthetic antioxidant BHT) were placed in the reactor which was connected to the pressurized oxygen cylinder; first, it was washed with oxygen and afterwards filled with oxygen to reach the defined initial pressure (0.5 MPa). The experiments were carried out at a temperature of 110°C in triplicate. The relative standard deviation was in the range of 0.1 to 3%; in rare cases when it was higher, additional measurements were performed. The induction period (*IP*) of oxidation was read at the time

when the pressure began to decrease abruptly (the end was measured from the cross-section point of tangents of the first part and the subsequent part of the curve recording the pressure changes, as evident from Figure 1). The protection factor (*PF*) value of sunflower oil in case of using essential oils and their antioxidant activities (*AA*) were calculated by the following formulas:

$$PF = \frac{IPX}{IPK}$$

$$AA = \frac{IPX - IPK}{IPBHT - IPK}$$

Where IPX is the induction period of a sample with an additive;
IPK is the induction period of a sample without an additive;
IPBHT is the induction period of a sample containing the synthetic antioxidant BHT;

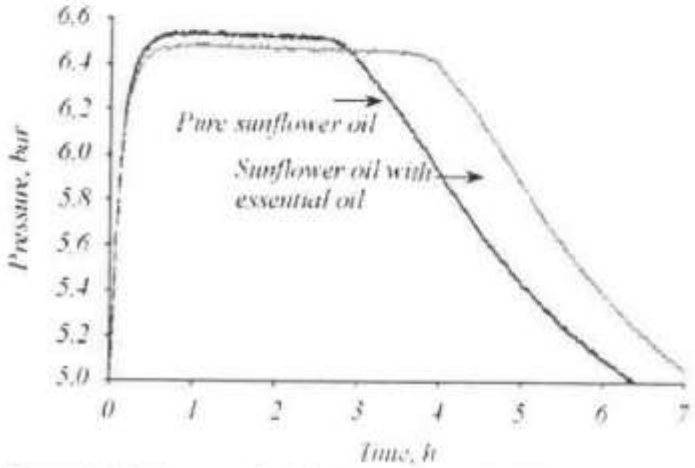


Figure 1: *Sunflower oil oxidation curves at 110 °C*

2.4 Antimicrobial Test

Nine food spoilage bacteria, including Gram-positive, *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and Gram-negative pathogens, namely *Salmonella typhymurium*, *Salmonella typhymurium D338*, *Salmonella enteritidis*, *Salmonella agona* and *Escherichia coli*, were used in this study. The antimicrobial properties were evaluated by the agar diffusion method. Bacteria were grown in plate count agar (CM 325, Oxoid) for 24 h at 37 °C. After cultivation, test culture cells were mixed using an *MSI* mini shaker (Wilmington, USA), and the cell suspensions were adjusted according to McFarland no. 0.5 standard. The suspension of bacteria cells in saline (2 mL) was introduced into the dissolved agar media; 10 mL of each bacteria culture was pipetted into a 90 mm Petri dish. Eight-millimeter diameter wells were pushed in the agar and filled with methanolic solutions of oils. The plates were incubated overnight at 37 °C. A 10 µL volume of 1.0 and 0.5% methanolic solutions of the essential oils was added to the agar wells. Pure methanol was used as a control in the blank samples. After incubation, the

inhibition zones were measured with calipers to an accuracy of 0.1 mm, and the effect was calculated as a mean of three replicate tests. An appropriate reference antibiotic disc was applied on each plate depending on the test micro organisms (ceftazidine/clavulanic acid, 30/10 μg; *BBL, CAZ/CLA, 231753*); they served as a positive control, as they are effective against all species used in the study.

3 RESULTS AND DISCUSSION

The Oxipress method, which was used to evaluate the antioxidant activity of essential oils, is a very convenient procedure performed without any chemicals. Typical curves of oxidation are presented in Figure 1. The change of pressure at the end of the induction period can be rather precisely measured. As shown in Figure 1, when adding plant essential oil to sunflower oil, the pressure drop was delayed for some period of time, indicating the antioxidative effect of the added substance. The effects of the applied essential oils at 0.05, 0.1 and 0.2% concentrations are summarized in Table 1. The essential oil of *M. didyma* was found to be the most effective natural antioxidant measured compared to the other essentials oils tested. The effectiveness of these essentials oils decreased in the following order: *M. didyma* > *C. grandiflora* > *M. odorata* > *T. balsamita*. Essential oils are complex mixtures of volatile natural compounds, and their total antioxidant capacity depends on the presence of the most active components and their concentration in the oil. For instance, such phenolic compounds as thymol, which is present in many essential oils, particularly in those isolated from plants belonging to the genus *Thymus*, possess high antioxidant strength due to the presence of the phenolic hydroxyl group. It was also suggested that the presence of an electron donating group *ortho* to the hydroxyl moiety may be crucial for effective antioxidant activity. *M. didyma* essential oil contained up to 50% thymol which may explain its comparatively high antioxidant activity. The chemical composition of the essential oil obtained from *M. didyma* had been analysed previously. The antioxidant activity of the oil was evaluated by the DPPH test and was found to be comparable to Trolox, and by the lipid peroxidation test where the activity of the oil was similar to that of BHT.[12] Antioxidant activity of the essential oils was dose dependent. In the case of the highest dose (0.2%) of *M. didyma*, *C. grandiflora* and *M. odorata*, it exceeded the antioxidant capacity of BHT added at a concentration of 0.02%. The main components in the essential oils of *C. grandiflora*, *T. balsamita* and *M. odorata* were menthone, β-thujone and anethol, respectively.

Antimicrobial activity of the methanol solutions of essentials oils was evaluated *in vitro* against nine bacteria species known to cause infections in humans (Table 2). Gram-positive bacteria are more sensitive than Gram-negative, especially *Bacillus cereus*, which was the most sensitive species in the case of *M. didyma* essential oil at a concentration of 1%. *M. didyma* oil showed the inhibitory effect against all test cultures. Most likely the high percentage of the strong antimicrobial agent thymol in *M. didyma* essential oil was the main factor for its pronounced antimicrobial activity. It is in agreement with the previously published results; e.g., thymol-rich essential oils from *Thymus pulegioides* were strong antimicrobial substances against various pathogenic bacteria [14]. The fungicidal activity of *Thymus vulgaris* oil correlated also with the content of thymol [15]. The antifungal activity of *M. didyma* oil was evaluated against four phytopathogenic fungi through direct contact using the agar diffusion method and via the fungistatic action of the vapours

Table 1. *Antioxidant activity (AA) of essential oils compared to BHT and their effect on the stability of sunflower oil, expressed in protection factor values (PF)*

