Chemistry and Physics of Lipids 163 (2010) 373–380

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

RP-HPLC/MS-APCI analysis of odd-chain TAGs from *Rhodococcus erythropolis* including some regioisomers

Tomáš Řezanka[a,*], Olga Schreiberová[b], Tereza Krulikovská[b], Jan Masák[b], Karel Sigler[a]

[a] *Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague, Czech Republic*
[b] *Department of Fermentation Chemistry and Bioengineering, Institute of Chemical Technology Prague, Technická 3, 166 28 Prague, Czech Republic*

ARTICLE INFO

Article history:
Received 30 November 2009
Received in revised form 25 January 2010
Accepted 28 January 2010
Available online 4 February 2010

Keywords:
Rhodococcus erythropolis
RP-HPLC/MS-APCI
Odd-chain triacylglycerols

ABSTRACT

We used reversed phase liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (RP-HPLC/MS-APCI) for direct analysis of triacylglycerols (TAGs) from four different cultivations of *Rhodococcus erythropolis* CCM 2595. This technique enabled us to identify and quantify the specific molecular species of TAGs directly from lipid extracts of the bacterium, including the determination of their basic characteristics such as retention time and mass spectra. A total of 17 TAGs having at least one odd-numbered-chain FA (fatty acid) were found. This is the first time when odd-chain TAGs, predominantly with pentadecanoic, margaric, and margaroleic acids, were identified. Multiple linear regression analyses were used for predicting retention times of molecular species, predominantly of odd-chain TAGs. Cultivations on two different substrates at two different temperatures (20 and 30 °C) showed that only temperature had any effect on the content of TAGs. In cultivations with succinate as a carbon source the content of these TAGs increased by half (i.e., from 23.5 to 34.2%) when the cultivation temperature was lowered from 30 to 20 °C. The content of the PoPoMo, PoMoO and PPoMo (see Table 1) TAGs containing the odd-numbered-chain margaroleic acid rose from 0.0 to 7.1% while in cultivation on phenol as a carbon source the lowering of cultivation temperature caused an increase of these TAGs from 0.5 to 6.7%.

1. Introduction

Lipophilic compounds, i.e., lipids, represent the most frequent and most useful form of energy storage in an organism. This fact reflects their high caloric value and their anhydrous character. All these tenets hold mostly for eukaryotic organisms, i.e., animals, plants and also moulds, fungi and yeast. A completely different situation is found in prokaryotes, especially bacteria. Accumulation of specialized lipids such as polyhydroxyalkanoic acids or triacylglycerols (TAGs) has so far been documented only in actinomycetes, i.e., in genera *Mycobacterium* (Barksdale and Kim, 1977), *Streptomyces* (Olukoshi and Packter, 1994; Packter and Olukoshi, 1995), *Acinetobacter* (Makula et al., 1975; Scott and Finnerty, 1976), *Nocardia* and *Rhodococcus* (Alvarez et al., 1997). Individual species of the genus *Rhodococcus* are in addition capable of catalyzing transformations and degradation of various hydrophobic substances (Finnerty, 1992). In association with its fairly frequent cultivation, *Rhodococcus opacus* was found to be able to accumulate up to 76% of TAGs when grown on different carbon sources under nitrogen-limiting conditions (Alvarez et al., 1996).

A very interesting biotechnological aspect of production of bacterial oils is the formation of specialized TAGs, i.e., TAGs containing odd-numbered-chain fatty acids. It should be noted that conventional commercially available plant and animal TAGs contain only minimum amounts of odd-numbered-chain FAs, with the exception of TAGs from ruminant milk and butter (Kalo et al., 2009), which are probably of bacterial origin.

Though bacterial TAGs have received considerable attention as concerns their cultivation and the content of fatty acids, only one study (Waltermann et al., 2000) has addressed their structure. Knowledge of TAG structure is important with regard to nutritional functions, quality control, technological characteristics (in particular viscosity and melting temperature), and authenticity establishment (Castilho et al., 2004). All the other above mentioned studies analyzed merely the FAs (fatty acids) in an isolated fraction of TAGs. The study by Waltermann et al. (2000) used three methods, i.e., stereospecific analysis by lipase, RP-HPLC, and MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time-of-Flight) that provide at least a partial picture of the structure of TAGs from *R. opacus* PD630.

