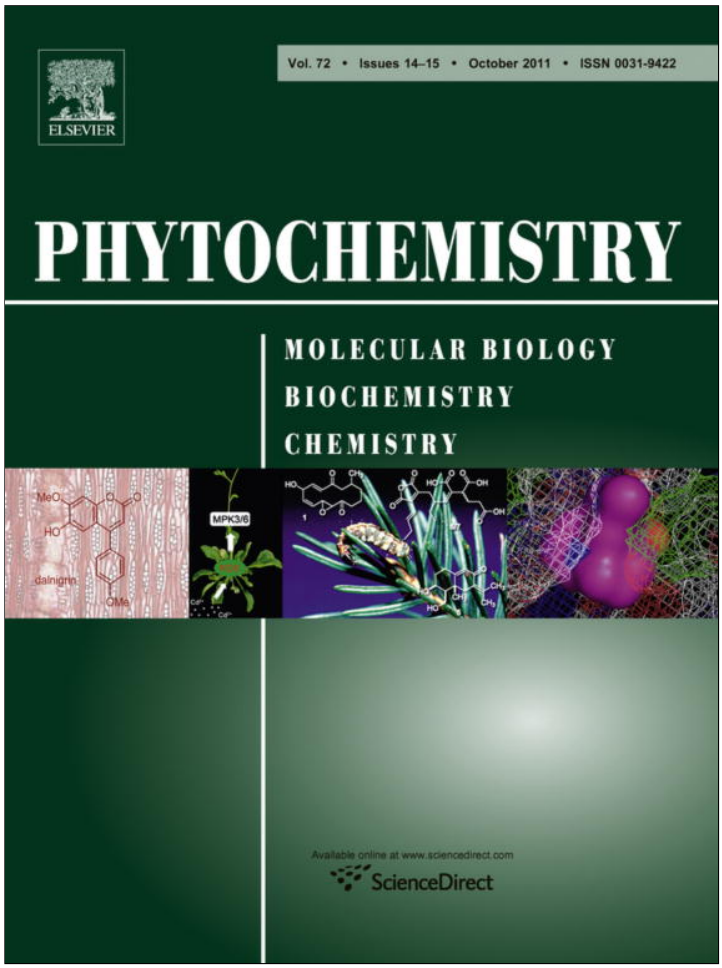

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The genus *Dracunculus* – A source of triacylglycerols containing odd-numbered ω-phenyl fatty acids

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ABSTRACT

Reversed phase liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (RP-HPLC/MS-APCI) was used to identify and quantify triacylglycerols (TAGs) having odd-numbered ω-phenylalkanoic acids from seeds of the flower plant *Dracunculus vulgaris*, and TAGs from the bacterium *Rhodococcus erythropolis* prepared by precursor directed biosynthesis from phenylalanine and having the corresponding even-numbered ω-phenylalkanoic acids.

Model compounds, which are not commercially available, were prepared by organic synthesis and this allowed us to extend the number of identified natural TAGs to nearly 140 molecular species. Both synthetic and natural compounds containing ω-phenylalkanoic acids were found to have antioxidant and free radical scavenging properties.

1. Introduction

Araceae are a large, predominantly tropical family of ca. 105 genera and ca. 2500 species (Mabberley, 1993; Mayo et al., 1997). *Dracunculus* is a genus of about 30 species of flowering plants in the family Araceae, native to Europe, Northern Africa and Western Asia, with the highest species diversity in the Mediterranean region. Little is known about the phytochemical characteristics of this plant species.

The genus *Dracunculus vulgaris* (Schott), which is spread throughout the Mediterranean area, grows mainly in the western part of Crete. It has large tuberous roots, spotted jagged leaves, tall stem (up to 150 cm) and dark purple spike inflorescences (up to 60 cm) enclosed in sheath-like spathes (up to 40 cm). *D. vulgaris*, the largest and most spectacular wild aroid of Greece, blooms in May and June. The reddish berry type fruits, which ripen in July, are used to treat internal hemorrhoids (Sfikas, 1992).

The seed lipids of genera of the subfamily Aroideae of the Araceae contain long-chain ω-phenylalkanoic and ω-phenylalkenoic acids, 13-phenyltridecanoic acid being the major component. This acid was found in all 35 species of 7 genera, among others also in two species of the genus *Dracunculus*, i.e. *Dracunculus canariensis* and *D. vulgaris* (Schmid et al., 1997).

The content of fatty acids (FAs) in the subfamily Aroideae was studied in detail by GC–MS on a polar capillary column, and monounsaturated ω-phenylalkenoic acids were found and characterized using dimethyl disulfide derivatization to locate the positions of their double bonds. Two groups of positional isomers of ω-phenylalkenoic acids (Δ^5 and Δ^7 from the phenyl ring) and also 13-phenyltridecadienoic acid were found (Meija and Soukup, 2004).

The *D. vulgaris* growing in Turkey has been analyzed by GC–MS and the seeds were found to contain, apart from common fatty acids found in plants, such as 16:0, 16:1ω−7, 18:1ω−9, 18:1ω−7 or 18:2ω−6, also 13-phenyltridecanoic acid in an amount of about 10% total FAs (Saglik et al., 2002).

The seed oil of *Arum maculatum*, another representative of family Araceae, was found to contain two ω-phenylalkanoic and two ω-phenylalkenoic acids, i.e. 13-phenyltridec-9-enoic (0.4%) and 15-phenyl-pentadec-9-enoic (1%) acids, which were identified by GC–MS as picolinyl esters (Christie, 2003).

Abbreviations: ACN, acyl carbon number; APCI, atmospheric pressure chemical ionization mass spectrometry; BHT, butylated hydroxy toluene; AG, diacylglycerol(s); DCC, dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ECN, equivalent carbon number; ESI-MS, electrospray ionization mass spectrometry; FA, fatty acid(s); FAME, fatty acid methyl ester; GC–MS, gas chromatography–mass spectrometry; HREIMS, high resolution electron ionization mass spectra; HR-FAB-MS, high resolution fast atom bombardment mass spectrometry; iPrOH, 2-propanol; LC–MS/APCI, liquid chromatography–mass spectrometry/atmospheric pressure chemical ionization mass spectrometry; MAG, monoacylglycerol(s); MeCN, acetonitrile; NMR, nuclear magnetic resonance; PEG-400, polyethylene glycol 400; Phe, phenylalanine; RP-HPLC/MS-APCI, reversed phase liquid chromatography–atmospheric pressure chemical ionization mass spectrometry; RT, retention time; TAG, triacylglycerol(s); THF, tetrahydrofuran; TLC, thin layer chromatography.

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Outside this family, ω-phenylalkanoic acids are found only rarely. 13-Phenyltridecanoic acid has been isolated and characterized from the ethanol extract of the sea buckthorn berries (*Hippophae rhamnoides*) (Singh et al., 2005). The extracts of leaves of *Trichilia claussenii* yielded a mixture of ω-phenylalkanoic and ω-phenylalkenoic acids from ω-phenyldecanoic to ω-phenylpentadecanoic acid, including two unsaturated FAs, ω-phenyltetradecenoic and ω-phenylpentadecenoic acids (Pupo et al., 1996).

Some bacteria were also found to contain ω-phenylalkanoic acids; thus a new strain of *Vibrio alginolyticus*, found in the alga *Cladophora coelothrix*, contained ω-phenyl fatty acids such as 10-phenyldecanoic, 12-phenyldodecanoic, and 14-phenyltetradecanoic acid, in an amount of 2.5% total FAs (Carballeira et al., 1997).

10-Phenyldecanoic (0.4%), 12-phenyldodecanoic (3.1%), 13-phenyltridecanoic (0.2%) and 14-phenyltetradecanoic (0.1%) acids were identified in the halophilic bacterium *Bacillus* sp. isolated from the saltpans in Bulgaria (Carballeira et al., 2001).

To our knowledge, no systematic analysis of triacylglycerols (TAGs) containing ω-phenylalkanoic or ω-phenylalkenoic acids in the molecule has as yet been performed. A possible exception is the study of the *Rhodococcus opacus* that grew on different carbon sources, e.g. phenylalanine, and was further incubated with phenyldecane. Phenyldecane was utilized for the biosynthesis of novel TAGs, which were identified by ESI-MS and ESI-MS/MS after separation on TLC plates (Alvarez et al., 2002). These TAGs contained odd- and even-numbered aliphatic fatty acids with chain lengths ranging from 13 to 19 carbon atoms. The main ions represented a mixture of TAGs in which one acyl group was replaced by a ω-phenyldecanoic acid residue, as documented by ESI-MS/MS.

Plant oils containing the most common FAs such as palmitic, stearic, oleic, linoleic and linolenic acids are usually analyzed by the APCI method (Rezanka and Sigler, 2007). Problems are encountered when analyzing oils containing FAs with unusual (i.e. not common) position and number of double bond(s) (Lisa et al., 2007), odd-chain FAs (Rezanka et al., 2010) or branched chain FAs (Schreiberová et al., 2010). Based on our preceding experience with the analysis of uncommon TAGs by LC–MS/APCI we analyzed the *D. vulgaris* seed oil, TAGs containing ω-phenylalkanoic acids, which were prepared by precursor directed biosynthesis using a bacterium of genus *Rhodococcus*, and also six TAGs prepared by organic synthesis.

2. Results and discussion

Table 1 gives the fatty acids isolated from *D. vulgaris* seeds. As shown in the table, apart from FAs found commonly in plant oils, such as palmitic, oleic or linolenic acids; major unusual acids were cis-vaccenic acid and ω-phenyltridecanoic acid, which were present in quantities above 10%, in agreement with previously published data (Meija and Soukup, 2004; Saglik et al., 2002; Schmid et al., 1997).

As stated in Section 1, analysis of TAGs from uncommon plant oils often presents problems, one of them being the lack of commercially available standards. We used two approaches to obtain the standards.