Additive	Concentration %	(PF)	Antioxidant Activity (AA)
Without additive	0.00	1.00	-
BHT	0.02	1,47	1,00
Monarda didyma	0,2	1,73	1,55
Monarda didyma	0,1	1,37	0,79
Monarda didyma	0,05	1,29	0,62
Calamintha grandiflora	0,2	1,63	1,34
Calamintha grandiflora	0,1	1,22	0,47
Calamintha grandiflora	0,05	1,18	0,38
Myrhis odorata	0,2	1,61	1,30
Myrhis odorata	0,1	1,2	0,43
Myrhis odorata	0,05	1,17	0,36
Tanacetum balsamita	0,2	1,41	0,87
Tanacetum balsamita	0,1	1,2	0,43
Tanacetum balsamita	0,05	1,1	0,21

Table 2. *Antimicrobial activity of the essential oils at different concentrations, expressed in the diameter of inhibition, mm; na – not active*

Bacterial Species	*M.didyma*		*C.grandiflora*		*M.odorata*		*T.balsamita*		Antibiotic
	1%	0,50%	1%	0,50%	1%	0,50%	1%	0,50%	sensi-disc
Bacillus subtilis	16,5	12,8	19,1	8,5	9,2	8,5	10,2	8,2	16,8
Bacillus cereus	40	13,1	9,8	10,6	10,6	9,9	11,4	8,8	29,0
Staphylococcus aureus	13,1	10,2	9,7	na	8,8	na	9	na	21,0
Staphylococcus epidermidis	14,7	11,5	10	na	9,1	na	8,5	na	25,8
Salmonella typhimurium	15	11	8,9	na	8,1	na	8,4	na	31,2
Salmonella typhymurium D338	16,2	12,2	9,3	8,8	9	na	9,7	na	34,0
Salmonella Enteritidis	15,2	11,5	10,1	9,1	8,1	na	8,9	na	32,2
Salmonella agona	13,8	10,7	8,4	na	na	na	na	na	20,8
Escherichia coli	18,9	14,4	10,3	na	10,2	na	9,7	na	26,4

using the micro-atmosphere method. The most sensitive fungus was *Rhizoctonia solani* in the first test.

T. balsamita and *M. odorata* oils at 0.5% possessed a weaker antimicrobial activity against the majority of test bacteria compared to the other essentials oils applied at the same concentration; they showed antibacterial activity only against *Bacillus cereus* and *Bacillus subtilis*. Comparing *Salmonella* spp., *Salmonella agona* was the most resistant species. The smallest zones of inhibition were measured for most of the applied essential oils. *C. grandiflora* oil applied at a concentration of 1% was the most effective antimicrobial agent against *Bacillus subtilis* compared to the other oils. The inhibitory effect of *M. didyma* oil (1%) against *Escherichia coli, Salmonella enteritidis, Salmonella typhymurium* and *Staphylococcus epidermidis* was about half that of the reference antibiotic compound; however, in case of *Bacillus subtilis*, the effect of *M. didyma* oil was equal to that of the antibiotic compound.

The composition of the yellow colored essential oil of *Calamintha grandiflora* obtained with a yield of 0,35% on a dry weight basis, conformed to that reported in the literature[11] where menthone, isomenthone and neo-isomenthol were the main volatiles (Table 3). Isomenthone was reported as the main compound of the essential oil (34.65%) while menthone was the main volatile identified in crushed leaves using SPME (21.16%) and Tenax (37.81%).

These preliminary results show that GC profiles of the essential oil (Figure 2) and the volatiles from leaves crushed using the ACF (Figures 3-4) had similarities for top and heart notes (menthone, isomenthone as major constituents) but also differences, especially for back note components due to the extraction technique used (hydrodistillation *vs* dynamic headspace).

4 CONCLUSIONS

The ACF (Artificial Crushing Finger) device combined with dynamic headspace sampling demonstrated real potential for the rapid evaluation of essential oils from aromatic plant leaves. Indeed, this novel system gave a fingerprint of fresh plant volatiles with top and heart notes similar to those of the essential oil (qualitatively and quantitatively).

A preliminary screening of the bioactivity of *Tanacetum balsamita, Calamintha grandiflora, Myrrhis odorata* and *Monarda didyma* essential oils showed the volatile fractions of these plants to possess antioxidant activity in vegetable oil and antimicrobial activity against various pathogenic bacteria. These results suggest the need for further study of these plants to assess their wider application in the production of valuable ingredients. Further studies should focus on the non-volatile fraction of the plant material.

Table 3. *Chemical composition of essential oil of Calamintha grandiflora and volatile compounds from crushed leaves. IR: retention Index.*

Identified compounds	IR	% EO	% SPME	% TENAX
Hexanal	853	0,00	2.53	0.00
cis-3-hexanol	870	0.00	0.18	0.00
alpha-thujene	930	0.00	1.46	0.00
alpha-pinene	939	0.03	2.38	6.43
Sabinene	975	0.20	4.17	18.87
beta-pinene	979	0.21	2.06	4.02
Myrcene	991	0.10	2.07	3.56
3-octanol	991	0.26	0.54	0.89
alpha-phellandrene	1003	0.00	0.07	0.00
alpha-terpinene	1017	0.00	0.83	0.00
Limonene	1029	0.72	0.86	4.37
beta-ocimene	1037	0.09	0.00	0.00
gama-terpinene	1060	0.00	1.37	0.00
Terpinolene	1089	0.03	0.58	0.00
Linalool	1091	0.05	0.00	0.00
p-Menth-8-en-3-ol	1150	0.25	0.00	0.00
Menthone	1153	4.48	21.16	37.81
Isomenthone	1162	34.65	0.25	6.67
Isomenthol	1183	7.78	5.75	4.38
neo-isomenthol	1187	20.17	30.17	5.22
alpha-terpineol	1198	0.00	0.00	0.00
Pulegone	1237	19.87	20.73	0.00
Piperitone	1253	1.30	0.38	0.00
Isomenthyl-acetate	1305	0.03	0.00	0.00
Copaene	1377	0.04	0.00	0.00
beta-bourbonene	1388	0.13	0.00	0.00
caryophyllene-Z	1409	2.02	0.67	5.76
Santalene	1447	0.37	0.00	0.00
Germacrene-D	1485	3.61	0.58	1.33
Bicyclo-germacrene	1500	1.81	0.32	0.70
alpha-farnesene	1506	0.11	0.00	0.00
gamma-cadinene	1514	0.12	0.13	0.00
Isogenol-acetate	1568	0.95	0.00	0.00
Caryophyllene-oxide	1583	0.44	0.00	0.00
alpha-cadinol	1654	0.20	0.00	0.00

