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Regioisomer separation and identification of triacylglycerols containing vaccenic and oleic acids, and α- and γ-linolenic acids, in thermophilic cyanobacteria *Mastigocladus laminosus* and *Tolypothrix* sp.

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ABSTRACT

Reversed phase liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (RP-HPLC/APCI-MS) was used for direct analysis of triacylglycerols (TAGs) from different strains of the cyanobacteria *Mastigocladus laminosus*, *Tolypothrix cf. tenuis* and *Tolypothrix distorta*. This technique enabled us to identify and quantify the specific molecular species of TAGs directly from lipid extracts of the cyanobacteria. The regioisomeric series of TAGs having α-linolenic and γ-linolenic and also oleic and *cis*-vaccenic acids were separated by RP-HPLC and identified by APCI-MS. *M. laminosus* produced only a few molecular species of TAGs, including both isomers of octadecenoic (oleic and vaccenic) acid, while *T. distorta* contained tens of molecular species of TAGs having FAs with up to four double bonds (stearidonic acid and including also its positional isomer, i.e. 3,6,9,12-octadecatetraenoic acid) and both positional isomers (α and γ) of linolenic acids. Individual strains of both cyanobacteria exhibited different contents of polyunsaturated fatty acids (*Tolypothrix* sp.) and different distribution of positional isomers of monoenoic fatty acids in TAGs (*M. laminosus*).

1. Introduction

Like other potentially technologically or nutritionally useful microorganisms, cyanobacteria have also been subjected to screening for lipid production. Unfortunately, the amounts of total lipids found in cyanobacteria are relatively low (Hu et al., 2008). According to Hu et al. (2008) and Alvarez and Steinbuchel (2002) naturally occurring cyanobacteria have not been observed to accumulate non-polar lipids or triacylglycerols (TAGs), which represent the most frequent and most useful form of energy storage in eukaryotic organisms such as animals, plants and also algae (Dubois et al., 2007). On the other hand, uncharacterized non-polar lipids were identified in many cyanobacteria, e.g. in *Scytonema julianum* or *Chroococcidiopsis* sp. (Antonopoulou et al., 2005a,b), and in *Pseudanabaena* sp. or *Oscillatoria splendida* (Deloura et al., 1987). Rezanka et al. (2003) and Ward et al. (1994) identified non-polar lipids in still other cyanobacteria by two-dimensional TLC.

Already in 1993, Taranto et al. (1993) analyzed the lipids of the cyanobacterium *Nostoc commune* by TLC and identified not only non-polar lipids, but also TAGs, unfortunately without their quantification. Also, Bychek and Bychek (1997) identified in *N. commune* non-polar lipids, predominantly TAGs (up to 4.4 mg/1 g of dry biomass). Ramadan et al. (2008) identified TAGs as 25% of the dry weight of the cyanobacterium *Spirulina platensis* by TLC.

Separation of TAGs is usually performed by using RP-HPLC, and APCI is the most frequently used ionization technique for TAG structural determination because of easy coupling to non-aqueous mobile phase systems and high ionization efficiency for non-polar species (Byrdwell, 2005; Rezanka and Sigler, 2007). Problems are encountered when analyzing unusual TAGs containing FAs with no common position and number of double bond(s) (e.g. non-methylene interrupted *cis*-5,9,12-octadecatrienoic (pinolenic) acid) (Lísa et al., 2007) or polyunsaturated fatty acids (PUFAs) (Rezanka et al., 2011).

Analysis of regioisomers either in which the acyl chains are at different positions (i.e. AAB, ABA) or the double bond(s) in one or more of the acyl chains are at different positions is one of the most difficult tasks in TAG analysis. Yet TAGs containing α- and γ-linolenic acids, such as those from black currant, borage or evening primrose have been successfully separated (Leskinen et al., 2010a; Lísa et al., 2009). Regioisomers of TAGs containing positional isomers of octadecenoic (vaccenic and oleic) acid were also

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separated and identified, e.g. in buckthorn (Leskinen et al., 2010b), see footnote to Table 1.

Based on our preceding experience with the analysis of algal fatty acids (Rezanka and Podojil, 1984; Dembitsky et al., 1993) and uncommon TAGs by LC–MS/APCI we analyzed cyanobacterial strains containing the regioisomeric series of TAGs having α-linolenic and γ-linolenic (*Tolypothrix* sp.) and also oleic and *cis*-vaccenic acids (*Mastigocladus laminosus*). To our knowledge, no systematic analysis of cyanobacterial TAGs has as yet been performed.

2. Results and discussion

GC–MS analysis of the methyl esters obtained after hydrolysis of TAGs followed by esterification confirmed the eleven major fatty acids (Table 1). Ten of the 11 peaks were identified based on retention characteristics and mass spectra of FAMEs. Only one peak, i.e. FAME with retention time 32.7 min was not identified. The methyl esters of PUFAs on polar capillary column were eluted in the order of ω-6, ω-3, etc., (Christie and Han, 2010) and it was possible to determine at least tentative positional isomers. We assumed, on the basis of retention time, that this peak contains a methyl ester of 18:4ω-6 FA. This compound has double bonds in a different position than the methyl ester of stearidonic acid (18:4ω-3) that has a peak with retention time 34.1 min.

The methyl esters of the methylene-interrupted tetraenes give molecular ions at very low abundance. The same is true for ions $[M-31/32]^+$ i.e. loss of a methoxyl or methanol from molecular ion. Diagnostically important for methyl esters of PUFAs are ω ions, i.e. ion at *m/z* 150 characteristic for a ω-6 terminal group and one at *m/z* 108 characteristic for a ω-3 terminal group (Brauner et al., 1982; Fellenberg et al., 1987). Based on retention times and mass spectra of FAME, we tentatively determined that the peak at equivalent chain-lengths 19.21 has the structure 18:4ω-6 and the peak at equivalent chain-lengths 19.55 has the structure 18:4ω-3.