The first approach was the use of precursor directed biosynthesis from phenylalanine, using the bacterium *Rhodococcus erythropolis*. This yielded a mixture of TAGs containing ω-phenylalkanoic acids. The bacterium is capable of utilizing different starter units for synthesizing FAs (e.g. branched amino acids (Schreiberová et al., 2010) or pivalic acid (Rezanka et al., in press)) and thus also TAGs. This method yielded a natural mixture of TAGs enriched in TAGs containing ω-phenylalkanoic acids. The other approach included classical organic synthesis, which afforded in the first step ω-phenyltridecanoic and ω-phenyltetradecanoic acid. Both these acids have been found in major proportions in the natural sources that we analyzed – ω-phenyltridecanoic acid in *D. vulgaris* seed oil and ω-phenyltetradecanoic in *R. erythropolis* mycelium, see Table 1. Neither is commercially available.

Table 1
Fatty acid compositions (%) of *D. vulgaris* and *R. erythropolis* as determined by GC–MS.

FA	Abbreviations	*D. vulgaris* %[a]	FA	Abbreviations	*R. erythropolis* %
14:0[b]	M	0.5 ± 0.2	14:0	M	9.2 ± 1.3
15:0	X	0.4 ± 0.2	15:0	X	8.0 ± 0.9
16:0	P	16.2 ± 0.7	16:0	P	14.4 ± 1.5
16:1*n*−7	Po	5.1 ± 0.3	16:1*n*−7	Po	17.0 ± 1.2
17:0	M	0.3 ± 0.2	17:0	Ma	4.2 ± 0.8
18:0	S	3.5 ± 0.1	17:1*n*−8	Mo	9.2 ± 0.6
18:1*n*−9	O	19.2 ± 1.4	18:0	S	2.4 ± 0.4
18:1*n*−7	–[c]	11.6 ± 0.9	18:1*n*−9	O	23.7 ± 2.1
18:2*n*−6	L	24.6 ± 2.1	Ph-12:0	P12	1.3 ± 0.4
18:3*n*−3	Ln	2.2 ± 0.8	Ph-14:0[d]	P14	10.6 ± 1.2
20:0	–	0.4 ± 0.2			
20:1*n*−9	–	0.2 ± 0.1			
20:1*n*−7	–	0.2 ± 0.1			
22:0	–	1.4 ± 0.4			
22:1*n*−9	–	0.2 ± 0.1			
22:1*n*−7	–	0.5 ± 0.2			
24:0	–	0.5 ± 0.2			
Ph-11:0	P11	0.2 ± 0.1			
Ph-13:0[d]	P13	10.1 ± 1.1			
Ph-13:1		0.4 ± 0.2			
Ph-15:0		1.4 ± 0.5			
Ph-15:1		0.9 ± 0.4			

[a] Arithmetic means from 5 analyses ± standard deviation.
[b] In the shorthand nomenclature, first number is number of carbon atoms in the chain, second number is number of cis (*Z*) double bonds. The position of the terminal double bond is denoted in the form (*n*−*x*), where *n* shows that the numbering is from the end of the acyl chain (CH_3 group) and *x* is the number of carbon atoms from methyl end to double bond, assuming that all double bonds are methylene-interrupted. Thus linoleate and α-linolenate are 18:2*n*−6 and 18:3*n*−3, respectively.
[c] TAGs differing only in positional isomers of Fas such as oleic (18:1*n*−9) and cis-vaccenic (18:1*n*−7) acids were not separated.
[d] For structures see Fig. 8.

The two ω-phenylalkanoic acids were prepared by a modified procedure including the direct reaction of acid chloride with Grignard reagents, first described by Hase and Ohura (1954), and modified by Scheiper et al. (2004). Briefly, slow inverse addition of a Grignard reagent to a cold (−78 °C) THF solution of excess acid chloride afforded ketones in excellent yields. Although the direct addition of Grignard reagents to acid chlorides is limited by the subsequent reaction of the product, i.e. ketone to form tertiary alcohols, the latter reaction can be suppressed by the addition of the catalytic quantities of $Fe(acac)_3$, which significantly enhances the yields and the reaction can be performed in the presence of functionalities normally incompatible with Grignard reagents (Dieter, 1999).

Wolff–Kishner reduction (i.e. a chemical reaction that fully reduces a ketone to an alkane) in the Huang–Minlon modification involves heating the oxo-esters (**4** or **5**), potassium hydroxide, and hydrazine hydrate together in ethylene glycol in a one-pot reaction to form $–CH_2–$ group (acids **6** or **7**), see Fig. 1. We further synthesized 6 different TAGs as standards (see Table 2) used to verify the basic chromatographic and mass spectral characteristics. The standards were synthesized by conventional procedures, as simply as possible and with the highest yield (Lie Ken Jie and Lam, 1995; Schreiberová et al., 2010). Commercially available 1-palmitin, 1,2-dipalmitin, and 1,3-dipalmitin yielded a total of 6 TAGs by mild organic synthesis performed at 25 °C for 24 h under catalysis with dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) (see Section 4). Synthesized TAGs were analyzed by RP-HPLC/MS-APCI and HR-FAB-MS.

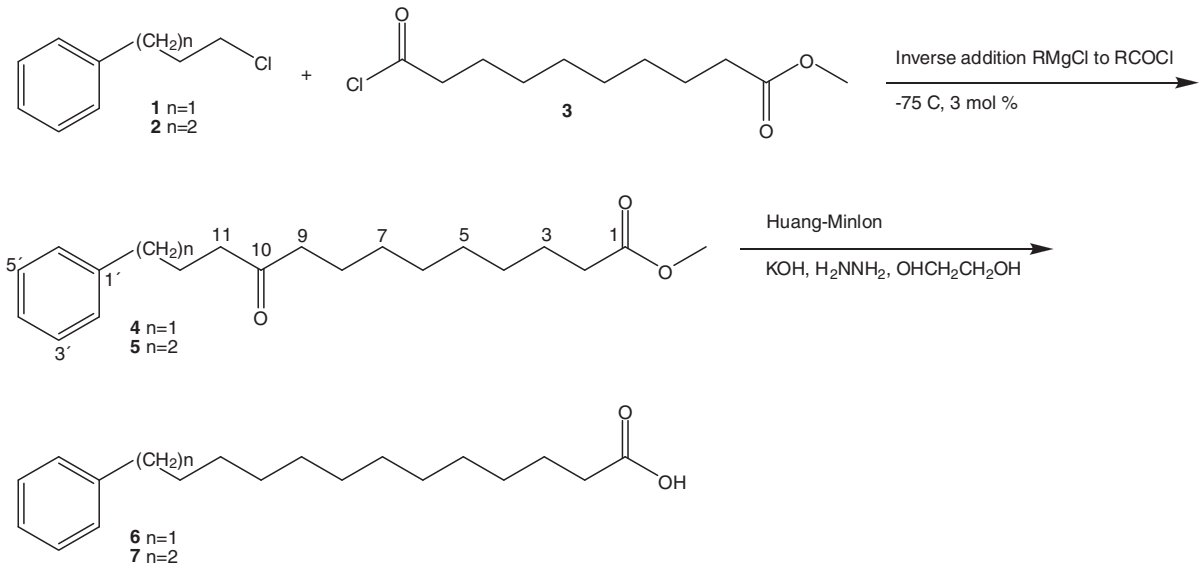

Fig. 1. Synthesis of ω-phenyltridecanoic and ω-phenyltetradecanoic acids.

Table 2
Synthetic TAGs.

Alcohol (1 μmol)	Acid (μmol)	TAG	Yield (%)	Purity (%)	HR-FAB-MS		
					Chemical formula	*m/z* obtained	*m/z* calculated
1-Monopalmitin	P13(2.10)	PP13P13	75	⩾99	$C_{57}H_{94}NaO_6$	897.6952	897.6948
1-Monopalmitin	P14(2.10)	PP14P14	73	⩾99	$C_{59}H_{98}NaO_6$	925.7265	925.7261
1,2-Dipalmitin	P13(1.05)	PPP13	76	⩾99	$C_{54}H_{96}NaO_6$	863.7110	863.7105
1,2-Dipalmitin	P14(1.05)	PPP14	69	>98	$C_{55}H_{98}NaO_6$	877.7264	877.7261
1,3-Dipalmitin	P13(1.05)	PP13P	68	>98	$C_{54}H_{96}NaO_6$	863.7106	863.7105
1,3-Dipalmitin	P14(1.05)	PP14P	70	>98	$C_{55}H_{98}NaO_6$	877.7268	877.7261

Separation of TAGs containing 0, 1 or 2 ω-phenylalkanoic acids in the molecule presented no problems (Fig. 2) but the separation of TAGs of AAB and ABA types (AAB or ABA denotes a TAG containing two different fatty acids A and B) was not successful even when using two 25 cm columns in series (efficiency of ~52,000 plates/50 cm). As TAGs containing long-chain ω-phenylalkanoic acids have not yet been analyzed by RP-HPLC, we tried unsuccessfully to predict their retention times by our previously used method, i.e. multiple linear regression analyses (Rezanka et al., 2010). Partial success was noted when predicting the retention times using the method described by Lin et al. (1998) that involves the use of calculated contributions of functional groups or chain shortenings of FAs to the retention time (RT) of TAGs. Here we found that ω-phenylalkanoic acid usually behaves as a long-chain FA with four double bonds. Although ω-phenylalkanoic acid has only three double bonds, its degree of unsaturation is four thanks to the aromatic ring. Although the success of the prediction of retention times of appropriate TAG was only partial, the determination of the retention times was relatively satisfactory. The PPA and PAP TAGs were synthesized as described above. RP-HPLC analysis revealed that the retention times of the two positional isomers differ only negligibly, i.e. by 0.2 min, from that of both natural and synthetic PPP14, see Table 3. The good agreement of contributions of increment of four bonds for ω-phenylalkanoic acids can be illustrated on some molecular species from Table 3. For instance, TAGs such as LLL, OOP12 or OP14Po, and LLnO having equivalent carbon numbers (ECN) 42 and also acyl carbon number (ACN) 54 and a total equivalent of six double bonds (aromatic ring and two double bonds in case of TAGs with ω-phenylalkanoic acids) have nearly identical retention times, eluting within 1.4 min. Likewise, other molecular species, e.g. LLnPo, P14PoPo, and LnLnP or LOPo, PPP14, and LLP have very similar retention characteristics (see Table 3). The structure of molecular species was determined from APCI mass spectra by manual identification of the prepared standards and of natural samples after RP-HPLC separation.