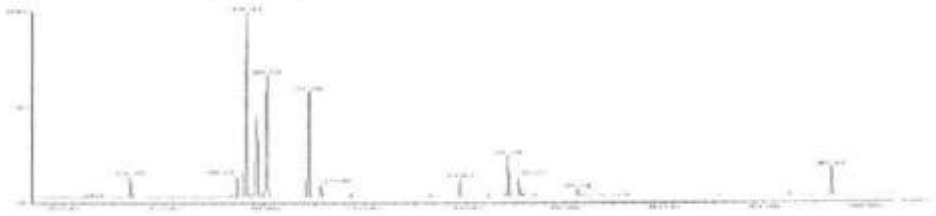


Figure 2. *Aromatic profile of essential oil of Calamintha grandiflora*

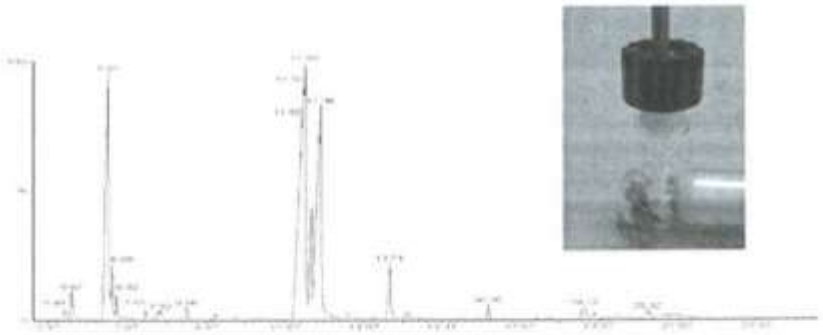


Figure 3. *Aromatic profile of crushed leaves using a Tenax trap*

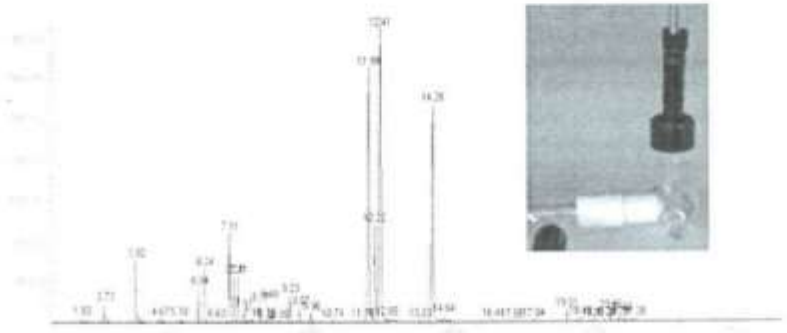


Figure 4. *Aromatic profile of crushed leaves using a SPME*

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ACTIVITY OF NATURAL ANTIOXIDANTS EXTRACTED FROM GREATER CALAMINT, SWEET CICELY AND COLTSFOOT CULTIVATED IN LITHUANIA AND IN FRANCE

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Abstract
The residues obtained after hydrodistillation of three aromatic plants (*Calamintha grandiflora*, *Myrrhis odorata* and *Tussilago farfara*) were separated into solid and liquid fractions. The liquid fraction was dried while the solid fraction was extracted with acetone, methanol or ethanol. Two main types of antioxidant activity tests were employed: i) assays to evaluate oxidation of fats, oils and other fat containing foods (Oxipress); ii) assays to evaluate radical scavenging activity in model systems (DPPH, FRAP). The antioxidant activities were expressed as gallic acid equivalents (GAE) with the aim of standardizing these methods and that allow data comparisons. The three antioxidant assay methods give different antioxidant activity trends. An initial screening of ethanol extracts from the three aromatic plants for antioxidant activity in oil reported them as the best. Despite hydrodistillation residues presented strong antioxidant activities, the total phenolics content in acetone, methanol, ethanol leaves extracts for *Tussilago farfara* was about twice lower than others plants extracts.
Key words: Aromatic plants, antioxidant activity, by-products valorization

Introduction

Agricultural and industrial residues/by-products obtained during processing of raw plants represent potential natural sources for antioxidants that are rarely exploited (wastes could be represent up to 99.5% of the raw material). Taking into account this situation our aims are analysing and evaluating such by-products as possible sources for food antioxidants. Chemical and biological diversity of aromatic and medicinal plants depending on such factors, as cultivation area, climatic conditions, genetic modifications and others is an important impetus to study flora present in different growing sites, countries and geographical zones. Aromatic and medicinal plants are used since ancient times for different purposes, e.g. for flavouring of foods, for preservation and for disguising unpleasant odours. Mostly this is linked with the discovery of synthetic drugs, which usually are more effective. Nevertheless a big part of the world population still widely relies on traditional herbal medicine. Many medicinal plants contain large amounts of antioxidants such as polyphenols, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower occurrence and lower mortality rates of several human diseases (Anderson et al. 2001). Herbs are most often defined as any part of a plant that is used in the diet for its aromatic properties (Hacskaylo, 1996; Davidson and Jaine, 2006; Smith and Winder, 1996). Recently, however, herbs have also been identified as sources of various phytochemicals, many of which possess important antioxidant activity (Velioglu et al., 1998; Larson, 1988; Kähkönen et al., 1999). Two main types of antioxidant activity tests were employed assays to evaluate oxidation of fats and assays to evaluate radical scavenging activity in model systems. This work is aimed at a preliminary screening for radical scavenging activity of extracts from aromatic plants namely *Calamintha grandiflora, Myrhis odorata* and T*ussilago farfara* has been performed using DPPH, FRAP radical scavenging assays as well as the total phenolic content estimated by the Folin-Ciocalteu assay. Residue extracts from these plants were added to sunflower oil, and oxidative deterioration was measured by the Oxipres method.

Materials and Methods

Plant Material. The following plants greater calamint, sweet cicely and coltsfoot (*Calamintha grandiflora, Myrrhis odorata and Tussilago farfara)* were harvested from the collection of

medicinal plants at Kaunas Botanical Garden (Vytautas Magnus University, Lithuania) and in Midi- Pyrénées (south west of France), during April-August, 2009.

Sample Preparation. Preparation of Extracts. The residues (by-product) of hydrodistillation were separated into solid and liquid fractions. The solid residue was dried at 30 °C and extracted with acetone, methanol and ethanol while the liquid fraction (water extract) was divided in two parts, one of which was spray-dried and the other freeze-dried. The yield of extracts varied from 8.0 to 17.1%. The extractions were performed in triplicate.