TAG separation by HPLC is usually performed by using RP-HPLC (Byrdwell, 2004, 2005; Rezanka and Sigler, 2007). APCI is the most frequently used ionization technique for TAG analysis because of easy coupling to non-aqueous mobile phase systems and high ionization efficiency for non-polar species (Lisa et al., 2007).

* Corresponding author. Tel.: +420 241 062 300; fax: +420 241 062 347.
E-mail addresses: rezanka@biomed.cas.cz, tomas.rnk@seznam.cz (T. Řezanka).

0009-3084/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.chemphyslip.2010.01.007

Unlike the TAGs in plant oils or animal fats, bacterial TAGs have received hardly any attention. For this reason, based on our long-term experience in the analysis of molecular species of lipids including TAGs (Rezanka and Podojil, 1985; Rezanka, 1990; Rezanka et al., 1986, 2008a,b), we employed the method used previously with success, i.e., RP-HPLC/MS-APCI, also for the analysis of TAGs from *Rhodococcus erythropolis* CCM 2595.

2. Results and discussion

Table 1 summarizes all FAs identified in individual TAGs of *R. erythropolis* together with their trivial names, abbreviations, CN, DB, and ECN.

Table 2 lists the *m*/*z* values of a range of protonated molecular ions ($[TAG]^+$) and their typical diacylglycerol ($[M+H\text{-}RCOOH]^+$, i.e., $[DAG]^+$) ions observed in the APCI mass spectra, including their chromatographic characteristics obtained by using two columns in series for identification of 48 TAGs in different cultivations of *R. erythropolis*. This separation is shown in Fig. 1.

Table 1
Names of FAs from TAGs of cultivated *R. erythropolis*, including their abbreviations, carbon numbers (CN), double bond (DB), and equivalent carbon numbers (ECN).

Systematic name	Trivial name	Abbreviation	CN:DB	ECN
Tetradecanoic	Myristic	M	14:0	14
Pentadecanoic	–	X	15:0	15
Hexadecanoic	Palmitic	P	16:0	16
cis-9-Hexadecenoic	Palmitoleic	Po	16:1	14
Heptadecanoic	Margaric	Ma	17:0	17
cis-10-Heptadecenoic	Margaroleic	Mo	17:1	15
Octadecanoic	Stearic	S	18:0	18
cis-9-Octadecenoic	Oleic	O	18:1	16

RP-HPLC without water in mobile phase has been widely used for the separation of complex natural lipid samples (Byrdwell, 2004, 2005; Mottram, 2005; Rezanka and Sigler, 2007). The retention of TAGs in RP-HPLC increases with increasing ECN. When using optimum conditions for separation, TAGs with the same ECN can be separated. They form so-called critical pairs such as MOO or PPoO or PoPoS.

Table 2
TAGs identified in different *R. erythropolis*, their retention times RT, equivalent carbon numbers (ECN), the masses of protonated molecules, and characteristic fragment ions of TAGs.[a].