Table 3 presents all TAGs identified in *D. vulgaris* and *R. erythropolis*, along with the values important for identification of individual molecular species of these TAGs. The two natural sources gave a total of 139 molecular species of TAGs.

The APCI mass spectra of an AAA type TAG are very simple. For instance, triolein, e.g. OOO, exhibits four clusters of ions, i.e. $[M+H]^+$ ($[TAG]^+$, i.e. $[OOO]^+$) at *m/z* 885, $[M-RCOO]^+$ ion ($[DAG]^+$, i.e. $[OO]^+$) at *m/z* 603 arising by the loss of oleate, $[RCOO+58]^+$ called also ($[MAG]^+$, i.e. $[O]^+$) at *m/z* 339, and $[RCO]^+$ ion (FA^+, i.e. O^+) at *m/z* 265, see also Table 3.

The mass spectrum of ABA type TAG such as PP13P (Fig. 3), which contains two FA, exhibits, in addition to $[M+H]^+$ at *m/z* 841, also two acylium ions, with *m/z* at 239 (P^+) and *m/z* 273 ($P13^+$), respectively. The spectrum naturally features also ions of the type $[MAG]^+$ at *m/z* 313 ($[P]^+$) and *m/z* 347 ($[P13]^+$), respectively. Like with OOO, also $[DAG]^+$ ions caused by the loss of ω-phenyltridecanoate at *m/z* 551 and palmitate at *m/z* 585 are present, resulting in $[PP]^+$ (type $[AA]^+$) and $[PP13]^+$ (type $[AB]^+$) ions, respectively.

Similarly, the mass spectrum of an ABC type TAG, i.e. TAG containing three different FA, such as OP14Po, see Fig. 4, shows three triplets of ions. Three $[M-RCOO]^+$ ions at *m/z* 575, 597, and 625, corresponding to loss of ω-phenyltridecanoate to give $[OPo]^+$, loss of oleate to give $[P14Po]^+$, and loss of palmitoleate to give $[OP14]^+$,

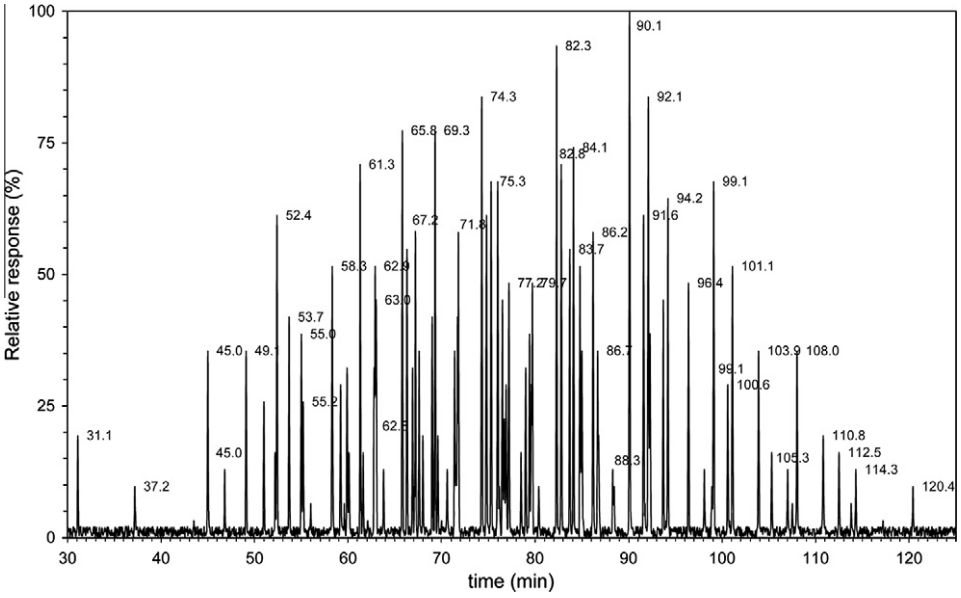

Fig. 2. RP-HPLC/MS-APCI chromatogram of the TAGs from the flower plant *D. vulgaris*. Numerals above peaks show the retention time of appropriate peak in minutes.

Table 3
Relative quantities (%) of TAGs identified in *D. vulgaris* (D) and *R. erythropolis* (R), their retention times, acyl carbon number, the masses of protonated molecules, and characteristic fragment ions of TAGs.[a]

TAG	RT	M+H	DAG	*m/z*	DAG	*m/z*	DAG	*m/z*	ECN	ACN:*n*	%^{D}	%^{R}
LnP13P13	31.1	897	LnP13	607	P13P13	619			34	56:11	0.6	–
LnLnP13	37.2	885	LnLn	595	LnP13	607			35	55:10	0.3	–
LnLnLn	43.5	873	LnLn	595					36	54:9	0.1	–
LLnP13	45.0	887	LLn	597	LnP13	607	LP13	609	37	55:9	1.1	–
P12PoPo	45.1	823	PoPo	547	P12Po	569			38	50:6	–	1.5
LnP13Po	46.8	861	LnP13	607	P13Po	583	LnPo	571	37	53:8	0.4	–
PP13P13[b]	49.1	875	P13P13	619	PP13	585			38	54:8	1.1	–
LLnLn	51.0	875	LnLn	595	LLn	597			38	54:8	0.8	–
P13PoPo	52.2	837	PoPo	547	P13Po	583			39	51:6	0.5	–
LLP13	52.4	889	LP13	609	LL	599			39	55:8	1.9	–
LnOP13	53.7	889	LnO	599	OP13	611	LnP13	607	39	55:8	1.3	–
LP13Po	55.0	863	LP13	609	P13Po	583	LPo	573	39	53:7	1.2	–
LnPP13	55.2	863	LnP	573	PP13	585	LnP13	607	39	53:7	0.8	–
LP15P15	56.0	955	P15P15	675	LP15	637			40	60:10	0.2	–
PP14P14[c]	57.0	903	P14P14	647	PP14	599			40	56:8	–	–
LLLn	58.3	877	LLn	597	LL	599			40	54:7	1.6	–
LLnPo	59.2	851	LLn	597	LnPo	571	LPo	573	40	52:6	0.9	–
PoPoLn	59.6	825	PoLn	571	PoPo	547			40	50:5	0.2	–
LnLnO	59.9	877	LnO	599	LnLn	595			40	54:7	1.0	–
P14PoPo	60.0	851	P14Po	597	PoPo	547			40	52:6	–	1.8
LLP15	60.1	917	LP15	637	LL	599			41	57:8	0.5	–
LOP13	61.3	891	LO	601	OP13	611	LP13	609	41	55:7	2.2	–
LnLnP	61.6	851	LnP	573	LnLn	595			40	52:6	0.5	–
LnPP15	62.1	891	LnP	573	PP15	613	LnP15	635	41	55:7	0.1	–
PPoP13	62.8	839	PPo	549	PoP13	583	PP13	585	41	51:5	1.0	–
LPP13	62.9	865	LP	575	PP13	585	LP13	609	41	53:6	1.6	–
OP13Po	63.0	865	OP13	611	P13Po	583	OPo	575	41	53:6	1.4	–
LnP13S	63.8	891	LnP13	607	P13S	613	LnS	601	41	55:7	0.4	–
LLL	65.8	879	LL	599					42	54:6	2.4	–
OOP12	66.1	879	OO	603	OP12	597			42	54:6	–	2.0
OP14Po	66.2	879	OP14	625	P14Po	597	OPo	575	42	54:6	–	2.1
LLPo	66.3	853	LL	599	LPo	573			42	52:5	1.7	–
LPoPo	66.9	827	LPo	573	PoPo	547			42	50:4	1.0	–
PoPoPo	67.1	801	PoPo	547					42	48:3	0.3	2.1
PPoP14	67.1	853	PPo	549	PoP14	597	PP14	599	42	52:5	–	1.7
LLnO	67.2	879	LLn	597	LnO	599	LO	601	42	54:6	1.8	–
LnOPo	67.6	853	LnO	599	OPo	575	LnPo	571	42	52:5	1.1	–
LOP15	68.0	919	LO	601	OP15	639	LP15	637	43	57:7	0.6	–
LLnP	69.0	853	LLn	597	LnP	573	LP	575	42	52:5	1.3	–
OOP13	69.3	893	OO	603	OP13	611			43	55:6	2.4	–
LnPPo	69.6	827	LnP	573	PPo	549	LnPo	571	42	50:4	0.6	–
LnLnS	70.0	879	LnLn	595	LnS	601			42	54:6	0.1	–
LPP15	70.6	893	LP	575	PP15	613	LP15	637	43	55:6	0.4	–

(*continued on next page*)

Table 3 (*continued*)