Evaluation of Antioxidant Activity. DPPH Radical Scavenging Assay. The RSC of aromatic plants extracts against stable 2,2-diphenyl-2-picrylhydrazyl hydrate radical DPPH (Sigma-Aldrich, Germany) was determined by a slightly modified spectrophotometric method of (Brand-Williams et al. 1995). A 2 ml aliquot of $DPPH^{\bullet}$ solution was mixed with a 50 µl of extract solution in 1 cm path length disposable microcuvette. The decreasing absorbance was read during 30 min reaction time at 1 min intervals until the absorbance curve reached the plateau phase. The amount of extract required to decrease the initial $DPPH^{\bullet}$ concentration in the reaction mixture by 50% is referred as an effective concentration, IC_{50}. *FRAP assay.* The ferric reducing power (FRAP) assay was performed in a Biotek EL808 microplate reader (Vermont, USA). The working FRAP reagent was prepared by mixing the acetate buffer (300 mM pH 3.6): 10 mM TPTZ (2.4.6-tripyridyl-s-triazine) (Sigma-Aldrich, Germany) in 40 mM HCl: 20 mM $FeCl_3 6H_2O$ in the ratio 10:1:1 (Benzie and Strain, 1996). Firstly, 300 µl of freshly prepared FRAP reagent was heated to 37 °C and an absorbance (A0) of a blank reagent was read at 593 nm. Then 10 µl of 0.1% sample solution in water and 30 µl H_2O were added (final dilution of samples in the reaction mixture was 1:34) and the absorbance (A) were recorded every 1 min during the whole monitoring period which lasted up to 30 min The change in the absorbance (ΔA593nm) between the final reading and A_0 was calculated for each sample and related to the ΔA593nm of a FeII reference solution which was measured simultaneously.

Total Amount of Phenolic Compounds. The concentration of phenolic compounds in extracts was determined by Folin–Ciocalteu method (Folin & Ciocalteu 1927). For the preparation of a calibration curve 1 ml reference gallic acid solutions in ethanol (aliquots of 0.025, 0.075, 0.100, 0.175, and 0.350 mg ml^{-1}) were mixed with 5 ml of standard Folin-Ciocalteu reagent and diluted with distilled water (1:10) and 4 ml of 7.5% sodium carbonate solution in distilled water. The absorption was read after 30 min at 765 nm. The concentration of phenolics compounds (*C*) was expressed in milligrams of gallic acid equivalents (GAE) per gram of plant extract.

Method of OXIPRES. Infused oil were prepared by mixing sunflower oil with different concentrations of extracts (0.05%, 0.1%, 0.2%) 5 g of infused oil was weighted (nearest 0.001 g) into a reactor tube which then placed thermostated (110 °C) and under oxygen atmosphere (5 bars). Pressure changes were recorded by using OXIPRESS apparatus (Mikrolab Aarhus). The protection factor (*PF*) value of sunflower oil in case of using plants extracts and their antioxidant activities (*AA*) were calculated by the following formulas:

$$PF = \frac{IP_X}{IP_K}; \quad (1)$$

$$AA = \frac{IP_X - IP_K}{IP_{BHT} - IP_K}; \quad (2)$$

where: IP_X – induction period of sample with additive, h;
IP_K - induction period of sample without additive, h;
IP_{BHT} – induction period of sample with BHT, h.

Results and Discussion

The antioxidant activity of the extracts was determined using a DPPH and FRAP scavenging assay. DPPH assay is known to give reliable information concerning the antioxidant ability of the tested compounds (Huang et al., 2005). The principle of the assay is based on the color change of the DPPH solution from purple to yellow as the radical is quenched by the antioxidant (Parthasarathy et al., 2009). The results of DPPH scavenging assay by different plant extracts are summarized in the Table 1. The acetone extracts were the most effective DPPH radical scavengers for *Calamintha grandiflora*. IC_{50} values 0.112 mg ml^{-1}. The IC_{50} values of *Myrhis odorata* ranged between 0.828 and 0.982 mg ml^{-1}. The lowest IC_{50} value (highest antioxidant activity) of 0.049 mg ml^{-1} was obtained for *Tussilago farfara* Leaves-AE (France). The free radical scavenging activity of spray dried water extract (*Calamintha grandiflora*-SD) exhibited slightly lower IC_{50} value against DPPH radicals (0.158 mg ml^{-1}). The DPPH radical seems to be more reactive with leaves acetone extract for *Tussilago farfara* (France). The roots extracts for *Tussilago farfara* had very low antiradical activity comparing with other extracts of blossom and leaves. Methanol and ethanol extracts were more active against the DPPH radical than acetone for *Myrhis odorata*.

Table 1

DPPH scavenging assay of *Calamintha gradiflora*, *Myrhis odorata* and *Tussilago farfara*

Plant extracts	DPPH IC_{50} mg·ml^{-1}	
	Lithuania	France
***Calamintha grandiflora*-AE**	–	0.112±0.004
***Calamintha grandiflora*-MeOH**	–	0.227±0.008
***Calamintha grandiflora*-EtOH**	–	0.221±0.004
***Calamintha grandiflora*-FD**	–	0.217±0.001
***Calamintha grandiflora*-SD**	–	0.158±0.006
***Myrrhis odorata* -AE**	0.982±0.075	0.828±0.067
***Myrrhis odorata* -MeOH**	0.160±0.003	0.140±0.002
***Myrrhis odorata* -EtOH**	0.107±0.001	0.123±0.005
***Myrrhis odorata* -FD**	0.117±0.002	0.115±0.001
***Myrrhis odorata* -SD**	0.241±0.004	0.226±0.003
***Tussilago farfara* Blossom-AE**	0.270±0.001	0.387±0.007
***Tussilago farfara* Blossom-MeOH**	0.201±0.012	0.352±0.039
***Tussilago farfara* Blossom-EtOH**	0.154±0.017	0.219±0.011
***Tussilago farfara* Leaves-AE**	0.103±0.005	0.049±0.003
***Tussilago farfara* Leaves-MeOH**	0.091±0.005	0.234±0.019
***Tussilago farfara* Leaves-EtOH**	0.315±0.006	0.175±0.024
***Tussilago farfara* Roots-AE**	0.877±0.041	0.877±0.021
***Tussilago farfara* Roots-MeOH**	0.184±0.052	0.461±0.029
***Tussilago farfara* Roots-EtOH**	0.231±0.066	0.415±0.031

Based on the result obtained from the analyses DPPH it is important to notice that, there is not significant similitude between the same plants of different climates and country (Lithuania and France), the only important difference is obtained by the use of different solvent extraction. On the other hand, the results obtained from the FRAP analyses showed in the figure 1 above, we can find that the plants located in Lithuania has more antioxidant activity that the plants located in the Midi- Pyrénées zone. The FRAP is versatileand can be readily applied to both aqueous, alcohol and acetone extracts of different plants. In this assay, the antioxidant activity is determined on the basis of the ability to reduce ferric (III) iron to ferrous (II) iron. The results

were expressed as mg ferrous iron equivalents per ml of sample The ferric reducing power (FRAP) assay of the alcohol extracts was in the range of 0.147–0.986 mg Fe (II)/ml (Figure 1).