We used picolinyl esters for the complete identification because these esters permit the location of the double bonds. Different positional isomers were found to give different mass spectra. Picolinyl ester of 18:4ω-3 acid has been measured many times (http://lipidlibrary.aocs.org/) and it is possible to distinguish between ω-3 and ω-6 positional isomers of 18:4 FAs. Mass spectra of picolinyl esters of both acids are given in Fig. 1 and Fig. 1S (Supplementary Material). Picolinyl ester of 18:4ω-3 acid showed a gap of 26 Da between *m/z* 312 and 338, which locates the double bond in position 15 while the gap between *m/z* 272 and 298 locates double bond in position 12, etc., see Fig. 1S. It is however easier to spot gaps of 40 Da for the double bond and an associated methylene group, i.e. gaps from *m/z* 178 to 218 to 258 to 298 to 338. The spectra of our compound (picolinyl ester of 18:4ω-3) and the same substance shown on the website (http://lipidlibrary.aocs.org/) are very similar, if not identical. This clearly implies the structure of 18:4ω-3, i.e. the later-eluted peak.

Similar principles were applied to the first eluted peak, i.e. 18:4ω-6 picolinyl ester (Fig. 1). The mass spectra show differences between the two picolinyl esters, i.e. different values of the 26 Da and/or 40 Da gaps. The gap of 26 Da between *m/z* 270 and 296 locates the double bond in position 12 while the gap between *m/z* 230 and 256 locates a double bond in position 9, etc. Again, it is easier to spot gaps of 40 Da for the double bond and a neighboring methylene group, i.e. only three gaps from *m/z* 176 to 216 to 256 to 296. The position of the first double bond, i.e. Δ^3, was not identified, because the mass spectrum is not informative for the three position, i.e. no 26 amu gap or characteristic ion was observed. Yet on the basis of our values (retention time, the presence of ion at *m/z* 150 in methyl ester and mass spectrum of picolinyl ester) we identified this compound as 18:4ω-6 acid. This compound, though it seems very unlikely, has so far been only synthesized (Pabon et al., 1965) and not isolated from nature (Chemical Abstracts search of November 1, 2011).

Fig. 2 shows the RP-HPLC with APCI detection of total ion current (from 200 to 1000 Da) of TAGs from *Tolypothrix distorta* collected in Tatra Mountains. More than 90 molecular species of TAG were identified; many of them are highly unsaturated, having up to 11 double bonds, as found by using RP-HPLC data from previous analyses (Rezanka et al., 2011). Although the major peaks were separated on the baseline, the separation of some peaks was insufficient. From the mass spectra it was suggested that several peaks contained two or more overlapping species, see also the analysis of similar TAGs containing PUFAs (Hori et al., 1993, 1994; Lísa et al., 2009).

Preliminary identification, later confirmed by mass spectra, was based on elution order of TAGs, which best expresses the ECN (equivalent carbon number, ECN = ACN-2*x*DB (ACN = acyl carbon number; DB = number of double bond(s))), (Momchilova et al., 2004; Mu and Hoy, 2000). Most TAGs having the same value of ECN can be separated, including the partial separation of critical pairs of TAGs, i.e. LLSt (RT = 49.6 min) and LnLLn (RT = 48.7 min) with ECN = 38. The retention characteristics of TAGs having identical ECN are strongly dependent on the FAs composition in individual TAGs, determined mainly by the number of double bond(s) and acyl chain lengths. The retention time of TAGs having the same ECN value increases with decreasing number of double bond(s) in acyl chains (Dermaux et al., 1999); thus on replacing oleic acid by palmitic acid the retention time of PPP is 75.3 min, while that of POP is 74.1 min. Identification of TAGs containing PUFAs requires LC–MS analysis because other identification methods do not give full value and accurate results; see Rezanka et al. (2011).

Positive ion APCI mass spectra of TAGs having PUFAs typically exhibited $[M+H]^+$ ion and fragment ions of type $[M+H-RCOOH]^+$

Table 1
Proportions of major fatty acids in TAGs of cultivated cyanobacterial strains, including their systematic and trivial names, abbreviations and fatty acid compositions (%).

Systematic name	Trivial name	Abbreviation	*M. laminosus* Carslbad	*M. laminosus* Sofia	*Tolypothrix* sp. Prague	*Tolypothrix* sp. Tatra M.
Tetradecanoic	Myristic	M	0.0	0.0	1.0 ± 0.13	0.9 ± 0.32
Hexadecanoic	Palmitic	P	36.4 ± 1.17[a]	35.8 ± 2.11	17.5 ± 1.37	16.1 ± 1.34
9*Z*-Hexadecenoic	Palmitoleic	Po	29.2 ± 1.87	29.4 ± 1.61	11.2 ± 1.24	7.1 ± 0.67
Octadecanoic	Stearic	S	2.3 ± 0.19	2.4 ± 0.16	6.3 ± 0.78	2.7 ± 0.35
9*Z*-Octadecenoic	Oleic	O	21.0 ± 1.45	19.8 ± 1.73	18.7 ± 0.86	12.1 ± 1.42
11*Z*-Octadecenoic	Vaccenic[b]	V	9.4 ± 0.65	11.0 ± 0.98	0.0	0.0
All *Z* 9,12-octadecadienoic	Linoleic	L	1.7 ± 0.17	1.6 ± 0.12	28.9 ± 2.31	30.1 ± 2.37
All *Z* 6,9,12-Octadecatrienoic	γ-Linolenic	γLn	0.0	0.0	2.5 ± 0.29	5.1 ± 1.22
All *Z* 9,12,15-octadecatrienoic	α-Linolenic	αLn	0.0	0.0	10.8 ± 0.37	19.8 ± 0.88
All *Z* 6,9,12,15-octadecatetraenoic	Stearidonic	St	0.0	0.0	2.9 ± 0.21	5.4 ± 0.41
All *Z* 3,6,9,12-octadecatetraenoic	γ-Stearidonic	γSt	0.0	0.0	0.2 ± 0.08	0.7 ± 0.19

[a] Results are given as mean ± S.D. for three separate determinations.
[b] The correct name of 11*Z*-octadecenoic acid is *cis*-vaccenic or asclepic acid.