TAG	RT	M+H	DAG	*m/z*	DAG	*m/z*	DAG	*m/z*	ECN	ACN:*n*	%^{D}	%^{R}
LP13S	71.4	893	LP13	609	P13S	613	LS	603	43	55:6	1.1	–
P13PoS	71.5	867	P13Po	583	PoS	577	P13S	613	43	53:5	0.5	–
PPP13[b]	71.7	841	PP	551	PP13	585			43	51:4	1.3	–
PP13P[c]	71.7	841	PP	551	PP13	585			43	51:4	–	–
OPP13	71.8	867	OP	577	PP13	585	OP13	611	43	53:5	1.8	–
OOP14	72.7	907	OO	603	OP14	625			44	56:6	–	2.3
LLO	74.3	881	LL	599	LO	601			44	54:5	2.6	–
LOPo	74.8	855	LO	601	OPo	575	LPo	573	44	52:4	1.9	
OPP14	75.2	881	OP	577	PP14	599	OP14	625	44	54:5	–	2.0
LnOO	75.3	881	LnO	599	OO	603			44	54:5	2.1	–
P14PoS	75.5	881	P14Po	597	PoS	577	P14S	627	44	54:5	–	1.2
PPP14[b]	75.7	855	PP	551	PP14	599			44	52:4	–	1.6
PP14P[c]	75.7	855	PP	551	PP14	599			44	52:4	–	–
LLP	76.0	855	LL	599	LP	575			44	52:4	2.1	–
LP15S	76.2	921	LP15	637	P15S	641	LS	603	45	57:6	0.3	–
LPPo	76.5	829	LP	575	PPo	549	LPo	573	44	50:3	1.4	–
OPoPo	76.7	829	OPo	575	PoPo	547			44	50:3	0.7	2.4
LLnS	76.9	881	LLn	597	LnS	601	LS	603	44	54:5	0.9	–
MoPoPo	77.0	815	MoPo	561	PoPo	547			43	49:3	–	1.8
LnOP	77.2	855	LnO	599	OP	577	LnP	573	44	52:4	1.5	–
XPoPo	77.3	789	XPo	535	PoPo	547			43	47:2	–	1.7
OPP15	78.5	895	OP	577	PP15	613	OP15	639	45	55:5	0.5	–
MOPo	78.8	803	MO	549	OPo	575	MPo	521	44	48:2	–	2.0
LnPP	79.0	829	LnP	573	PP	551			44	50:3	1.0	–
PPoPo	79.4	803	PPo	549	PoPo	547			44	48:2	1.2	2.0
PP13S	79.6	869	PP13	585	P13S	613	PS	579	45	53:4	0.9	–
OP13S	79.7	895	OP13	611	P13S	613	OS	605	45	55:5	1.5	–
MPPo	79.8	777	MP	523	PPo	549	MPo	521	44	46:1	–	1.7
MMO	80.1	777	MM	495	MO	549			44	46:1	–	1.7
PPP15	80.4	869	PP	551	PP15	613			45	53:4	0.3	–
MMP	80.8	751	MM	495	MP	523			44	44:0	–	1.3
MoOPo	81.4	843	MoO	589	OPo	575	MoPo	561	45	51:3	–	2.0
MoPPo	81.6	817	MoP	563	PPo	549	MoPo	561	45	49:2	–	1.7
LOO	82.3	883	LO	601	OO	603			46	54:4	2.9	–
OOPo	82.8	857	OO	603	OPo	575			46	52:3	2.2	2.6
MOO	83.3	831	OO	603	MO	549			46	50:2	–	2.3
LLS	83.7	883	LL	599	LS	603			46	54:4	1.7	–
OP14S	84.0	909	OP14	625	P14S	627	OS	605	46	56:5	–	1.5
LOP	84.1	857	LO	601	OP	577	LP	575	46	52:3	2.3	–
PP14S	84.1	883	PP	551	P14S	627			46	54:4	–	1.1
OPPo	84.8	831	OP	577	PPo	549	OPo	575	46	50:2	1.6	2.2
LnOS	85.0	883	LnO	599	OS	605	LnS	601	46	54:4	1.1	–
BLnLn	85.1	935	BLn	657	LnLn	595			46	58:6	0.1	–
PoPoS	85.4	831	PoPo	547	PPoS	577			46	50:2	–	1.5
MOP	86.2	805	MO	549	OP	577	MP	523	46	48:1	–	2.0
LPP	86.2	831	PP	551	LP	575			46	50:2	1.8	–
PPPo	86.7	805	PP	551	PPo	549			46	48:1	1.1	1.9
LnPS	86.8	857	LnP	573	LnS	601	PS	579	46	52:3	0.6	–
MoMoO	87.4	857	MoMo	575	MoO	589			46	52:3	–	–
MPoS	87.7	805	MPo	521	PoS	577	MS	551	46	48:1	–	1.7
MoOO	88.0	871	OO	603	MoO	589			47	53:3	–	1.2
OOX	88.3	845	OO	603	OX	563			47	51:2	–	2.4
P13SS	88.3	897	P13S	613	SS	607			47	55:4	0.4	2.2
OP15S	88.4	923	OP15	639	P15S	641	OS	605	47	57:5	0.3	–
MoOP	88.5	845	MoO	589	OP	577	MoP	563	47	51:2	–	1.9
OPX	88.8	819	OP	577	PX	537	OX	563	47	49:1	–	1.9
MMS	89.2	779	MS	551	MM	495			46	46:0	–	0.8
MPP	89.4	779	MP	523	PP	551			46	46:0	–	1.5
OOO	90.1	885	OO	603					48	54:3	3.1	3.1
PP15S	90.2	897	PP15	613	P15S	641	PS	579	47	55:4	0.1	–
LOS	91.6	885	LO	601	OS	605	LS	603	48	54:3	1.9	–
BLLn	91.7	937	BL	659	LLn	597	BLn	657	48	58:5	0.2	–
OOP	92.1	859	OO	603	OP	577			48	52:2	2.6	2.6
OPoS	92.3	859	OPo	575	PoS	577	OS	605	48	52:2	1.2	0.6
P14SS	92.7	911	P14S	627	SS	607			48	56:4	–	1.8
LPS	93.7	859	LP	575	PS	579	LS	603	48	52:2	1.4	–
LnSS	94.0	885	SS	607	LnS	601			48	54:3	0.1	–
OPP	94.2	833	OP	577	PP	551			48	50:1	2.0	2.1
PPoS	94.6	833	PPo	549	PoS	577	PPo	549	48	50:1	–	1.4
MOS	94.6	833	MO	549	OS	605	MS	551	48	50:1	–	1.4
MaOO	96.1	873	OO	603	MaO	591			49	53:2	–	2.1
OSX	96.3	847	OS	605	SX	565	OX	563	49	51:1	–	1.4
PPP	96.4	807	PPP	551					48	48:0	1.5	1.7
MPS	96.8	807	MP	523	PS	579	MS	551	48	48:0	–	1.1
MoPS	97.0	847	PS	579	MoS	591	MoP	563	49	51:1	–	1.1

Table 3 (*continued*)

TAG	RT	M+H	DAG	*m/z*	DAG	*m/z*	DAG	*m/z*	ECN	ACN:*n*	%[D]	%[R]
MaOP	97.3	847	OP	577	MaP	565	MaO	591	49	51:1	–	1.8
PoSS	98.1	861	PoS	577	SS	607			50	52:1	–	1.0
BLL	98.1	939	BL	659	LL	599			50	58:4	0.4	–
BLnO	98.9	939	LnO	599	BO	661	BLn	657	50	58:4	0.3	–
OOS	99.1	887	OO	603	OS	605			50	54:2	2.1	2.1
LSS	100.6	887	SS	607	LS	603			50	54:2	0.9	–
BLnP	100.8	913	LnP	573	BP	635	BLn	657	50	56:3	0.1	–
OPS	101.1	861	PS	579	OS	605	OP	577	50	52:1	1.6	1.7
PPS	103.9	835	PP	551	PS	579			50	50:0	1.1	1.4
MSS	105.1	835	SS	607	MS	551			50	50:0		0.7
BLO	105.3	941	BL	659	BO	661	LO	601	52	58:3	0.5	–
MaOS	106.2	875	OS	605	MaS	593	MaO	591	51	53:1	–	1.3
MoSS	106.7	875	MoS	591	SS	607			51	53:1	–	0.7
BLP	107.0	915	LP	575	BP	635	BL	659	52	56:2	0.4	–
MaPS	107.3	849	PS	579	MaS	593	MaP	565	51	51:0	–	0.8
BLnS	107.5	941	LnS	601	BS	663	BLn	657	52	58:3	0.2	–
OSS	108.0	889	OS	605	SS	607			52	54:1	1.1	1.3
PSS	110.8	863	SS	607	PS	579			52	52:0	0.6	0.9
BOO	112.5	943	OO	603	BO	661			54	58:2	0.5	–
MaSS	113.6	877	SS	607	MaS	593			53	53:0	–	0.5
BLS	113.8	943	LS	603	BS	663	BL	659	54	58:2	0.2	–
BOP	114.3	917	OP	577	BP	635	BO	661	54	56:1	0.4	–
SSS	117.2	891	SS	607					54	54:0	0.1	0.1
BOS	120.4	945	BO	661	BS	663	OS	605	56	58:1	0.3	–

[a] See Fig. 9.
[b] Natural and also synthetic TAG.
[c] Synthetic TAG.