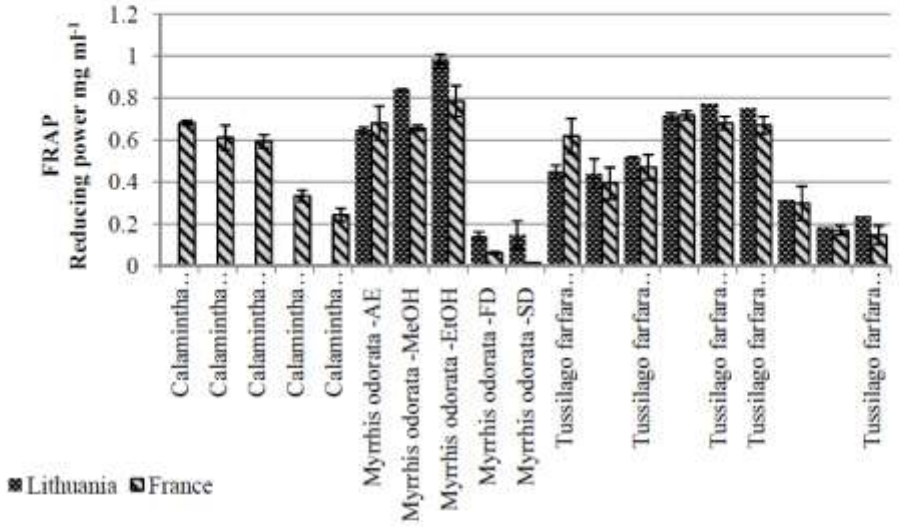


Figure 1. Ferric Reducing Capacity assay of *Calamintha gradiflora*, *Myrhis odorata* and *Tussilago farfara*

For the aqueous extracts, the antioxidant activity ranged from 0.014 to 0.334 mg Fe (II) ml^{-1}. Ethanol, methanol, acetone extracts for plants exhibited highest antioxidant capacity, than the spray dried and freeze dried water extracts.

Table 2

Total phenolic content of *Calamintha gradiflora*, *Myrhis odorata* and *Tussilago farfara*

Plant	Total phenolic compounds, mg g^{-1} plant extract (in GAE)				
	Acetone extract	Methanol extract	Ethanol extract	Freeze-dried extract	Spray-dried extract
Calamintha grandiflora	27.99±0.12	64.79±0.22	82.76±1.02	58.89±0.72	72.78±1.11
Myrrhis odorata (France)	39.19±0.32	85.25±1.32	68.97±0.30	27.25±0.02	55.03±0.04
Myrrhis odorata (Lithuania)	10.78±0.02	62.32±0.08	80.60±0.97	25.89±0.03	35.55±0.06
Tussilago farfara Blossom (France)	123.16±1.25	48.79±0.17	66.23±1.01	-	-
Tussilago farfara Leaves (France)	151.36±1.87	204.57±1.54	131.88±0.99	-	-
Tussilago farfara Roots (France)	13.58±0.01	41.64±0.03	11.30±0.06	-	-
Tussilago farfara Blossom (Lithuania)	94.79±0.98	48.79±0.03	157.25±0.02	-	-
Tussilago farfara Leaves (Lithuania)	102.00±0.01	144.33±1.20	157.25±0.08	-	-
Tussilago farfara Roots (Lithuania)	6.05±0.01	41.08±0.78	38.87±0.65	-	-

The amounts of total phenolic compounds in the herbs are presented in Table 2. The leaves extracts of *Tussilago farfara* possessed approximately twice-higher amounts of phenolics (102–204.57 GAE) than the other plant extracts. However, the total phenolics content in *Calamintha grandiflrora* acetone extract was lower than in methanol, ethanol extracts, and lower than in some other herbs having lower antioxidant activities.

Table 3

Antioxidant activity (*AA*) the extracts of *Myrhis odorata* and *Calamintha grandiflora* as compared with the effect of BHT and their effect on the stability of sunflower oil, expressed in protection factor values (*PF*)

Extracts	Conc %	IP		PF		AA	
		Lithuania	France	Lithuania	France	Lithuania	France
Myrrhis odorata -AE	0.20	2.17	2.19	1.09	1.10	0.08	0.09
	0.10	2.09	2.11	1.05	1.06	0.04	0.05
	0.05	2.05	2.07	1.03	1.04	0.02	0.03
Myrrhis odorata -MeOH	0.20	2.19	2.10	1.10	1.05	0.09	0.05
	0.10	2.07	2.02	1.04	1.01	0.03	0.01
	0.05	2.01	2.00	1.01	1.00	0.00	0.00
Myrrhis odorata -EtOH	0.20	2.30	2.30	1.15	1.15	0.14	0.14
	0.10	2.10	2.20	1.05	1.10	0.05	0.10
	0.05	2.02	2.07	1.01	1.04	0.01	0.03
Myrrhis odorata -FD	0.20	2.36	2.42	1.18	1.21	0.17	0.2
	0.10	2.21	2.34	1.11	1.17	0.10	0.16
	0.05	2.19	2.29	1.10	1.15	0.09	0.14
Myrrhis odorata -SD	0.20	2.51	2.21	1.26	1.11	0.25	0.10
	0.10	2.34	2.02	1.17	1.01	0.16	0.01
	0.05	2.26	2.01	1.13	1.01	0.13	0.00
Calamintha grandiflora-AE	0.20	–	2.48	–	1.24	–	0.35
	0.10	–	2.72	–	1.36	–	0.33
	0.05	–	2.68	–	1.34	–	0.23
Calamintha grandiflora-MeOH	0.20	–	2.81	–	1.41	–	0.39
	0.10	–	2.71	–	1.36	–	0.34
	0.05	–	2.58	–	1.29	–	0.28
Calamintha grandiflora-EtOH	0.20	–	2.98	–	1.49	–	0.47
	0.10	–	2.93	–	1.47	–	0.45
	0.05	–	2.78	–	1.39	–	0.38
Calamintha grandiflora-FD	0.20	–	2.61	–	1.31	–	0.29
	0.10	–	2.54	–	1.27	–	0.26
	0.05	–	2.30	–	1.15	–	0.14
Calamintha grandiflora-SD	0.20	–	2.78	–	1.39	–	0.38
	0.10	–	2.51	–	1.26	–	0.25
	0.05	–	2.37	–	1.19	–	0.18
BHT	0.02	4.07		2.04		1.00	
Sunflower oil	0.00	2.00		1.00		–	

This finding showed that the content of total phenolics in herbs is not a reliable indicator of their antioxidant activity. The structures of the individual constituents need to be elucidated and assessed in order to obtain more precise results and information. The oxipres method, which was used to evaluate the antioxidant activity (AA) of extracts, is a very convenient procedure performed without using any chemicals. The change of pressure at the end of the induction

period can be rather precisely measured. In case of adding plants extracts to sunflower oil, the pressure drop was delayed for some period of time, indicating the antioxidative effect of the added substance. The effects of the applied extracts at 0.05, 0.1 and 0.2% concentrations are summarized in Table 3. The extracts obtained from *Calamintha gradiflora* and *Myrhis odorata* were found to be the most effective natural antioxidants. The effect of extracts on the stability of Sunflower oil during accelerated oxidation was comparable with the effect of butylated hydroxytoluene (BHT) at the same concentration. The extracts of *Calamintha grandiflora* was the most effective natural antioxidant as compared with extracts of *Myrhis odorata*. It has also should be emphasized that no positive correlation was found between the total amount of phenolic compounds and AA. For instance, methanol extract of *Myrhis odorata* which were rich in the total phenolics possessed the lowest AA.