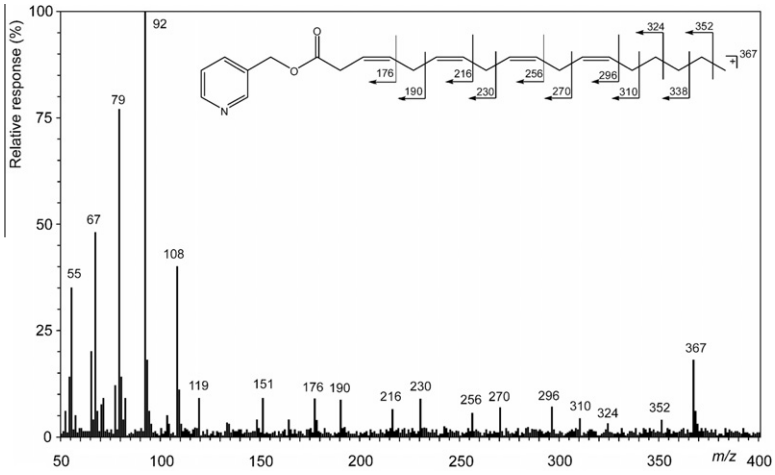

Fig. 1. Mass spectrum of picolinyl ester of 18:4ω-6 acid. For explanation of values see the text.

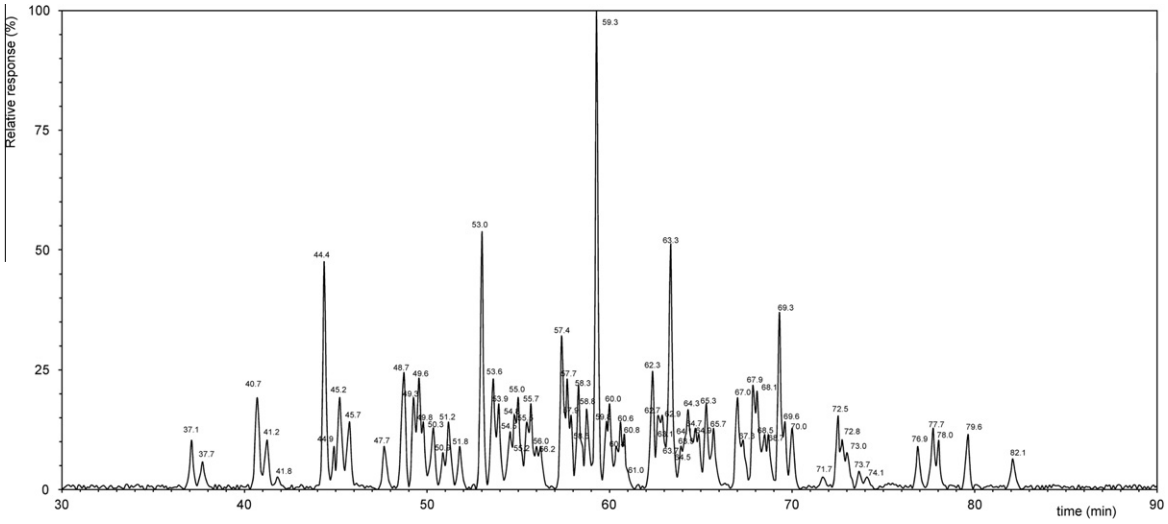

Fig. 2. RP-HPLC/APCI-MS chromatogram (TIC) of the TAGs from the cyanobacterium *T. distorta* collected in Tatra Mountains. Numerals above peaks show the retention time of appropriate peak in minutes.

([DAG]$^+$), resulting from the loss of an acyl chain from TAG. Monoacyl glycerol ([MAG]$^+$) is equivalent to [RCO+74]$^+$ ion. The positive ion APCI mass spectrum of LnLnLn (for abbreviations of FAs, see Table 1) shows the fragment ions [RCO]$^+$ at *m/z* 261, [RCO+74]$^+$ at *m/z* 335, and [M+H−RCOOH]$^+$ at *m/z* 595. These ions were observed with sufficient intensity to analyze the mass spectrum. Increasing number of double bonds in the TAGs was accompanied by increase in the intensity of the protonated molecular ion species [M+H]$^+$ while the intensities of [RCO]$^+$, [RCO+74]$^+$ and [M+H−RCOOH]$^+$ ions decreased (Lísa et al., 2007, 2009).

From the APCI mass spectrum of the peak at RT 37.1 min it is obvious that this is a StLnSt, as indicated by the following ions: [M+H]$^+$ at *m/z* 869, [M+H−RCOOH]$^+$ at *m/z* 591 (StSt), and *m/z* 593 (StLn), [RCO+74]$^+$ at *m/z* 333 [St]$^+$ and *m/z* 335 [Ln]$^+$, RCO$^+$ at *m/z* 259 (St) and *m/z* 261 (Ln), see also Fig. 3.

Identification of other molecular species of TAGs from another part of the chromatogram was more complicated, which best shows on the partially separated peak with retention time approximately 63.8 min (peak center), the front of this peak containing OLM with [M+H]$^+$ at *m/z* 829. The structure was established from the presence of the ions: [M+H−RCOOH]$^+$ at *m/z* 601 (OL), *m/z* 549 (OM), and *m/z* 547 (LM). Conversely, the latter part of this peak contained PLPo with [M+H]$^+$ at *m/z* 829, and its structure was confirmed by the following ions: [M+H−RCOOH]$^+$ at *m/z* 575 (PL), *m/z* 573 (PoL) and *m/z* 549 (PPo).

Though we used two RP-HPLC columns with a total of 52,000 theoretical plates, we could not separate some of the TAGs. Therefore, we used a SIM (selected ion monitoring) method, where for instance the interval 57.0–61.0 min is shown in Fig 4. SIM was measured for the following values: *m/z* 547 [PoPo,LM]$^+$, 571 [StP]$^+$, 573 [LPo,LnP, γLnP]$^+$, 575 [LP]$^+$, 577 [OP]$^+$, 595 [LnLn,LnγLn,LSt]$^+$, 597 [LLn,LγLn,StO]$^+$, 599 [LL,OLn,OγLn,SSt]$^+$, 601 [OL,SLn,SγLn]$^+$, and 603 [SL]$^+$, including total ion current (TIC). This method allowed us, usually in combination with tandem mass spectrometry, to generate [RCO]$^+$ and/or [RCO+74]$^+$ ions which would confirm acyl identities. This facilitates the identification of molecular species of TAGs which could not be separated by RP-HPLC.