TAG	RT^e	RT^c	ECN	ACN:n	$[M+H]^+$	$[DAG]^+$	*m*/*z*	$[DAG]^+$	*m*/*z*	$[DAG]^+$	*m*/*z*
PoPoO	50.7	53.8	42	50:3	829	PoO	575	PoPo	547	–	
PoPoMo	51.2	49.8	43	49:3	815	PoMo	561	PoPo	547		
XPoPo	51.6	50.0	43	47:2	789	XPo	535	PoPo	547	–	
MPoO	53.4	54.2	44	48:2	803	PoO	575	MPo	521	MO	549
PPoPo	53.8	54.2	44	48:2	803	PPo	549	PoPo	547	–	–
MPPo	54.3	54.6	44	46:1	777	PPo	549	MP	523	MPo	521
MMO	54.7	54.6	44	46:1	777	MO	549	MM	495	–	–
MMP	56.4	57.7	44	44:0	751	MP	523	MM	495	–	–
PoMoO	58.2	57.8	45	51:3	843	MoO	589	PoMo	561	PoO	575
PPoMo	58.4	58.3	45	49:2	817	MoP	521	PoMo	561	PoP	549
PoOO	60.9	61.9	46	52:3	857	OO	603	PoO	575	–	
MOO	61.3	62.4	46	50:2	831	OO	603	MO	549	–	
PPoO	61.9	62.4	46	50:2	831	PoO	575	PPo	549	PO	577
PoPoS	62.6	62.4	46	50:2	831	PoS	577	PoPo	547	–	
MPO	63.5	63.6	46	48:1	805	PO	577	MP	523	MO	549
PPPo	64.2	63.6	46	48:1	805	PPo	549	PP	551	–	
MPP	64.7	66.8	46	46:0	779	PP	551	MP	523	–	
MoMoO	65.1	61.9	46	52:3	857	MoO	589	MoMo	575	–	
MPoS	65.5	63.6	46	48:1	805	PoS	577	MPo	521	MS	551
MoOO	65.9	65.9	47	53:3	871	OO	603	MoO	589	–	
XOO	66.3	68.0	47	49:1	819	OO	603	XO	563	–	
PMoO	66.7	66.5	47	51:2	845	MoO	589	PMo	521	PO	577
XPO	67.1	66.5	47	51:2	845	PO	577	XP	537	XO	563
MMS	67.6	66.8	46	46:0	779	MS	551	MM	495		
OOO	69.0	69.9	48	54:3	885	OO	603	–	603	–	
POO	69.5	70.6	48	52:2	859	OO	603	PO	577	–	
PoSO	70.8	70.6	48	52:2	859	SO	605	PoS	577	PoO	575
PPO	72.2	72.5	48	50:1	833	PO	577	PP	551	–	
PoPS	72.6	72.5	48	50:1	833	PS	579	PoP	549	PoS	577
MOS	73.1	72.5	48	50:1	833	OS	605	MO	549	MS	551
MaOO	75.3	74.8	49	53:2	873	OO	603	MaO	591	–	
XOS	75.6	77.0	49	51:1	847	OS	605	XO	563	XS	565
PPP	76.2	75.8	48	48:0	807	PP	551	–		–	
MPS	76.7	75.8	48	48:0	807	PS	579	MP	523	MS	551
PMoS	77.1	77.0	49	51:1	805	MoS	591	PMo	521	PS	579
MaOP	77.2	77.0	49	51:1	847	OP	577	MaO	591	MaP	565
PoSS	78.2	81.4	50	52:1	861	SS	607	PoS	577	–	
OOS	79.4	78.9	50	54:2	887	OS	605	OO	603	–	
POS	81.4	81.4	50	52:1	861	OS	605	PO	577	PS	579
PPS	85.5	84.9	50	50:0	835	PS	579	PP	551	–	
MSS	86.7	84.9	50	50:0	835	SS	607	MS	551	–	
MaSO	87.8	85.9	51	53:1	875	SO	605	MaS	593	MaO	591
MoSS	88.3	85.9	51	53:1	875	SS	607	MoS	591		
PMaS	88.9	89.4	51	51:0	849	MaP	565	MaS	593	PS	579
SSO	89.6	90.4	52	54:1	889	SO	605	SS	607	–	
SSP	93.6	94.0	52	52:0	863	SP	579	SS	607	–	
MaSS	97.1	98.5	53	53:0	877	SS	607	MaS	593		

[a] ACN, acyl carbon number; ECN, equivalent carbon number; n, number of double bond(s); RT, retention time (min). RT^c, RT calculated, RT^e, RT experimental.