Conclusions

The test result in the DPPH analyses obtained in preliminary investigations show that the type of compounds that have a different antioxidant activity differ depending on the solvent used and do not depend in the zone of origin. This study suggests that the *Myrhis odorata*, *Calamintha gradiflora* and *Tussilago farfara* extracts contain valuable antioxidant active components, which might be helpful in preventing or slowing the progress of various oxidative stresses. We can find a practical application of these plants in different areas, particularly in formulation and production of food additives. It also can be concluded that methanol and ethanol extraction are a suitable way to prepare extracts with antioxidant activity in oil, this conclusion was based in the observations obtained by the analyses. Continues with this investigation a further analyses on the isolation and identification of antioxidants components of the plants mentioned previously will be realised.

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Essential oil composition of *Myrrhis odorata* (L.) Scop. leaves grown in Lithuania and France

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The chemical composition of essential oils of sweet cicely (*Myrrhis odorata* (L.) Scop.) leaves from two geographic areas (Lithuania and France) were determined by gas chromatography/mass spectrometry (GC/MS). Comparatively slight differences were observed in the chemical composition of essential oils from both selected origins. The yield of essential oil varied from 0.4±0.02% in Lithuanian to 0.5±0.01% in French origin. In total twenty and twenty-six components were identified in the hydrodistilled oils of Lithuanian and French origin, respectively. The essential oils of both origins were found to be rich in *E*-anethole (48.1±1.4% and 50.7±1.1%), methyleugenol (14.3±0.5% and 13.1±0.4%), *E*-nerolidol (10.2±0.8% and 12.0±0.3%) and germacrene-D (5.0±0.1% and 4.3±0.3%).

Keywords: *Myrrhis odorata* (L.) Scop.; geographic origin; essential oil composition; *E*-anethole.

Introduction

Sweet cicely (*Myrrhis odorata* (L.) Scop.) is an underrated plant deserving more attention as a culinary herb and as a sugar saver. *Myrrhis odorata* is a species particularly abundant in the mountainous regions of Europe. It has been grown as a medicinal plant in the monasteries, and now it is spread to most of Europe. Sweet cicely is a hardy perennial herb that blooms in spring, usually in May. The flavor is often described as being an intermediate between anise and celery (1). Nowadays, sweet cicely has only a small cooking importance. Since it is tolerant to cold, it is a useful plant in Scandinavia as an aromatic herb providing fresh fragrant leaves nearly all year. Because of the sweet fragrance, cicely fruits are a good substitute for anise, fennel or even licorice; the leaves may be used instead of chervil. *Myrrhis odorata* presents expectorant, diuretic and tonic properties and was proposed for malaria treatment (2).

The chemical composition of aromatic and medicinal plants depends on cultivation area, climatic conditions, vegetation phase, genetic modifications and other factors; therefore characterization of flora present in different growing sites, countries and geographical zones is an important task. Factors that determine the composition and the yield of essential oil are numerous and in some instances it is difficult to segregate these factors from each other, since many are interdependent and influence one another (3). These variables may include seasonal and maturity variation, geographical origin, genetic variation, growth stages, part of plant, post-harvest drying, and storage (3, 4). There are many reports in the literature showing the variation in the yield and chemical composition of the essential oil with respect to geographical regions. For instance, variations in the yield and chemical profile of essential oils were reported for *Rosmarinus officinalis* and *Tagetes minuta* populations, collected from different geographical locations (5–7). Such differences could be linked to the soil textures and possible adaption response of different populations, resulting in different chemical products being formed, without morphological differences being observed in the plants (6). To our knowledge, all studies (1, 8–10) on the essential oil composition of *M. odorata* were carried out on the aerial parts of the plant, and in particular their leaves, by using a hydrodistillation method for oil isolation.

The main aim of this study was to examine the composition of *M. odorata* essential oils of two different origins and to compare with previously published results on this species. To the best of our knowledge, no information is available on the chemical constituents of *M. odorata* essential oils from the plants grown in France and Lithuania.

Experimental

Plant material

The leaves of two accessions of sweet cicely (*M. odorata*) were collected at full flowering stage in

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Saint-Cyr-en-Val, region of France (47° 49′9.09″ N/1° 56′51.3″ E) during July 2009, and in Kaunas Botanical Garden of Vytautas Magnus University, Lithuania (54°52′14″ N/23° 54′40″ E), during August 2009. The range of temperatures was 8.7°–28.1°C for Lithuania and 9.8°–32.6°C in France. The mean temperature and rainfall amount during the month of leaf harvesting were slightly higher in Saint-Cyr-en-Val (19.3°C and 61.6 mm) than in Kaunas (17.8°C and 57.2 mm).

Essential oil extraction

Air-dried leaves (40 g) were mixed with 500 mL of distilled water and the essential oil was isolated by hydrodistillation in a Clevenger-type apparatus for 3 hours. The obtained oil was separated from the water and dried over anhydrous sodium sulfate. Isolation of the oil was performed in duplicate and the samples were stored in a freezer prior to further analysis.

GC/FID and GC–MS analysis of oil constituents

The oil was analyzed by gas chromatography (GC) using a Hewlett–Packard 6890 apparatus (Hewlett–Packard, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and electronic pressure control (EPC) injector. DB-5 column (30 m × 0.25 mm, 0.25 μm film thickness) was used. The flow of the carrier gas (N_2) was 1.0 mL/minute at a column pressure of 42 kPa. The analysis was performed using the following temperature program: oven temperature isothermal at 50°C for 5 minutes, increase at a rate of 5°C/minute and isothermal at 300°C for 5 minutes. The temperatures of the injector and detector were held at 280° and 300°C, respectively.