Other peaks in the chromatogram were identified in a similar manner. Poorly separated peaks in the intervals of 53.0–56.2 min are shown as an example. These peaks contained a total of 11 unseparated molecular species having ECN 40 and their structures

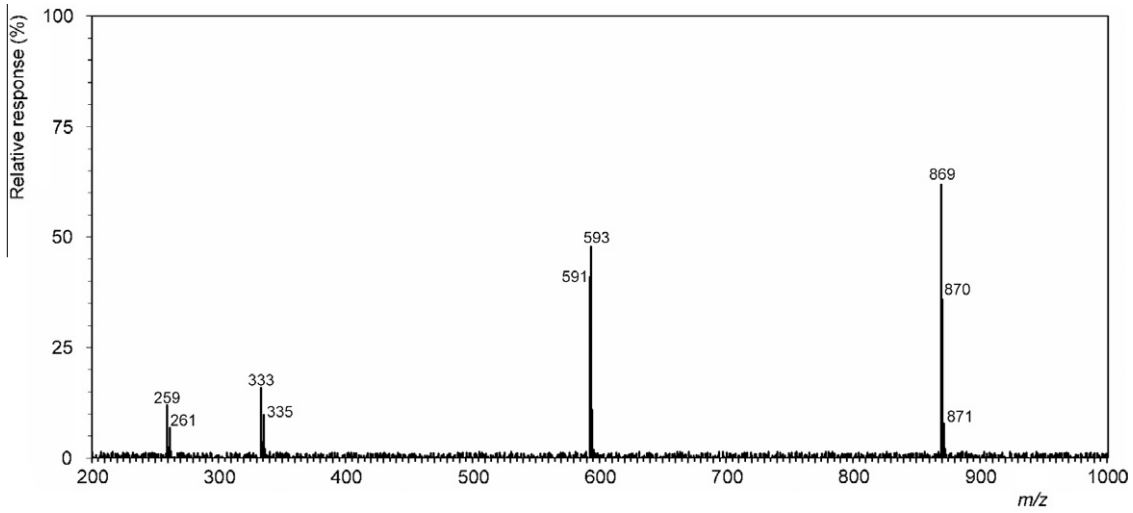

Fig. 3. Mass spectrum (APCI) of natural StαLnSt.

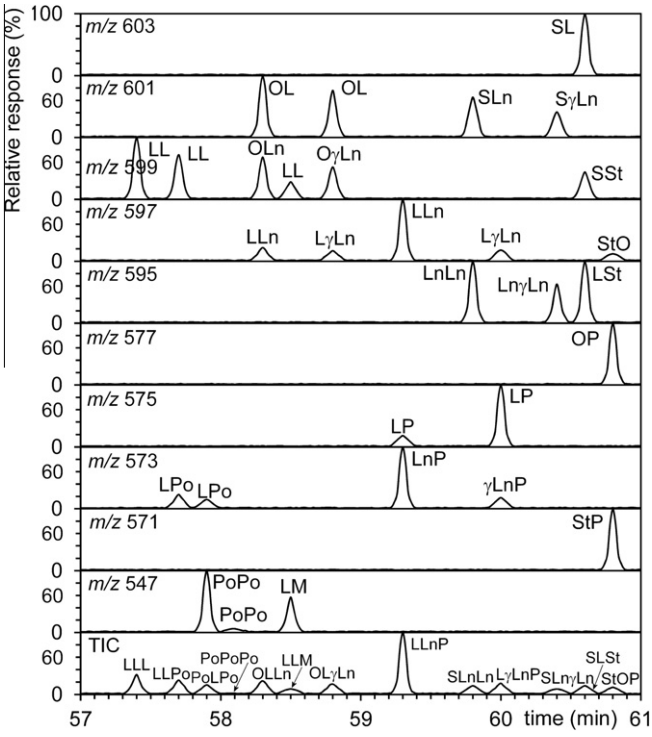

Fig. 4. Selected ion monitoring in the interval from 51.5 to 52.5 min. SIM was measured for the following values: *m/z* 547 [PoPo,LM]$^+$, 571 [StP]$^+$, 573 [LPo,LnP,γLnP]$^+$, 575 [LP]$^+$, 577 [OP]$^+$, 595 [LnLn,LnγLn,LSt]$^+$, 597 [LLn,LγLn,StO]$^+$, 599 [LL,OLn,OγLn,SSt]$^+$, 601 [OL,SLn,SγLn]$^+$, and 603 [SL]$^+$, including total ion current (TIC).

were determined based on the SIM and MS/MS as the following TAGs: LLLn, LLγLn, LnOLn, LnOγLn, OLSt, LnLnP, γLnOγLn, γLnLnP, StLP, γLnγLnP, and SLnSt.

Separation of regioisomers of TAGs containing positional isomers of FAs, such as oleic and vaccenic acids, is generally very difficult. Acheampong et al. (2011) identified oleic acid and vaccenic acid (asclepic acid) by capillary GC. Unfortunately, the MS^2 could not distinguish between TAGs having the same molecular mass, the same chain length of FA, and differing only in positions of the double bond. Based on the analysis of log capacity factor versus the total carbon atoms (log*k* vs. CN plots) it was found that OOV must coelute with OOO and thus the TAGs with Δ^9 and Δ^{11} fatty acids cannot be separated (e.g. OLL and VLL).

On the other hand, Leskinen et al. (2010b) separated PoPoV and PoPoO from each other, but they could not separate regioisomers PoVPo from PoPoV and PoOPo from PoPoO by RP-HPLC. To quantify their proportions, they used the ratio of ion intensities in DAG by RP-HPLC/ESI-MS/MS and corresponding calibration curves. However, this method requires available pure standards, whether commercial or synthesized, and therefore it cannot be used for a natural mixture of TAGs, which typically contains tens of TAGs. This high complexity of natural mixtures then may results in a mutual influencing and overlapping of peaks.