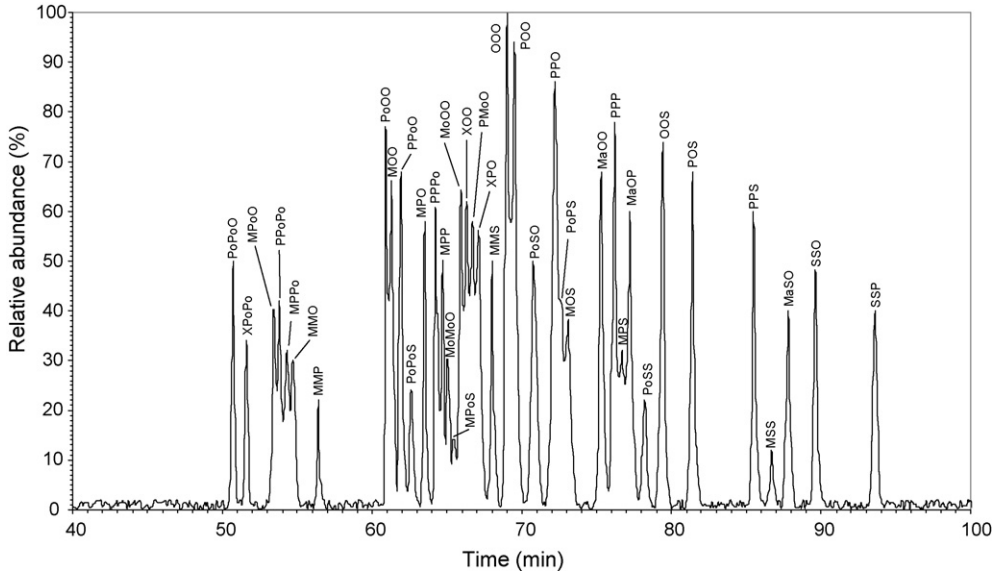

Fig. 1. RP-HPLC/APCI-MS chromatogram of the TAGs from *R. erythropolis* cultivated on succinic acid at 30 °C.

TAGs often occur as regioisomers, i.e., positional isomers on the glycerol backbone—see below. One of the first mentions of the possibility to separate regiosomers of TAGs by RP-HPLC was published by Mottram et al. (2001). The spectrum on the left-hand side of the chromatographic peak was found to correspond to SPS whilst the spectrum on the right-hand side of the same peak corresponded to SSP. Also another study (Lin et al., 1997) succeeded in separating synthetic TAGs such as POP (RT = 33.12 min) and PPO (RT = 33.16 min). Further advances were noted in other papers (Momchilova et al., 2004, 2006), which reported on the separation of some positionally isomeric disaturated and monounsaturated TAGs as intact species, e.g., as POP-PPO, by RP-HPLC. The separation on a column with acetonitrile-2-propanol (70:30, v/v) mobile phase at 0.8 ml/min took more than 5 h but the separation of PPO from POP was nearly complete (resolution = 0.9). In all cases the isomer with the unsaturated acyl residue in either 1- or 3-position was retained more strongly than the respective 2-isomer.

As demonstrated above, the separation of positionally isomeric TAGs as intact species by RP-HPLC is possible. Because our separation on a single column, in agreement with the literature, was not too successful (resolution = 0.45 and less, data not shown), we used two columns in series that were previously found useful (Rezanka et al., 2008a,b). This separation was successful, as documented in Figs. 2 and 3. The PoXPo to PoPoX ratio was found to be 100:61 while that of MaOO to OMaO was 100:25. Mixed unsaturated fatty acid regioisomers have not been systematically examined, especially by using identification by APCI. All studies have so far focused on their separation by normal phase HPLC or Ag^+-HPLC (Kuksis and Itabashi, 2005), not by RP-HPLC. Both literature data and our experience indicate that, by using sufficiently efficient columns, these regioisomers can be separated and identified. The findings offer a method for cross-checking the estimates of regioisomeric TAGs obtained by MS.

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, $[RCOO+58]^+$ also called ($[MAG]^+$, 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 OMaO (Fig. 4), which contains two FA, exhibits, in addition to $[M+H]^+$ at *m/z* 873, also two acylium ions, with *m/z* at 253 (Ma^+) and *m/z* 265 (O^+), respectively.