The GC–mass spectrometry (GC–MS) analyses were performed on a gas chromatograph HP 5890 (II) interfaced with an HP 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with electron ionization (EI) of 70 eV. An apolar HP-5MS capillary column (60 m × 0.25 mm, 0.25 μm film thickness) was used. The column temperature was programmed from 40° to 280°C at a rate of 5°C/minute. The carrier gas was He with a flow rate of 1.2 mL/minute. Scan time and mass range were 1 second and 50–550 *m/z*, respectively. The injected volume was 1 μL and the total run time was approximately 63 minutes. A second analysis with a polar HP Innowax (PEG) column (30 m × 0.25 mm, 0.25 μm film thickness) was also used. The flow of the carrier gas (N_2) was 1 mL/minute. The analysis was performed using the following temperature program: oven temperature isothermal at 50°C for 5 minutes, increase at a rate of 5°C/minute and isothermal at 300°C for 5 minutes. The injector and detector temperatures were held, respectively, at 280° and 300° C. Identification was based on a comparison of peak retention indices relative to (C8–C22) *n*-alkanes with literature data or authentic compounds available in our laboratory. Further identification was confirmed by matching recorded spectra with Wiley/NBS mass spectral library and other published mass spectra (11). Quantitative data were obtained from the electronic integration of the FID peak areas.

Statistical analysis

All the data were subjected to variance analysis using the GLM procedure of SAS (12). The mean pairwise comparisons were based on the Duncan test. For GC/MS, raw data were imported into the statistical software package MSStat (ANALYT MTC, Muehlheim, Germany, MS Statistical Software Version 3.02u); the software was looking for differences in the mass fragments (*m/z*), the retention time and the abundance.

Results and discussion

The yields of the oils were 0.4±0.02% (Lithuania) and 0.5±0.01% (France) (w/w), which was in accordance with literature (8, 9). The chemical composition of the oils of the two origins is presented in Table 1. Twenty and twenty-six components (Figure 1) were detected in Lithuanian and French oils, respectively. Four volatile compounds, namely *cis*-thujone, pinocarveol, carvacrol and α-bergamotene, which were identified in *M. odorata* French oil, were not detected in Lithuanian oil. Volatile constituents identified in the sweet cicely belong to the following classes of organic compounds: phenylpropanoids, terpenoids, alcohols and aldehydes. Phenylpropanoids were the most abundant components in *M. odorata* leaves and ranged between 63.3±0.7% and 68.2±0.6% of total oil, followed by terpenes (33.3 ±0.2% to 29.3±0.1%). Hydrocarbon monoterpenes are ranged from 3.7±0.1% to 4.1±0.0%, while oxygenated monoterpenes (1.2±0.0%) were detected only in the French origin plant. A higher percentage of hydrocarbon sesquiterpenes was determined in *M. odorata* from Lithuania (16.9±0.1%), than in French origin oil (11.7 ±0.1%), while the content of oxygenated sesquiterpenes was almost similar, 12.3±0.4% and 12.7±0.0%, respectively. The essential oil of sweet cicely contained a higher content of hydrocarbon sesquiterpenes than hydrocarbon monoterpenes. It is known that the parent sesquiterpenes are biosynthesized from geranyl diphosphate by its condensation with isopentenyl diphosphate. It can be suggested that monoterpenes are consumed by the plants faster at late vegetation phases compared with sesquiterpenes.

The major components in both *M. odorata* oils were *E*-anethole, methyleugenol and *E*-nerolidol, and the differences in their percentages were not remarkable. The odor of these compounds is described as sweet, green, anethole herbal anise, licorice

Table 1. Essential oil composition (%) of *Myrrhis odorata* (L.) Scop. leaves from Lithuania and France.

No	Compounds	RI[a,b]	RI[b]	Content GC area % Lithuania	Content GC area % France	Least significant difference (LSD)	Odor perception[c]
1	α-Pinene	936	1032	0.1±0.0a	0.1±0.0a	0.48	Fresh, camphor, sweet pine, earthy, woody
2	β-Myrcene*	986	1161	1.7±0.1a	1.8±0.1a	0.54	Peppery, terpene, spicy, balsam, plastic
3	*p*-Cymene	1022	1280	0.2±0.0a	0.5±0.1a	0.44	Fresh, citrus, terpene, woody, spice
4	Limonene	1030	1203	1.7±0.0a	0.4±0.0b	0.09	Citrus, fresh, peppery
5	E-β-Ocimene	1032	1259	0.3±0.1b	0.8±0.0a	0.22	Sweet, herbal, green, celery spicy, floral
6	γ-Terpinene	1058	1236	0.3±0.0b	0.6±0.1a	0.22	Oily, woody, terpene lemon/lime, tropical, herbal
7	*cis*-Thujone	1092	1452	–	0.2±0.0	–	Thujonic, cedarleaf
8	Nonanal	1103	1400	0.2±0.0b	0.4±0.0a	0.11	Aldehydic rose, fresh, orris, orange peel, fatty peely
9	Camphor	1131	1532	–	0.3±0.0	–	Medicinal, camphoreous
10	iso-Menthone	1168	1573	–	0.2±0.1	–	Powerful, refreshing and clean minty, cool, peppermint, sweet
11	Pinocarveol	1169	1664	–	0.5±0.0	–	Herbal, camphor, woody, pine, balsam
12	Carvacrol	1192	2239	–	0.4±0.0	–	Spice, woody, camphor, thymol
13	Estragole*	1197	1670	0.9±0.1b	4.1±0.4a	1.31	Sweet, licorice, anise spice, green herbal fennel
14	*p*-Anisaldehyde	1271	2012	–	0.3±0.0	–	Sweet, powdery, mimosa, floral, hawthorn, balsam
15	*E*-Anethole*	1290	1828	48.1±1.4a	50.7±1.1a	5.38	Sweet, green, anethole herbal
16	Methyleugenol*	1401	1985	14.3±0.5a	13.1±0.4a	1.98	Sweet, fresh, warm, spicy, clove, carnation, cinnamon
17	β-Caryophyllene	1409	1612	9.7±0.3a	6.1±0.3b	1.22	Sweet, woody, spice, clove dry
18	α-Bergamotene	1430	1573	0.9±0.1a	0.5±0.0b	0.32	Woody, warm, tea
19	α-Humulene	1459	1687	0.9±0.2a	0.5±0.0a	0.75	Woody, herbal
20	Germacrene-D*	1485	1726	5.0±0.1a	4.3±0.3a	0.96	Woody, spice
21	β-Bisabolene	1496	1714	0.1±0.0a	0.1±0.0a	0.00	Balsamic, citrus, myrrh, opoponax, spicy
22	α-Farnesene	1504	1758	0.3±0.0a	0.2±0.0b	0.00	Citrus, herbal, lavender, bergamot, myrrh, neroli, green
23	*E*-Nerolidol*	1559	1961	10.2±0.8a	12±0.3a	2.72	Floral, green, waxy, citrus, woody
24	Spathulenol	1618	2153	2.1±0.5a	0.7±0.0a	1.51	Earthy, herbal, fruity
25	Hexadecanol	1864	2333	1.2±0.2a	0.7±0.0a	0.65	Waxy, floral
26	Phytol	2125	2622	1.4±0.1a	0.4±0.0b	0.44	Delicate, floral, balsam, powdery, waxy
Phenylpropanoids (%)				63.3±0.7a	68.2±0.6a	2.89	
Monoterpene hydrocarbons (%)				4.1±0.0a	3.7±0.1a	0.31	
Oxygenated monoterpenes (%)				–	1.2±0.0	–	
Sesquiterpene hydrocarbons (%)				16.9±0.1a	11.7±0.1b	0.81	
Oxygenated sesquiterpenes (%)				12.3±0.4a	12.7±0.0a	2.11	
Others (%)				3.0±0.1a	2.4±0.0b	0.41	
Total (%)				99.6±0.2a	99.9±0.1a	0.97	