In our case, gradient elution of TAGs having two positional isomers of FAs in the molecule, i.e. two O or two V moieties (e.g. POO from PVV) was not successful. Separation of two regioisomeric pairs TAGs such as VPV and PVV or OPO and POO, respectively, could be accomplished only by isocratic elution using the mobile phase ACN-iPrOH (65:35, v/v). Unfortunately, this separation took hours, a time span unacceptable in the analysis of a natural sample (this analysis is shown in Fig. 5). Standards should be used for good measure of complete identification, see above.

In the case of TAGs having a single octadecenoic FA we did not succeed in separating these TAGs. APCI tandem mass spectra of both synthetic TAGs, i.e. VPV and OPO and POO and PVV, were completely identical; the determination of all four molecular species (VPV, PVV, OPO, and POO) would therefore always require a comparison of the retention times in a natural TAG with a synthetic (commercial) TAG standard. Leskinen et al. (2010b), who were not sure which of the two acids is present in the TAGs, used the name octadecenoic acid (Od). These authors also refrained from dealing with other TAGs in sea buckthorn oil, obviously because they did not have appropriate standards for TAGs other than those with vaccenic acid which they have identified. The above data clearly show that TAG regioisomers containing acyl chains of different double bond positional isomers cannot be resolved based on their mass spectra. A possible solution would be to use the technique described by Hsu and Turk (1999, 2008), who used tandem mass spectrometry up to MS^5 for determination of double bond(s) in FAs of TAGs and phospholipids. The above data clearly show that any combination of FAs with positional double bond(s) in chain acyls of TAG cannot be resolved based on mass spectrum (Hsu and Turk, 1999).

It was found that *M. laminosus*, which has been isolated from a hot spring near Sofia, has a very similar composition of fatty acids,

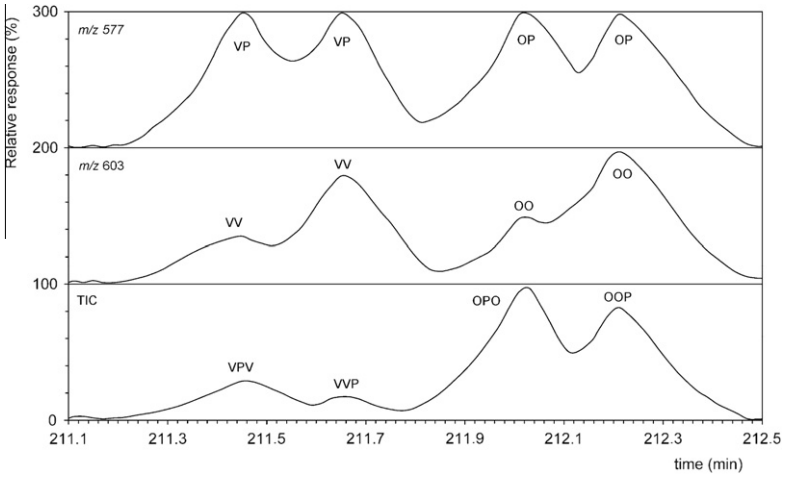

Fig. 5. Selected ion monitoring in the interval from 51.5 to 52.5 min. A natural sample of TAGs containing VPV, VVP, OPO, and OOP was measured. SIM was measured for the following values: *m*/*z* 603 $[OO;VV]^+$, and *m*/*z* 577 $[VP;OP]^+$.

but not TAGs, as *M. laminosus* isolated from Carlsbad (Table 2) (Fig. 2S, Supplementary Material). The disparity of TAGs is manifested mainly in the dissimilarity of regioisomers containing positional isomers of fatty acids, i.e. O and V (Table 3). Considering the similarity in fatty acid composition between *M. laminosus* from Carlsbad and Sofia, but much lower content of V compared to O containing TAGS from Sofia (Table 3), there must be other TAGs in the Sofia isolate in which V is greater than O, although these have not been determined.

Leskinen et al. (2010b) described changes in the proportions of TAGs in sea buckthorn oil as dependent on the origin and weather conditions. It should be noted that the sea buckthorn oil contains a much higher amount of vaccenic acid than our cyanobacteria and TAGs with vaccenic acid have to be present in a larger amount.

Cyanobacterial oils from *T. cf. tenuis* and from *T. distorta* are among of the most complex oils that are found in nature. They are characterized by a high content of both linolenic acid (α- and γ-, i.e. the double bond positional isomers) and stearidonic acid. A similarly complex composition of the TAGs occurs only in currant seed oils (blackcurrant or alpine currant) (Lísa et al., 2009; Laakso, 1997). TAGs were again identified and separated by two RP-HPLC columns, see also Section 4. Double bond positional isomers were resolved and we found again, in accord with previously published data (Lísa et al., 2009), that the difference in retention times between TAGs containing γ-Ln and α-Ln is approximately 0.6 min. It can best be seen in the four TAGs, i.e. αLnαLnαLn (retention time 44.4 min), γLnαLnαLn (44.9 min), γLnαLnγLn (45.4 min), and γLnγLnγLn (46.1 min). Also, the TAGs containing two octadecatrienoic acid moieties (e.g. αLnαLnSt, γLnαLnSt, and γLnγLnSt) were separated into three peaks. The elution order was αLnαLnSt > γLnαLnSt > γLnγLnSt, which is fully in line with already published data (Lísa et al., 2009; Laakso, 1997).

The chromatogram of oil from *T. distorta* collected in the Tatra Mountains is presented in Fig. 1, and the identification of the components is given in Table 2. TAGs contained both α- and γ-linolenic acids as well as stearidonic acid, including its positional isomer. Total TAGs were separated into more than 90 chromatographic peaks. Identified TAGs featured predominantly 52 or 54 acyl carbons and up to 11 double bonds in the acyl chains. In addition, some molecular species with 50 or less acyl carbons, mainly with palmitic and myristic acids, were identified.