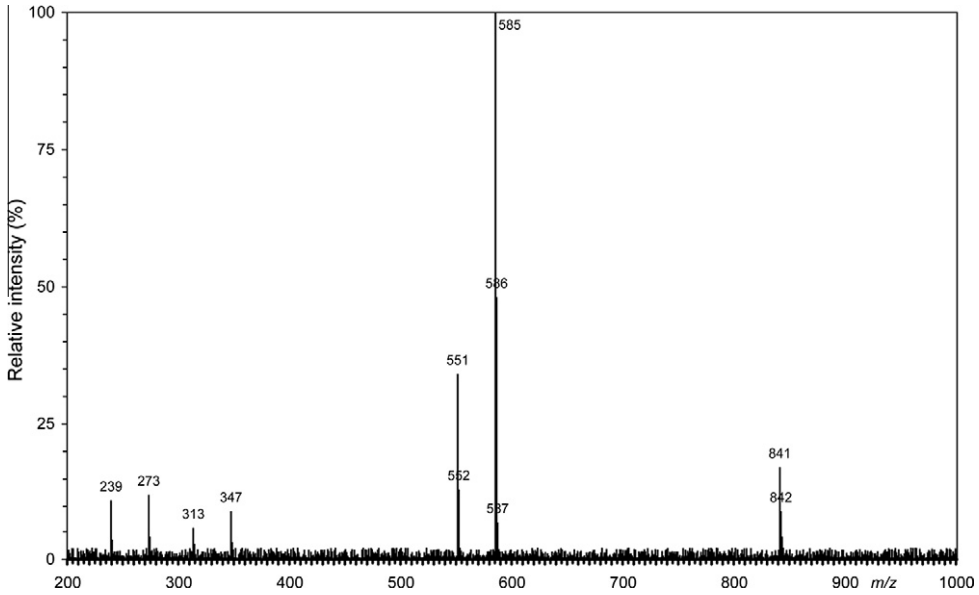

Fig. 3. Mass spectrum (APCI) of synthetic PP13P.

respectively. Another triplet, $[MAG]^+$ at *m/z* 311, 339, and 361 is formed by $[Po]^+$, $[O]^+$, $[P14]^+$ ions. The last triplet belongs to ions $[Po]^+$ (*m/z* 237), $[O]^+$ (*m/z* 265), and $[P14]^+$ (*m/z* 287).

As previously described (Mottram, 2005; Byrdwell, 2005; Fauconnot et al., 2004; Leskinen et al., 2007), the relative intensities of the $[M-RCOO]^+$ ions, i.e. $[DAG]^+$, can be used for determining the position of FA in TAG. This is due to the fact that the loss of FA in the *sn*−2 position is assumed to be energetically less favorable than the loss of FA from the *sn*−1 or *sn*−3 position. We therefore performed the chemical synthesis of two pairs of model compounds, i.e. PPP13 and PP13P, and PPP14 and PP14P, respectively. As a rule, the cleavage of these bifunctional TAGs gives rise to an isomeric pair of the same $[DAG]^+$ ions, i.e. $[AA]^+$ and $[AB]^+$. The ratio of $[AA]^+$:$[AB]^+$ was found to be lower for the ABA isomer, since formation of the 1,2-isomer of the $[AB]^+$ ion is energetically more favorable than the generation of the analogous 1,3-$[AB]^+$ ion from the AAB isomer. For this reason the intensity of ions $[AA]^+$ and $[AB]^+$ should be in a 2:1 ratio irrespective of the position of the A or B acyl substitution on the glycerol backbone; however, this did not happen. The ratio $[PP]^+$:$[PP13]^+$ observed for PP13P was only 34:100, indicating that the formation of the 1,3-$[PP]^+$ ion is energetically unfavorable (Fig. 3).

On the other hand, PPP13 TAG featured the $[PP]^+$:$PP13]^+$ ratio of 77:100 and the above rule confirms the structure of this positional isomer. Consequently, the two regioisomers show two different spectra (Fig. 5).

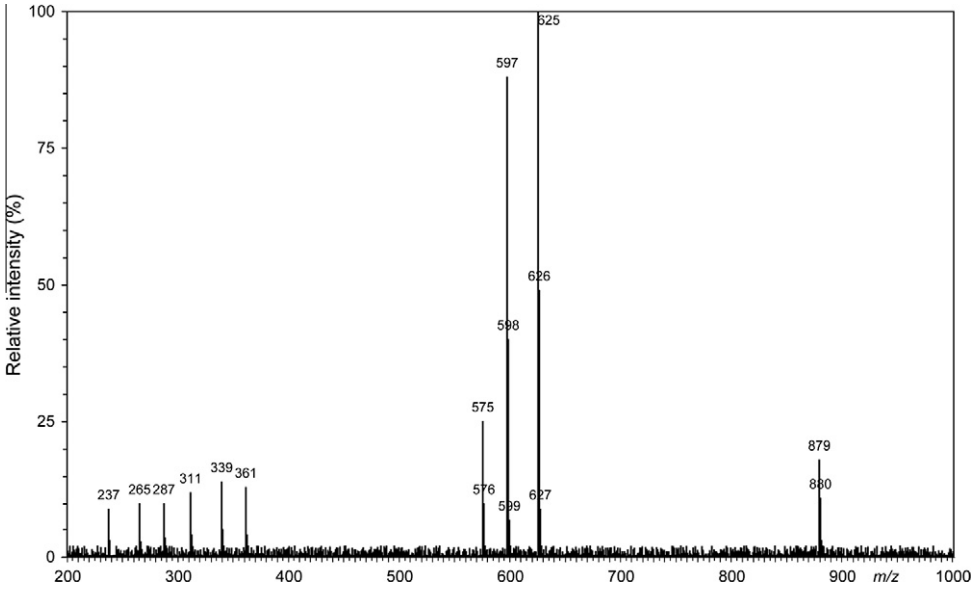

Fig. 4. Mass spectrum (APCI) of natural OP14Po.

Unfortunately, as mentioned above, we did not succeed in separating these two positional isomers even in the standards, i.e. PPP13 and PP13P. The natural TAG containing two palmitic and one ω-phenyltridecanoic acid has the $[PP]^+$:$[PP13]^+$ ratio of 58:100; this points to a mixture of two positional isomers, PPP13 and PP13P. The same results were obtained with two other synthesized positional isomers, PPP14 and PP14P (mass spectra not shown). Thus, it can be stated that, as the chromatographic separation of positional isomers of ω-phenylalkanoic acids is not successful, the positional isomers will not be unambiguously identified.

To show that even a complex biological material such as *D. vulgaris* seed oil can yield mass spectra that make it possible to identify TAGs, we present in Figs. 6 and 7 the spectra of PP13P13 and also OP15S. Identification of PP13P13, i.e. TAG obtained both by organic synthesis and from *D. vulgaris* seed oil is again based on four types of ions, $[M+H]^+$, $[DAG]^+$, $[MAG]^+$, and RCO^+. The *m/z* values of individual ions are given in Fig. 6 and in Table 3. Identification of TAGs having three different FAs in the molecule, such as OP15S, presents also no problem – see Fig. 7 and Table 3. We did not succeed in identifying the position of individual FAs in the TAG but assume that, because of its low abundance, the ion at *m/z* 605 with the structure $[OS]^+$ is 1,3-diacylglycerol and the corresponding TAG is therefore 1-oleoyl-2-ω-phenylpentadecanoyl-3-stearoyl-glycerol. The exact determination of structure of this and other TAGs requires the separation of the TAGs such that the peaks will contain chemical species, not mixtures of positional isomers. Furthermore, at least some of the TAGs should be synthesized. Identification of other TAGs was performed as described above and given in Table 3.

LC–MS analysis of TAGs from *D. vulgaris* revealed a total of 33 TAGs containing at least a single ω-phenylalkanoic acid. In keeping with the GC–MS analysis of FAs, see Table 3, the most abundant were Ph-13:0 and Ph-15:0, while the amounts of the others were below 1% total FAs. TAGs were found to contain only these two

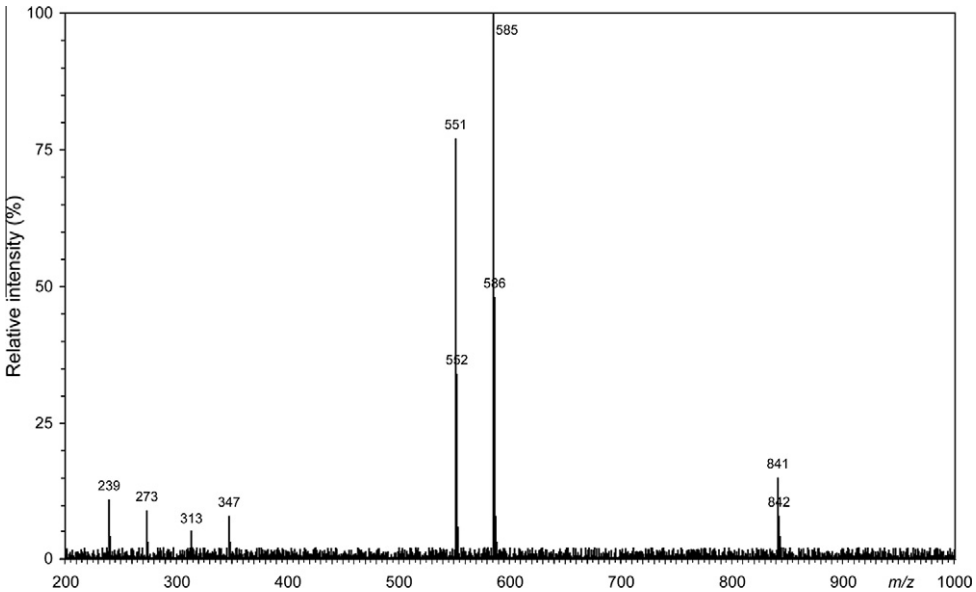

Fig. 5. Mass spectrum (APCI) of synthetic PPP13.