Notes: RI[a,b], RI[b], retention indices calculated using respectively an apolar column (HP-5) and polar column (HPInnowax). *Authentic standards were co-injected for the compounds identification. Data are means ± SD of three replicates and values with different letters (a–b) are significantly different at $p<0.05$. [c]Odor description adapted from www.thegoodscentscompany.com.

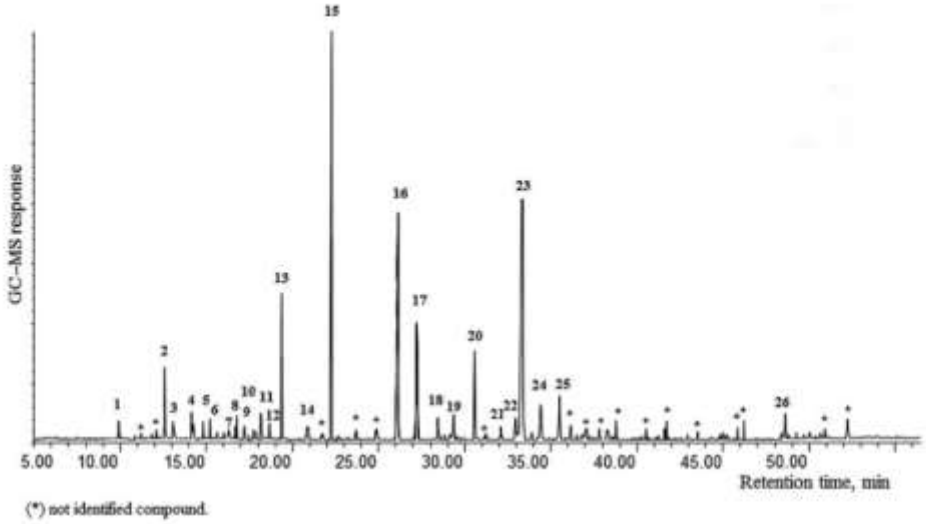


Figure 1. Gas chromatography–mass spectrometry chromatogram of *Myrrhis odorata* (L.) essential oil from France.

(*E*-anethole), clove, spicy (methyleugenol) and floral, green, waxy, citrus, woody (*E*-nerolidol), and they are found in such important spices as *Illicium verum, Foeniculum vulgare* and *Piper claussenianum* (13–15). From this point of view, *M. odorata* from France and Lithuania may be preliminary assigned to the same *E*-anethole, methyleugenol and *E*-nerolidol chemotype. However, some qualitative and quantitative significant differences in oil composition of two remote origins may be observed (Table 1). *cis*-Thujone, camphor, isomenthone, pinocarveol, carvacrol and *p*-anisaldehyde were detected only in *M. odorata* oil of French origin; the content of estragol in it was more than four times higher than in the oil of Lithuanian origin plant material (Table 1). The odor of estragol is described as anise or licorice so the French oil had a stronger anise or licorice flavor than the Lithuanian essential oil. On the other hand, the percentage of β-caryophyllene, germacrene-D, spathulenol and limonene was significantly higher in the oil from Lithuania (Table 1) and this oil had fresh, sweet, woody, spice, clove dry, earthy, herbal, fruity odor.

Comparison of the percentages of the main components in *M. odorata* oils analyzed in our study and previously reported in literature are summarized in Table 2. *E*-Anethole was the dominating constituent (82–85%) in *M. odorata* oils from Finland (1), Russia (8) and north USA (10) and it may be assumed that the plants from these countries were purely *E*-anethole chemotype. It should be noted that the plants from north USA contained reasonable amount of methyleugenol, which was not found in Finnish and Russian origin sweet cicely, while *E*-nerolidol was not detected in Russian and Serbian chemotypes. Completely different *M. odorata* chemotype was reported in Serbia (Prokletije region) with *p*-cymene the major compound constituting 62.0% in the total oil (9). This variation in component level it is surprising but not impossible,

Table 2. Comparison of main essential oil constituents in *Myrrhis odorata* (L.) Scop. leaves from different geographic origins.

Compound	Lithuania[a]	France[a]	Finland[b]	Russia[c]	Serbia[d]	North USA[e]
β-Myrcene	1.7	1.8	1.1	–	–	–
p-Cymene	0.2	0.5	–	–	62.0	–
Limonene	1.7	0.4	0.1		2.2	0.3
Estragole	0.9	4.1	2.6	–	–	1.7
E-Anethole	48.1	50.7	83.1	82.0	–	85.5
β-Caryophyllene	9.7	6.1	1.0	1.7	0.7	–
Methyleugenol	14.3	13.1	–	–	–	9.1
Germacrene-D	5.0	4.3	–	2.9	–	–
E-Nerolidol	10.2	12.0	1.0	–	–	–

Notes: [a]Our study; [b]Ref. (1); [c]Ref. (8); [d](Ref. (9); [e]Ref. (10).

because chemical polymorphism is significantly characteristic of many Umbelliferae family species (16). The oil reported from Serbia may be would not have the typical sweet, anise, licorice odor of *M.odorata* because *p*-cymene had a stronger fresh, citrus, terpene, woody, spice smell. These differences may be dependent on climatic conditions prevailing during plant development and their genetic potential. Indeed, the composition of essential oils as well as other chemical molecules of plants may vary considerably depending on annual climatic changes and genotype (17, 18). These parameters influence the total content of essential oils and their composition. Unfortunately, weather conditions were not reported in studies performed in Finland, Serbia and USA.

Conclusion

The present study provides new information about essential oil chemical composition of *M. odorata* from two geographical origins (Lithuania and France) not studied before. The percentage of the main oil compounds in the studied *M. odorata* collected in remote habitats was quite similar; however, comparison of the obtained data with previously studied *M. odorata* oils revealed that they are different plant chemotypes. Therefore, further investigations are necessary to confirm our results by studying a collection of accessions from different geographic regions cultivated under the same climatic conditions. This could allow identification of the genetic potential, which could help improve *M. odorata* breeding programs and diversification of its industrial uses.

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