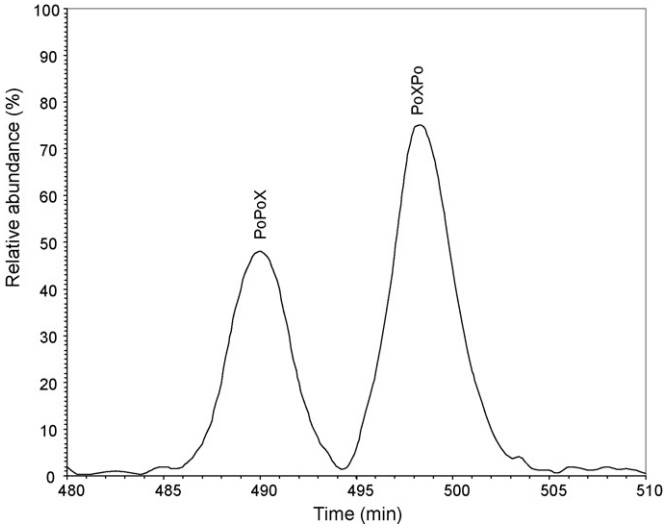

Fig. 2. Separation of two regioisomers, i.e., PoXPo and PoPoX by isocratic RP-HPLC/APCI-MS.

The spectrum naturally features also ions of the type $[MAG]^+$ at *m/z* 327 ($[Ma]^+$) and *m/z* 339 ($[O]^+$), respectively. Like with OOO, also $[DAG]^+$ ions caused by the loss of margarate at *m/z* 603 and oleate at *m/z* 591 are present, resulting in $[OO]^+$ (type $[AA]^+$) and $[MaO]^+$ (type $[AB]^+$) ions, respectively.

Similarly, the mass spectrum of an ABC type TAG, tj. TAG containing three different FA, such as MaSO, see Fig. 5, shows three

Table 3
The identities and masses of common fragments from lower part of mass spectra.

RCO^+	*m/z*	MAG $[RCO+74]^+$	*m/z*
M	211	M	285
X	225	X	299
Po	237	Po	311
P	239	P	313
Mo	251	Mo	325
Ma	253	Ma	327
O	265	O	339
S	267	S	341

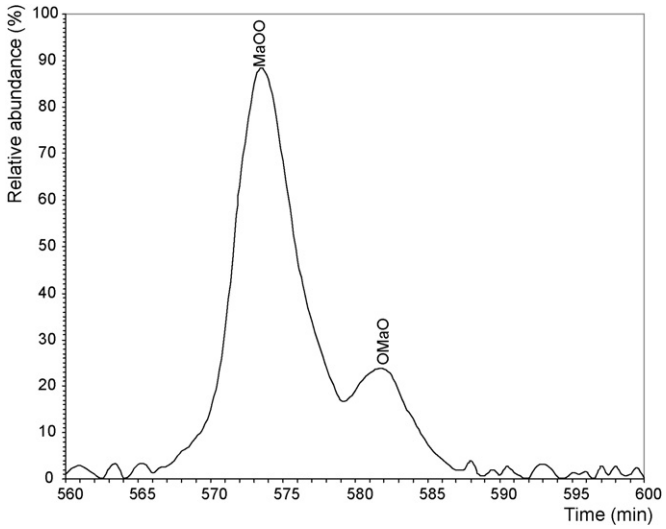

Fig. 3. Separation of two regioisomers, i.e., OMaO and MaOO by isocratic RP-HPLC/APCI-MS.

triplets of ions. Three $[M-RCOO]^+$ ions at m/z 591, 593, and 605, corresponding to loss of stearate to give $[MaO]^+$, loss of oleate to give $[MaS]^+$, and loss of margarate to give $[SO]^+$, respectively. Another triplet, $[MAG]^+$ at m/z 341, 329, and 327 is formed by $[S]^+$, $[O]^+$, $[Ma]^+$ ions. The last triplet belongs to ions S^+ (m/z 267), O^+ (m/z 265), and Ma^+ (m/z 253).

Some authors observed (Mottram, 2005; Byrdwell, 2005; Fauconnot et al., 2004; Leskinen et al., 2007) that the relative intensities of the $[M-RCOO]^+$ ions, i.e., $[DAG]^+$ could be used to obtain information on the positions of FA within the TAG. These authors noted that in the APCI-MS mass spectrum of an ABC type TAG, the least abundant $[M-RCOO]^+$ ion corresponds to loss of the FA from the *sn*-2 position. 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 demonstrated this effect in the spectrum of MaSO (Fig. 5), in which the least abundant $[M-RCOO]^+$ ion ($[DAG]^+$ ion, i.e., $[MaO]^+$ at m/z 591) is formed by the loss of stearate from the *sn*-2 position. The two more abundant $[DAG]^+$ ions are due to loss of margarate (*sn*-1(3)) and oleate (*sn*-3(1)) giving $[SO]^+$ and $[MaS]^+$ ions, respectively.