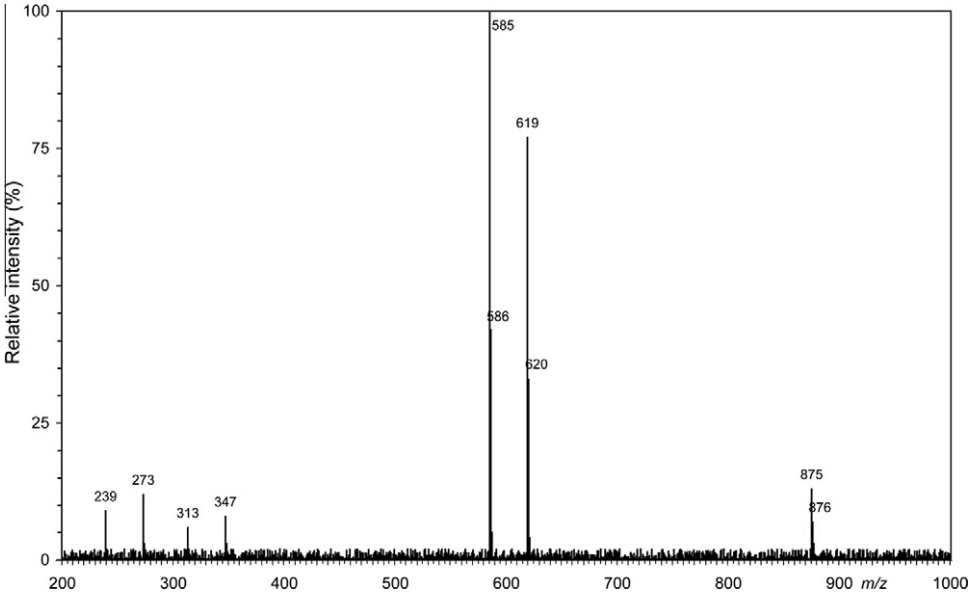

Fig. 6. Mass spectrum (APCI) of synthetic PP13P13.

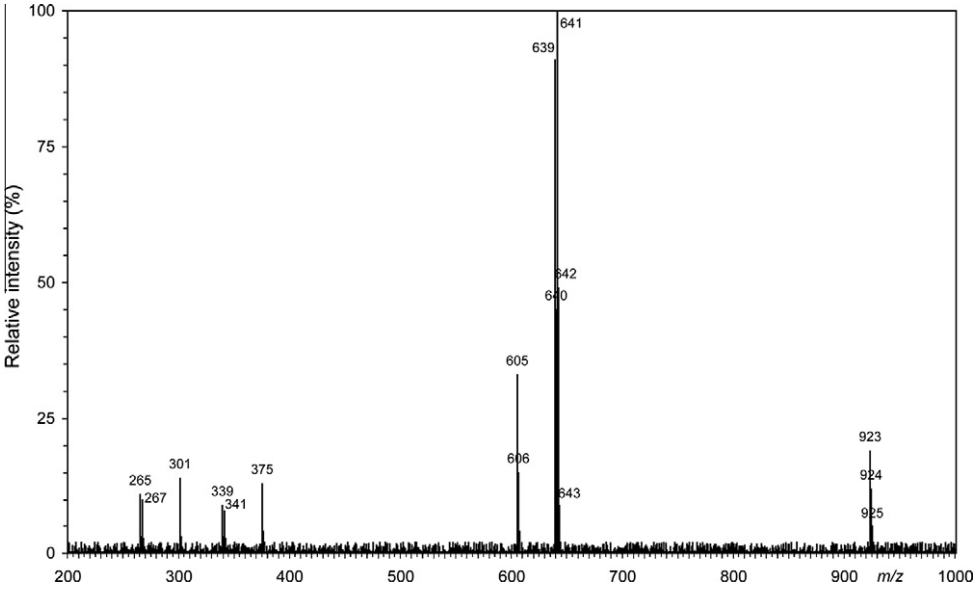

Fig. 7. Mass spectrum (APCI) of natural OP15S.

FA; if some TAGs contain also minor acids such as Ph-11:0 or monoenoic ω-phenylalkanoic acids, then these TAGs are below the detection limit, which was 0.1% total TAGs.

Only three TAGs containing two ω-phenylalkanoic acids were identified and their proportion was a mere 1.9% total TAGs. Table 3 shows that none of the TAGs is preferred, and the distribution of FAs is random. When compared with *R. erythropolis*, the TAGs from *D. vulgaris* have lower ECN, i.e. they contain more unsaturated FAs, preferentially α-linolenic and linoleic acids, which are completely missing in the bacterium.

The mass spectra of synthesized and natural TAGs documented that at least four molecular species (PPP13, PPP14, PP13P, and PP14P) the biosynthesis of asymmetrical TAGs, i.e. TAGs that do not have ω-phenylalkanoic acids in *sn*−2 position of glycerol backbone.

An interesting feature is that the contents of TAGs in *D. vulgaris* and *R. erythropolis* differed so widely that the two organisms shared only a single TAG, P13SS. This can be ascribed to the totally different metabolism, i.e. a different starter unit for the biosynthesis of ω-phenylalkanoic acids.

The abundance of the major TAGs containing ω-phenylalkanoic acids, such as LOP13 or OOP13, reaches the abundance of major TAGs of LLO or LOO types that are common components of plant oils (Holcapek et al., 2003).

Due to their beneficial effects on human health, natural substances of plant, and increasingly also microbial origin able to eliminate effects of free radicals in tissues, or compounds with high antioxidant activity are receiving an ever increasing attention (Korkina, 2007; Olsson et al., 2006). Among lipophilic compounds exerting these effects are ω-phenylalkanoic acids and TAGs containing them (Korkina, 2007; Ruberto and Baratta, 2000).

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging and antioxidant activities were determined by the coupled oxida-

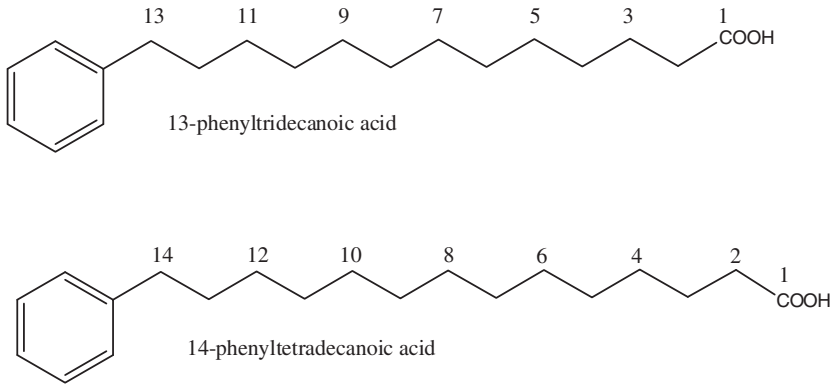

Fig. 8. Structures of two major phenolic fatty acids, i.e. Ph-13:0 and Ph-14:0.

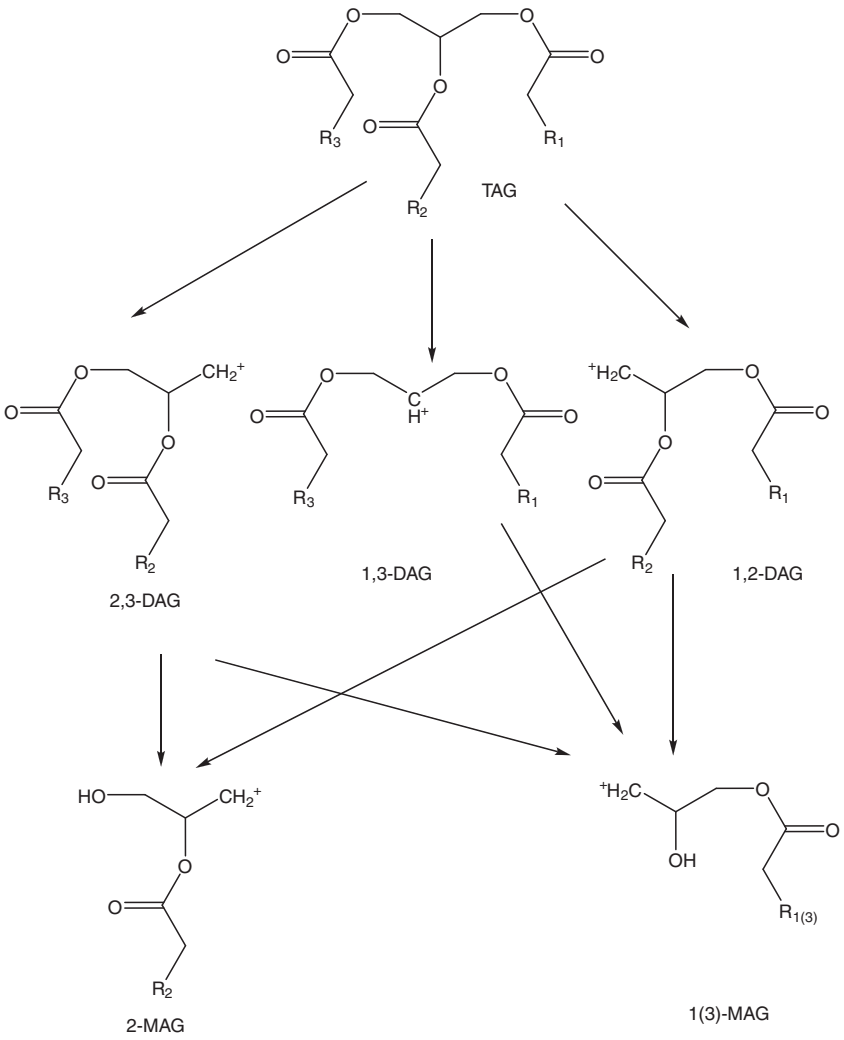

Fig. 9. Structures of the fragment ions from TAGs.

tion of linoleic acid and β-carotene in two mixtures of natural TAGs from *D. vulgaris* seed oil and *R. erythropolis* cultures growing in a medium containing phenylalanine as the sole source of carbon under protein synthesis restrictive conditions (Alvarez et al., 2002). These activities of natural ω-phenyl fatty acids and TAGs containing them were compared with those of the standard BHT (butylated hydroxy toluene), six synthetic compounds, i.e. two ω-phenylalkanoic acids (ω-phenyltridecanoic acid and ω-phenyltetradecanoic acid), and four TAGs (PPP13, PP13P, PP13P13, and PPP14) (Table 4). The BHT standard exhibited 100% activity in both tests whereas both free acids and TAGs having one ω-phenylalkanoic acid in the molecule had about 30% activity. The activity of PP13P13 was about 50% relative to BHT, natural mixtures exhibited a 20–30% efficiency. Several trends could be observed: both ω-phenyltridecanoic acid and ω-phenyltetradecanoic acids had about the same activity in both tests,

Table 4
Comparison of DPPH radical scavenging and antioxidant activities of FAs and TAGs containing ω-phenyl acids with the standard (BHT).