The profiles of the TAGs of two cyanobacteria collected in Tatra Mountains and in Prague are presented also in Table 2. As an example, three of the most unsaturated fractions – 54:10 (αLnαLnSt, γLnαLnSt, and γLnγLnSt), 54:9 (αLnαLnαLn, γLnαLnαLn, αLnLSt, γLnαLnγLn, γLnLSt, and γLnγLnγLn) and 54:8 (StPSt, αLnLαLn, γLnLαLn, LLSt, γLnLγLn, αLnOSt, and γLnOSt) (total carbon in acyl chains:total double bonds), accounted for 18.4% of all Tatra Mountains TAGs, but only for 10.2% of Prague TAGs. The percentages indicate a higher degree of unsaturation of Tatra Mountains TAGs, which may be due, in our opinion, to a lower average temperature in the mountains, because the place where the cyanobacterium collected was at the altitude of 1750 m (Kopper Pass) while the Prague botanical garden is located at an altitude of less than 200 m. The fatty acid composition (Table 1) also indicates a higher degree of unsaturation in the Tatra isolate.

Fatty acids bound to the secondary hydroxyl of glycerol can be identified by the abundance ratios of fragment ions $[M+H-RCOOH]^+$, because the cleavage of FA in this position is more energy-consuming than from position *sn*-1 and/or *sn*-3 (Mottram, 2005; Byrdwell, 2005). This corresponds to a lower abundance of fragment ions from loss of *sn*-2 position. Relative abundances $[M+H-RCOOH]^+$ also depend on the number of double bonds and the length of chains in the TAG molecule. Accurate differentiation of regiosiomers by MS is impossible without the use of pure regioisomeric standards.

In both TAGs, the fraction species 54:8 is one of the main fractions, in which a combination of two octadecatrienoic and linoleic acids is the most abundant (Table 2). The abbreviations refer to the primary/secondary hydroxyl only and therefore we found from mass spectra that L is bound in the *sn*-2 position, while α- and/or γ-Ln in position *sn*-1 or *sn*-3. In the second possible combination, i.e. TAGs with at least one St, we found that position *sn*-2 is dominantly esterified by FAs having a maximum of two double bonds, not by St or α-Ln (γ-Ln). The same result, i.e. preferential binding of linoleic acid in *sn*-2 position of TAGs, was found also with the 54:9 fraction.

In the further fraction studied, i.e. 54:10, which contains two molecules of octadecatrienoic acid and one molecule of stearidonic acid, it was again found that St is bound to the *sn*-1 or *sn*-3 and not the *sn*-2.

Fraction 54:11 allowed us to demonstrate what effect altitude has on the unsaturation of FAs. The sum of the two TAGs, i.e. StαLnSt and StγLnSt in the cyanobacterium collected from the Tatra Mountains is 1.2%, while in the cyanobacterium collected in Prague it is only 0.3% of total TAGs.

Unfortunately, we failed to separate individual regioisomers of TAGs containing two positional isomers of octadecatetraenoic acid,

Table 2
Relative quantities (%) of molecular species of TAGs identified in *Mastigocladus* and *Tolypothrix* sp., their retention times and equivalent carbon number.

ECN	TAG	RT	*M. laminosus* Carslbad	*M. laminosus* Sofia	*Tolypothrix* sp. Prague	*Tolypothrix* sp. Tatra M.
32	StLnSt	37.1			0.2	0.8
32	StγLnSt	37.7			0.1	0.4
34	LnLnSt	40.7			1.1	1.5
34	γLnLnSt	41.2			0.3	0.8
34	γLnLnγSt	41.8			0.0	0.2
36	LnLnLn	44.4			2.2	3.5
36	γLnLnLn	44.9			0.3	0.7
36	LnLSt	45.2			1.0	1.5
36	γLnLnγLn	45.4			0.2	0.4
36	γLnLSt	45.7			0.7	1.1
36	γLnγLnγLn	46.1			0.0	0.1
36	StPSt	47.7			0.2	0.7
38	LnLLn	48.7			1.2	1.9
38	γLnLLn	49.3			1.1	1.5
38	LLSt	49.6			0.3	1.8
38	γLnLγLn	49.8			0.9	1.1
38	LnOSt[a]	50.3			0.6	1.0
38	γLnOSt	50.9			0.2	0.6
38	StPLn	51.2			0.7	1.1
38	StγLnP	51.8			0.2	0.7
40	LLLn	53.0			4.5	4.2
40	LLγLn	53.6			2.0	1.8
40	LnOLn	53.9			1.5	1.4
40	LnOγLn	54.5			1.1	1.0
40	OLSt	54.8			1.4	1.3
40	LnLnP	55.0			1.7	1.5
40	γLnOγLn	55.2			0.7	0.6
40	γLnLnP	55.5			1.2	1.1
40	StLP	55.7			1.5	1.4
40	γLnγLnP	56.0			0.8	0.7
40	LnSSt	56.2			0.8	0.7
42	LLL	57.4			2.8	2.5
42	LLPo	57.7	0.6	0.7	2.0	1.8
42	PoLPo	57.9	1.0	1.2	1.3	1.2
42	PoPoPo	58.1	0.5	0.6	0.1	0.1
42	OLLn	58.3			1.9	1.7
42	LLM	58.5			0.8	0.7
42	OLγLn	58.8			1.4	1.3
42	LnLP	59.3			8.6	7.8
42	SLnLn	59.8			1.2	1.1
42	γLnLP	60.0			1.5	1.4
42	SLnγLn	60.4			0.8	0.7
42	SLSt	60.6			1.2	1.1
42	StOP	60.8			1.0	0.9
42	SγLnγLn	61.0			0.3	0.3
44	OLL	62.3	0.9	0.8	2.3	2.1
44	OLPo	62.7	0.8	0.7	1.4	1.3
44	OOLn	62.9			1.3	1.2
44	PoOPo	63.1	12.5	13.7	0.8	0.7
44	LLP	63.3	0.3	0.2	4.5	4.1
44	OOγLn	63.5			0.9	0.8
44	OLM	63.7			0.2	0.2
44	PLPo	63.9	0.4	0.5	0.8	0.7
44	SLLn	64.1			0.9	0.8
44	LnOP	64.3			1.4	1.3
44	PPoPo	64.5	13.1	12.5	0.9	0.8
44	SLγLn	64.7			1.1	1.0
44	γLnOP	64.9			1.0	0.9
44	PLM	65.1			0.3	0.3
44	PLnP	65.3			1.5	1.4
44	SOSt	65.5			0.6	0.5
44	PγLnP	65.7			1.1	1.0
44	PMM	66.3			0.1	0.1
46	OLO	67.0	0.4	0.3	1.7	1.5
46	OOPo	67.3	16.4	14.5	0.9	0.8
46	SLL	67.9			1.9	1.7
46	OLP	68.1	0.5	0.6	1.8	1.6
46	OOM	68.3			0.1	0.1
46	POPo	68.5	15.7	16.2	1.0	0.9
46	SOLn	68.7			1.0	0.9
46	PLP	69.3	0.8	0.9	3.6	3.3
46	PPoP	69.6	11.6	12.4	1.2	1.1
46	POM	69.8			0.2	0.1
46	SLnP	70.0			1.1	1.0