The rule about the energetically less favorable loss of FA from position *sn*-2 and formation of $[M-RCOO]^+$ i.e., 1,3-$[DAG]^+$ ions was applied also to bifunctional TAGs such as AAB or ABA. 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 generating the analogous 1,3-$[AB]^+$ ion from the AAB isomer (Figs. 4 and 6).

If these two fragments were energetically equivalent, then 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 (see, e.g., Figs. 4 and 6).

Ratio $[XPo]^+$:$[PoPo]^+$ observed for PoXPo is only 100:35, indicating that the formation of the 1,3-$[PoPo]^+$ ion is energetically unfavorable. This is probably due to the 1,3-ion forming a resonance structure containing a five-membered ring that is less stable than the six-membered rings forming the resonance of the 1,2- and 2,3-ions (Holcapek et al., 2003). Consequently, the two regioisomers show two different spectra (Fig. 6).

A similar situation was found in the other pair of regioisomers, see Fig. 3. The two compounds, i.e., OMaO and MaOO, were separated but the elution time was almost 6 h. The mass spectra also unambiguously demonstrate the structure of separated TAGs. Dif-

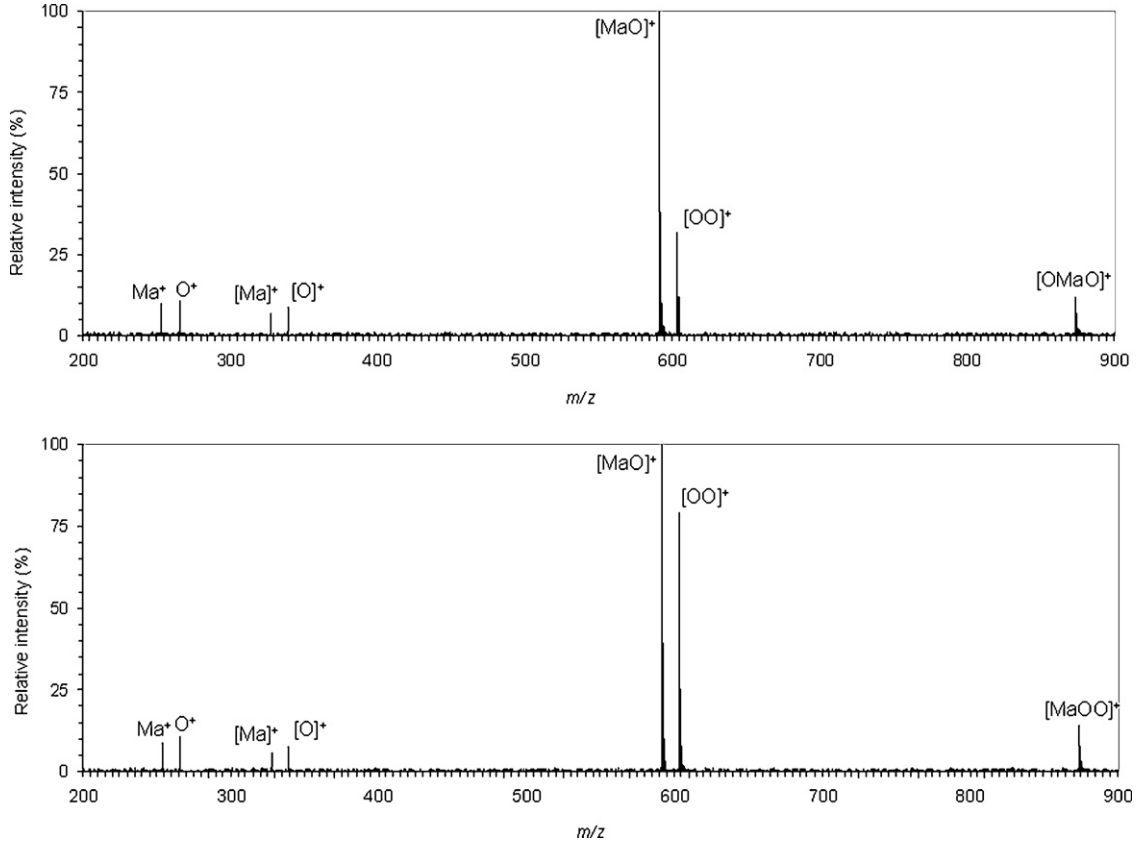

Fig. 4. Mass spectra (APCI) of two molecular species of TAGs, i.e., OMaO and MaOO.

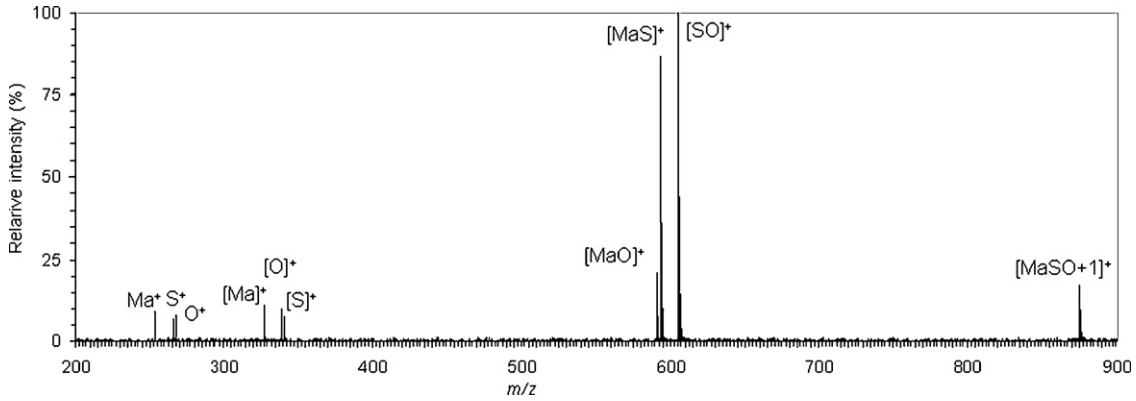

Fig. 5. Mass spectrum (APCI) of a molecular species of TAG, i.e., MaSO.

ferent abundances of ions $[MaO]^+$ and $[OO]^+$ were again found, their ratio for MaOO being 100:79 and for OMaO 100:32 (Fig. 4). This demonstrated that RP-HPLC, though with relatively long elution times, can be used for separating and then identifying by MS-APCI even regioisomers of odd-numbered-chain TAGs.