Compounds	% Antioxidant activity	% DPPH radical scavenging activity
BHT	100 ± 6	100 ± 5
ω-Phenyltridecanoic acid	45 ± 5	46 ± 4
ω-Phenyltetradecanoic acid	45 ± 4	46 ± 5
PPP13	38 ± 5	39 ± 5
PP13P	38 ± 4	40 ± 6
PP13P13	57 ± 5	60 ± 5
PPP14	37 ± 4	38 ± 5
TAGs of *D. vulgaris*	30 ± 4	38 ± 4
TAGs of *R. erythropolis*	20 ± 4	25 ± 3
Canola oil	11 ± 3	9 ± 4
Soybean oil	18 ± 4	17 ± 5

Each assay was repeated three times and the average result and standard deviation were calculated.

positional isomers of PPP13, PP13P and homologs PPP13 and PPP14 were equally active. The TAG having two ω-phenylalkanoic acids had activity exceeding 50% of the standard. Natural oils had the lowest activity, probably because they contain many other TAGs with saturated FAs. The oil from *D. vulgaris* was more active owing to the presence of polyunsaturated FAs, which are missing in *R. erythropolis*. Two plant oils, i.e. canola (rape seed) and soybean had lower radical scavenging and antioxidant activities than all other TAGs or oils. The activity of our natural oils reached 1/5 to 1/3 of BHT – much more than published, e.g., for *Artemisia* and oregano essential oils (Lopes-Lutz et al., 2008).

GC–MS analysis of fatty acids from *D. vulgaris* and from *R. erythropolis* provided data on the proportion of ω-phenylalkanoic acids. The plant *D. vulgaris* contained odd-numbered whereas the bacterium *R. erythropolis* even-numbered ω-phenylalkanoic acids (Table 1). This difference in the chain length can be explained as being due to the manner in which ω-phenylalkanoic acids are biosynthesized in plants. Plant phenolic compounds are synthesized via the phenylpropanoid pathway (starting from shikimic acid) and share a common building block, the C6–C3 unit (Harborne, 1980). Similarly, according to Pupo et al. (1996), the ω-phenylalkanoic acids can be biosynthesized through direct condensation of polyketide chains of different lengths with C6–C3 units, providing a starter unit. This leads to the elongation of the odd-numbered precursor having 9 carbon atoms by $n \times$ malonyl-CoA extensions, i.e. extension by an even number of carbon atoms that produces in all cases a final odd-chain acid.

On the other hand, in the bacterium, which does not under normal conditions synthesize ω-phenylalkanoic acids (Rezanka et al., 2010; Alvarez et al., 2002), addition of phenylalanine leads to the production of even-numbered ω-phenylalkanoic acids, which is also confirmed by the presence of even acids in two bacteria, *Bacillus* and *Vibrio* (Carballeira et al., 1997, 2001). The biosynthesis involves the known mechanism in which Phe is degraded to phenylacetyl-CoA (Smith and Tsai, 2007), which serves as a starter unit for the biosynthesis of ω-phenylalkanoic acids with chain length greater than C2 (Smith and Stern, 1983). The process takes place under both anaerobic (Schneider et al., 1997) and aerobic (Teufel et al., 2010) conditions; this is in keeping with our data on *R. erythropolis*, which has aerobic metabolism.

3. Conclusion

To our knowledge, this is the first time when TAGs containing ω-phenylalkanoic acids were identified by RP-HLPC/MS-APCI. A total of 95 molecular species of TAGs were identified in *D. vulgaris* and a mere 61 molecular species in *R. erythropolis*. This difference is given by the far richer spectrum of FAs in the plant and the fact that these FAs include unsaturated FAs, see Table 3. In agreement with the presumed biosynthesis of ω-phenylalkanoic acids, *D. vulgaris* contained TAGs containing exclusively odd-numbered ω-phenylalkanoic acids while *R. erythropolis* TAGs contained only even counterparts thanks to the starter unit, i.e. phenylacetic acid derived from Phe. Both natural TAG mixtures exhibited antioxidant and free radical scavenging activity. We documented that RP-HLPC/MS-APCI can be used to identify unusual TAGs containing rare FAs, and may therefore be employed for analyzing oils from unusual sources.

4. Experimental

4.1. Standards and instrumentation

Acetonitrile, 2-propanol, Mg turnings, THF, hexane, dichloromethane, glycerol, 4-dimethylaminopyridine (DMAP), *N*,*N*′-dicyclohexylcarbodiimide (DCC), DL-α-palmitin (1-palmitin), 1,3-dipalmitin (1,3-dipalmitoyl-glycerol), and 1,2-dipalmitin (1,2-dipalmitoyl-rac-glycerol) were purchased from Sigma–Aldrich (Prague, CR). High resolution MS were recorded using a VG 7070E-HF spectrometer (70 eV). HR-FAB-MS (positive ion mode) were obtained with a PEG-400 matrix. NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^{1}H) and 125.7 MHz (^{13}C).

4.2. Synthesis

4.2.1. Synthesis of methyl 10-oxo-13-phenyltridecanoate (**4**) and of methyl 10-oxo-14-phenyltetradecanoate (**5**)

To a suspension of magnesium (turnings, 24 mg, 1 mmol) in THF (10 ml), a solution of alkylphenyl chloride (143 μl, 1 mmol (**1**); 163 μl, 1 mmol (**2**), respectively) in THF (5 ml) was added dropwise under mild reflux with vigorous stirring under nitrogen, and stirring was continued for 1 h under reflux. After cooling, the mixture (Grignard reagent) was added via syringe to a solution of acid chloride (224 μl, 1.0 mmol (**3**)) and $Fe(acac)_3$ (11 mg, 0.03 mmol) in THF at −78 °C under argon, causing an immediate color change from bright red to dark brown-black. After being stirred for 15 min at that temperature, the reaction was quenched with saturated aqueous NH_4Cl (10 ml) and the mixture was extracted with tert-butyl methyl ether (3× 20 ml) after reaching ambient temperature. The combined organic layers were dried over $MgSO_4$, the solvent was evaporated, and the residue was purified by TLC (hexane–EtOAc, 10:1) providing the title compounds as a white solid (262 mg, 79%; 242 mg, 73%).

^{1}H NMR of **4**, ($CDCl_3$, 500 MHz) δ ppm: 1.27–1.47 (8H, m, H-4–H-7), 1.51–1.70 (4H, m, H-3, H-8), 1.81–2.01 (2H, m, H-12), 2.36 (2H, t, J = 7.3 Hz, H-2), 2.36–2.43 (4H, m, H-9, H-11), 2.53–2.72 (2H, m, H-13), 3.66 (3H, s, OCH_3), 7.23–7.37 (5H, m, H-2′–H-6′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ ppm: 22.7 (C-8), 25.1 (C-3), 25.5 (C-12), 29.0–29.6 (C-4–C-7), 34.1 (C-2), 36.0 (C-13), 38.8 (C-9), 42.5 (C-11), 51.4 (OCH_3), 125.5 (C-4′), 128.2 (C-3′, C-5′), 128.3 (C-6′), 130.2 (C-2′), 142.9 (C-1′), 174.2 (C-1), 211.0 (C-10); IR (neat) ν cm^{-1} 2950, 2800, 1735 (ester carbonyl), 1710 (oxo group); HREIMS (m/z): 318.2201 $[M]^+$, calc. for $[C_{20}H_{30}O_3]^+$ 318.2195.

^{1}H NMR of **5**, ($CDCl_3$) δ ppm: 1.27–1.47 (10H, m, H-4–H-8), 1.51–1.70 (4H, m, H-3, H-9), 1.81–2.01 (2H, m, H-13), 2.37 (2H, t, J = 7.3 Hz, H-2), 2.36–2.43 (4H, m, H-10, H-12), 2.53–2.72 (2H, m, H-14), 3.66 (3H, s, OCH_3), 7.23–7.37 (5H, m, H-2′–H-6′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ ppm: 22.7 (C-9), 25.1 (C-3), 25.5 (C-13), 29.0–29.6 (C-4–C-8), 34.1 (C-2), 36.0 (C-14), 41.8 (C-10), 42.5 (C-12), 51.4 (OCH_3), 125.5 (C-4′), 128.2 (C-3′, C-5′), 128.3 (C-6′), 130.2 (C-2′), 142.9 (C-1′), 174.2 (C-1), 211.0 (C-11); IR (neat) ν cm^{-1} 2950, 2800, 1735 (ester carbonyl), 1710 (oxo group); HREIMS (m/z): 332.2356 $[M]^+$, calc. for $[C_{21}H_{32}O_3]^+$ 332.2351.

*4.2.2. Synthesis of 13-phenyltridecanoic acid (**6**) and 14-phenyltetradecanoic acid (**7**)*

To a solution of methyl oxo-acid (222 mg, 232 mg, both of 0.7 mmol) obtained as above in diethylene glycol (10 ml), 80% hydrazine hydrate (1 ml) and potassium hydroxide (700 mg) were added and stirring was continued for 2 h under reflux. The reaction mixture was concentrated, poured into water (10 ml), acidified with dilute hydrochloric acid and extracted with chloroform. The organic extract was washed with water, dried over anhydrous $MgSO_4$ and evaporated in vacuo. The residue was recrystallized from hexane to afford the title compounds as white leaflets; yield: 160 mg, 80%; 165 mg, 78%, mp of **6** 48.5 °C, lit. data 48–49 °C (Hase and Ohura, 1954), mp of **7** 68.0 °C, lit. data 68–69 °C (Hase and Ohura, 1954).