Table 2 (*continued*)

ECN	TAG	RT	*M. laminosus* Carslbad	*M. laminosus* Sofia	*Tolypothrix* sp. Prague	*Tolypothrix* sp. Tatra M.
46	PPM	70.2			0.9	0.1
48	OOO	71.7	0.5	0.4	1.0	0.2
48	SLO	72.5	0.7	0.8	1.3	1.2
48	OOP	72.8	9.2	9.4	0.8	0.7
48	SOPo	73.0	0.2	0.3	0.7	0.6
48	SLP	73.7	0.1	0.2	0.3	0.3
48	SLnS	73.9			0.3	0.1
48	POP	74.1	9.6	8.6	0.2	0.2
48	PPP	75.3	0.6	0.7	0.2	0.1
48	SPM	75.5			0.1	0.1
50	SOO	76.9	1.2	1.5	1.4	1.3
50	SLS	77.7			1.1	1.0
50	SOP	78.0	0.5	0.6	0.9	0.8
50	SPP	79.6	1.0	0.9	1.0	0.9
52	SOS	82.1	0.9	0.8	0.6	0.5

[a] TAGs containing oleic acid can also contain vaccenic acid.

Table 3
Regioisomer compositions in% of TAGs containing one palmitic and two octadecenoic acids for *M. laminosus* from Carlsbad and Sofia.

Regioisomer	*M. laminosus* from Carlsbad	*M. laminosus* from Sofia
POO	1.2	4.1
OPO	0.7	3.9
PVV	3.9	0.8
VPV	3.4	0.6

probably due to the low occurrence of 18:4ω-6 and also due to the complexity of present TAGs. It should be noted that the theoretical number of combinations of molecular species of TAGs containing one octadecatrienoic and two octadecatetraenoic acids is 16 molecular species, i.e. 16 chromatographic peaks.

3. Conclusion

This is the first report on the production of TAGs by cyanobacteria, both the thermophilic species *M. laminosus* and the two species of *Tolypothrix* growing at room temperature. These TAGs were identified by RP-HPLC/MS-APCI. In keeping with previously published data (Gombos et al., 1992) on the effect of temperature on the content of FAs and thus also TAGs increasing temperature in *Tolypothrix* (i.e. habitat temperature and not the culture temperature) was found to cause a decrease in the unsaturation of FAs. The content of unsaturated FAs in *T. distorta* collected from a high altitude locality (Tatra Moutains) was higher than in the low-altitude specimen. We also succeeded in separating and identifying regioisomers of TAGs containing oleic and vaccenic acids from *M. laminosus*, which have so far been described only in sea buckthorn (*Hippophae rhamnoides*) oil (Leskinen et al., 2010b). We also identified for the first time 3,6,9,12-octadecatetraenoic acid in a natural source and determined its structure through its picolinyl ester, see Fig. 1. Another locality-related difference was found in *M. laminosus*, which displayed different ratios of regioisomers containing oleic and vaccenic acids.

4. Experimental

4.1. Standards and instrumentation

Acetonitrile, 2-propanol, THF, hexane, dichloromethane, glycerol, 4-dimethylaminopyridine (DMAP) and EDCI (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide), and *cis*-vaccenic acid (*cis*-11-octadecenoic acid) were purchased from Sigma–Aldrich (Prague, CR). 1,2-Olein-3-palmitin, 1,3-olein-2-palmitin, 1-monopalmitin, and 2-monopalmitin were purchased from Larodan Fine Chemicals AB (Sweden).

A Perkin–Elmer (Perkin–Elmer, Norwalk, CT, USA) model 1310 IR spectrophotometer was used for scanning IR spectroscopy as neat films. High resolution MS spectra were recorded using a VG 7070E-HF spectrometer (70 eV). 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. Cultivation

T. cf. tenuis Kützing, strain Lhotsky/CCALA C-130 (Culture Collection of Autotrophic Organisms, Institute of Botany, Trebon, Czech Republic) was isolated from Czech Republic, Prague, Botanical garden of Charles University, basin, and is deposited in CCALA as no. 197. *T. distorta* Kützing ex Bornet et Flahault, strain Hindak 1963/152, isolated from Kopper Pass soil, Belanske Tatry Mountains, Slovakia, is deposited in CCALA as no. 198. All the strains were cultivated as batch cultures, in nutrient solution after Zender as described by Staub (1961), irradiated from one side by florescent tubes (day) under 35 μmol photons m^{-2} s^{-1}, at 25 °C. CO_2 was supplied only by diffusion via cotton stopper and the bottles of 200 mL were shaken 3×/day. The yield of lyophilized cells was 78 mg (*T. cf. tenuis*) and 69 mg (*T. distorta*), respectively

M. laminosus, strain Lukavsky 2008/29 was isolated from Bulgaria, Pancharevo (a small town near Sofia) hot spring (see Lukavsky et al., 2011), *M. laminosus*; strain Lukavsky et Rezanka 2011/1 was isolated from Karlovy Vary from Mlynsky hot spring (receiver no. 81). The strains are deposited in the collection of the first author. All the strains were cultivated in D nutrient solution after Castenholz (1969) at about 45 °C, under irradiation by incandescent bulbs, 53 μmol photons m^{-2} s^{-1}; the 200 mL bottles were shaken 3×/day and CO_2 was supplied only by diffusion via cotton stopper. The yield of lyophilized cells was 107 mg (Bulgarian *M. laminosus*) and 96 mg (Czech *M. laminosus*), respectively.

4.3. Preparation of mixed FA composition TAGs

The esterification of glycerol derivatives with acids was described previously (Haraldsson et al., 2000; Halldorsson et al., 2003). The general procedure for the synthesis of TAGs containing one palmitic and two vaccenic acids was as follows: under an inert atmosphere (argon), a solution of 1-palmitoylglycerol (2-palmitoylglycerol) (36 mg, 0.1 mmol) and vaccenic (31 mg, 0.11 mmol) as a free acid in dichloromethane (1.0 mL) was added to DMAP (6.1 mg, 50 μmol) and EDCI (18.6 mg, 0.12 mmol) in dichlorometh-

ane (1.0 mL). The resulting solution was stirred at room temperature for 15 h. The reaction was stopped by passing the reaction mixture in ether/dichloromethane (10:90) through a short column packed with silica gel. Solvent removal in vacuo afforded the pure product as yellowish oil. The yield of the target TAGs was (46.3 mg, i.e. 52% for PVV and 49.9 mg, i.e. 56% for VPV, respectively). NMR spectrum of PVV:

^{1}H NMR (500.1 MHz, $CDCl_3$, δ, ppm) 5.31–5.39 (m, 4H, =CH), 5.25–5.28 (m, 1H, $CH_2CH(O–)CH_2$), 4.29 (dd, J = 12.0, 4.3 Hz, 2H, –$OCH_2CH(O)CH_2O$–), 4.14 (dd, J = 11.9, 5.7 Hz, 2H, –$OCH_2CH(O)$-CH_2O–), 2.34 (t, J = 7.6 Hz, 4H, CH_2COO in V), 2.30 (t, J = 7.6 Hz, 2H, CH_2COO in S), 2.01–2.14 (m, 8H, =$CHCH_2CH_2$), 1.52–1.72 (m, 6H, CH_2CH_2COO), 1.17–1.40 (m, 64H, CH_2), and 0.88 (t, J = 6.9 Hz, 9H, CH_3). ^{13}C NMR (125.7 MHz, $CDCl_3$, δ, ppm) 173.2 (2C, *C*=O in *sn*-1/3), 172.8 (1C, *C*=O in *sn*-2), 129.3–130.9 (4C, =*C*H), 68.9 (1C, *C*HO–), 62.1 (2C, CH_2–O), 34.2 (1C, CH_2COO in *sn*-2 V), 34.1 (2C, CH_2COO in *sn*-1/3), 31.9 (3C, $CH_2CH_2CH_3$), 29.1–30.1 (26C, CH_2), 27.2 (4C, CH_2CH=), 25.0 (3C, CH_2CH_2COO), 22.9 (3C, CH_2CH_3), 14.2 (3C, CH_3), IR ν_{max} 3020 (s, C–H), 2920 (vs, C–H), 2850 (vs, C–H), 1735 (vs., C=O) cm^{-1}. HREIMS (*m*/*z*): 886.7681 [M]$^+$, calc. for $[C_{55}H_{102}O_6]^+$ 858.7676.

The ^{1}H NMR spectra of all four TAGs, i.e. POO, OPO, PVV and VPV are practically identical, minor differences in chemical shifts were obtained only in ^{13}C NMR spectra in agreement with the literature (Haraldsson et al., 2000).

4.4. Isolation of TAGs

The extraction procedure was based on the method of Bligh and Dyer (1959), except that 2-propanol was substituted for methanol, since isopropanol does not serve as a substrate for phospholipases (Kates, 1986). The alcohol–water mixture of the lyophilized cells of both cyanobacterial strains was cooled and one part chloroform was added and the lipids were extracted for 30 min. Insoluble material was sedimented by centrifugation and the supernatant was separated into two phases. The aqueous phase was aspirated off and the chloroform phase was washed three times with two parts 1 M KCl each. The resulting chloroform phase was evaporated to dryness under reduced pressure.

Total lipid extracts were applied to Sep-Pak Cartridge Vac 35 cc (Waters; with 10 g of aminopropylsilica-based polar bonded phase), and from the cartridge were subsequently eluted non-polar lipids with 40 mL of chloroform–methanol (7:3). The eluate of non-polar lipids was reduced in volume, mixed with 1 mL of hexane and the mixtures were stirred for 15 min. The solution was filtered, 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 HPLC column.

4.5. FAMEs analysis

The total 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 BF_3/MeOH (Rezanka, 1993).

Gas chromatography–mass spectrometry of FAME was done on a GC–MS system consisting of Varian 450-GC, Varian 240-MS ion trap detector with electron impact ionization, and CombiPal autosampler (CTC, USA). 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. The structures of FAMEs were confirmed by comparison of retention times and fragmentation patterns with those of the standard FAMEs (Supelco, Prague).

4.6. Picolinyl esters of FAs (Rezanka, 1990)

A solution of potassium *tert*-butoxide in tetrahydrofuran (0.5 mL, 1.0 M) was added to nicotinyl alcohol (1 mL). After mixing, the appropriate fatty acid methyl esters (~0.5 mg) in dry dichloromethane (1 mL) were added, and the mixture was held at 40 °C for 30 min in a closed vial. After cooling to room temperature, water and hexane were added, and the organic phase was collected, dried over anhydrous sodium sulfate, and evaporated.

A fatty acid picolinyl ester mixture was analyzed on the instrument described above. Injection temperature (splitless injection) was 100 °C, and an Ultra-1 capillary column (Supelco, Czech Republic) 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 *m*/*z* 50–500.

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 i.d., 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 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2012.02.028.

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