^{1}H NMR of **6**, ($CDCl_3$) δ ppm: 7.18–7.28 (5H, m, H-2′–H-6′), 2.60 (2H, t, J = 7.7, H-13), 2.34 (2H, t, J = 7.6, H-2), 1.63 (4H, m, H-3, H-12), 1.27 (16H, m, H-4–H-11); ^{13}C NMR ($CDCl_3$, 125 MHz) δ ppm: 24.8 (C-3), 29.1–29.6 (C-4–C-11), 31.5 (C-12), 34.0 (C-2), 36.0 (C-13), 125.5 (C-4′), 128.2 (C-3′, C-5′), 128.4 (C-6′, C-2′), 143.0 (C-1′), 179.2 (C-1); HREIMS (m/z): 290.2251 $[M]^+$, calc. for $[C_{19}H_{30}O_2]^+$ 290.2246.

^{1}H NMR of **7**, ($CDCl_3$) δ ppm: 7.18–7.28 (5H, m, H-2′–H-6′), 2.60 (2H, t, J = 7.7, H-14), 2.34 (2H, t, J = 7.6, H-2), 1.63 (4H, m, H-3, H-13), 1.27 (18H, m, H-4–H-12); ^{13}C NMR ($CDCl_3$, 125 MHz) δ ppm: 24.8 (C-3), 29.1–29.6 (C-4–C-12), 31.5 (C-13), 34.0 (C-2), 36.0 (C-14), 125.5 (C-4′), 128.2 (C-3′, C-5′), 128.4 (C-6′, C-2′), 143.0 (C-1′), 179.2 (C-1); HREIMS (m/z): 304.2408 $[M]^+$, calc. for $[C_{20}H_{32}O_2]^+$ 304.2402.

4.3. Preparation of mixed FA composition TAGs

The esterification of glycerol derivatives with acids was described previously (Ziegler and Berger, 1979). Briefly, a rapidly stirred suspension of 1,3-diacylglycerol or 1,2-diacylglycerol (10 μmol), the acid (11 μmol), and a catalytic amount of 4-dimethylaminopyridine (2 μmol) in anhydrous CH_2Cl_2, (5 ml) was treated with *N*,*N*′-dicyclohexylcarbodiimide (12 μmol). The resulting mixture was stirred under N_2 for 24 h at room temperature, diluted with CH_2Cl_2, and filtered to remove precipitated 1,3-dicyclohexyl urea. The filtrate was washed with 0.5 N HCl, saturated aqueous $NaHCO_3$, H_2O, and brine and dried ($MgSO_4$). The solvent was removed in vacuo, and the remaining residue was purified by TLC (silica gel H, developed in hexane/diethyl ether/acetic acid (70:30:1, by vol.). The yields of appropriate TAGs are in Table 2. The purity (see also Table 2) of individual synthesized TAGs was determined by RP-HPLC, for the conditions see below. The TAGs PP13P13 and PP14P14 were synthesized in a similar manner but with different ratios of reagents, i.e. 1-palmitin (5 μmol) and ω-phenylalkanoic acids (11 μmol).

4.4. Cultivation

R. erythropolis CCM 2595 was obtained from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic). Cells were grown in Erlenmeyer flasks using a rotary shaker (100 rpm, 30 °C) in basic mineral medium (g/l: KH_2PO_4 0.17; $MnCl_2{\cdot}4H_2O$ 0.001; K_2HPO_4 0.13; $CaCl_2{\cdot}2H_2O$ 0.00026; $(NH_4)_2SO_4$ 0.71; $FeSO_4{\cdot}7H_2O$ 0.0006; $MgCl_2{\cdot}6H_2O$ 0.34; $Na_2MoO_4{\cdot}2H_2O$ 0.002; pH was adjusted to 7.0). The cultivation according to Alvarez et al. (2002) was modified as follows: in the first stage the cells were grown on phenylalanine (10 g/l) as a sole carbon source. Biomass from late exponential phase was centrifuged (9050 g, 10 min) and resuspended in fresh mineral medium with phenylalanine as a sole carbon source (10 g/l). The initial biomass concentration was adjusted to optical density of 2.5 at 400 nm. Chloramphenicol (0.2 g/l) was added to suppress protein synthesis and to prefer entry of phenylalanine into other metabolic pathways. After 24 h incubation the cells were collected by centrifugation (9050*g*, 10 min), washed twice with physiological solution and lyophilized (dry biomass yield 650 mg/l).

4.5. Isolation of TAGs

The seeds from of *D. vulgaris* (Schott) were collected in early summer 2010 along the road from Kares to Omalos, Crete, Greece. A voucher specimen is deposited by the first author. Two to three grams of seeds were crushed, mixed with 5 ml of hexane, and the mixture was stirred occasionally for 15 min. The solid particles were filtered out using a fine filter with 0.45 μm pores. Hexane was evaporated and the oil sample was dissolved in an acetonitrile–2-propanol–hexane mixture (1:1:1, v/v/v) to prepare 3% solution (w/v), 10 μl of this solution was injected for HPLC analysis.

The lyophilized cells of *R. erythropolis* were mixed with 10 ml of hexane and the mixture was stirred for 15 min. The cells were filtered off, hexane was evaporated and the oil samples were dissolved in an acetonitrile-2-propanol–hexane mixture (1:1:1, v/v/v), which was injected on the column.

4.6. FAMEs analysis

The TAGs (~5 mg) were saponified overnight in 10% KOH–MeOH at room temperature. A fatty acid fraction obtained from saponification was partitioned between alkali solution (pH 9) and Et_2O to remove basic and neutral components. The aqueous phase, containing fatty acids, was acidified to pH 2 and extracted with hexane. The fatty acid fraction was methylated using CH_2N_2 (Rezanka et al., 1986). GC–MS of fatty acid methyl ester (FAME) mixture was done on a Finnigan 1020 B in EI mode. Splitless injection was at 100 °C, and a fused silica capillary column (Supelcowax 10; 60 m × 0.25 mm i.d., 0.25 mm film thickness; Supelco, Prague) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C/min to 180 °C and at 2 °C/min to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. All spectra were scanned within the range of m/z 50–500. The structures of FAMEs were confirmed by comparison of retention times and fragmentation patterns with those of the standard FAMEs (Supelco, Prague) and our synthesized standards (ω-phenyltridecanoic and ω-phenyltetradecanoic acids).

4.7. RP-HLPC/MS-APCI

HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Agilent, USA) and two Hichrom columns HIRPB-250AM 250 × 2.1 mm ID, 5 μm particle size, in series. This setup provided us with a high-efficiency column – approximately 26,000 plates/250 mm. A quadrupole mass spectrometer system Navigator (Finnigan MAT, San Jose, CA, USA) was used for analysis. The instrument was fitted with an atmospheric pressure chemical ionization source (vaporizer temperature 390 °C, capillary heater temperature 260 °C, corona current 7 μA, sheath gas – high-purity nitrogen, pressure 0.45 MPa, and auxiliary gas (also nitrogen) flow rate 15 ml/min). Positively charged ions with m/z 200–1000 were scanned with a scan time of 0.5 s. The whole HPLC flow (0.35 ml/min) was introduced into the APCI source without any splitting. TAGs were separated using a gradient solvent program with acetonitrile (MeCN) and 2-propanol (iPrOH) as follows: initial MeCN/iPrOH (99:1, vol/vol); linear from 5 min to 120 min MeCN/iPrOH 30:70, vol/vol); held until 30 min; the composition was returned to the initial conditions over 10 min. A peak threshold of 0.07% intensity was applied to the mass spectra. Data acquisition and analyses were

performed using PC with MassLab 2.0 for Windows XP applications/operating software.

4.8. Biological activity

4.8.1. Determination of DPPH radical scavenging activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was measured according to the procedures described by Blois (1958), Chen and Ho (1997), and Kulisic et al. (2004). Briefly, 0.3 mM DPPH in methanol solution was prepared, and a 1.0 ml aliquot was added to 2.5 ml of compounds solution in MeOH (1.0 mg/ml). After incubation for 30 min in the dark at room temperature, the absorbance was measured at 517 nm, and the percentage of radical scavenging activity was calculated according to the equation $[1 - (A_{sample}/A_{control})] \times 100$, where A_{sample} is the absorbance of the test compounds and $A_{control}$ is the absorbance of the control reaction (containing all of the reagents except the test compounds). DPPH solution (1.0 ml) plus MeOH (2.5 ml) were used as a control. Each assay was repeated three times and the average result and standard deviation calculated.

4.8.2. Determination of antioxidant activity using β-carotene/linoleic acid assay

The antioxidant activity was determined by measuring the ability of the compounds to inhibit the conjugated diene hydroperoxide formation from linoleic acid and β-carotene coupled oxidation in an emulsified aqueous system, which loses its orange color when reacting with the radicals (Miller, 1971; Braca et al., 2001). The linoleic acid (20 mg, 99% purity) and Tween 20 (200 mg) were dissolved in $CHCl_3$ (1 ml) and a solution of 2 mg of β-carotene (95% purity) in $CHCl_3$ (1 ml) was added, solution was completely evaporated and distilled water (50 ml) saturated with oxygen by shaking for 30 min was added to form an emulsion. Tested compound(s) were dissolved in methanol (1.0 mg/ml) and 200 μl of this solution was added to β-carotene/linoleic acid emulsion (2.5 ml). Mixtures were subjected to oxidation by placing in an oven at 50 °C for 3 h. The absorbance was measured at 470 nm, and the relative antioxidant activity was calculated according to equation $AA\% = 100 \times [1 - (A_0 - A_t/A_{00} - A_{0t})]$, where A_0 is the absorbance at the beginning of the incubation, with compound; A_t is the absorbance after 3 h, with compound; A_{00} is the absorbance at beginning of the incubation, without compound (200 μl of MeOH and 2.5 ml of β-carotene emulsion); A_{ot} is the absorbance after 3 h, without compound. BHT was used as control. Each assay was repeated three times and the average result and standard deviation calculated.

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