As seen in Table 2, TAGs from *Rhodococcus* contain large amounts of odd-numbered-chain FAs. Since these unusual TAGs have been only exceptionally identified in the available literature, we attempted to predict the RT of odd-numbered-chain TAGs by several methods. The first method was that described by Lin et al. (1998) that involves the use of the previously calculated contributions of functional groups or chain shortenings of FAs to the RT of TAGs. However, the increments taken from this paper did not lead to any satisfactory RT data for our TAGs.

Another possibility was to use multiple linear regression analyses published by several authors (Perona and Ruiz-Gutierrez, 1999; Lopez-Hernandez et al., 2004; Najera et al., 1998). The agreement between experimental and calculated values always depends on the mathematical apparatus (equation) used. The experimental and calculated data are compared in Table 2. When using the following equation and standards of TAGs we obtained highly accurate values.

$$RT = b_0 + b_1[CN] + b_2[DB] + b_3[MUFA],$$

where [CN] is the sum of the carbon numbers of the FA residues attached to the glycerol backbone, [DB] is the number of double bonds contained in these residues, and [MUFA] is the number of monounsaturated FA present in the TAG molecule of interest.

The corresponding values of the constants in equation for RT in minutes are $b_0 = -134.17$, $b_1 = 4.36$, $b_2 = -5.48$, and $b_3 = -5.04$. Statistical significance at the 95% confidence level was obtained for all parameters in the regression model and very acceptable agreement of predicted and experimental values was obtained. The determination coefficient (R^2) is 0.98, which indicates the high quality of

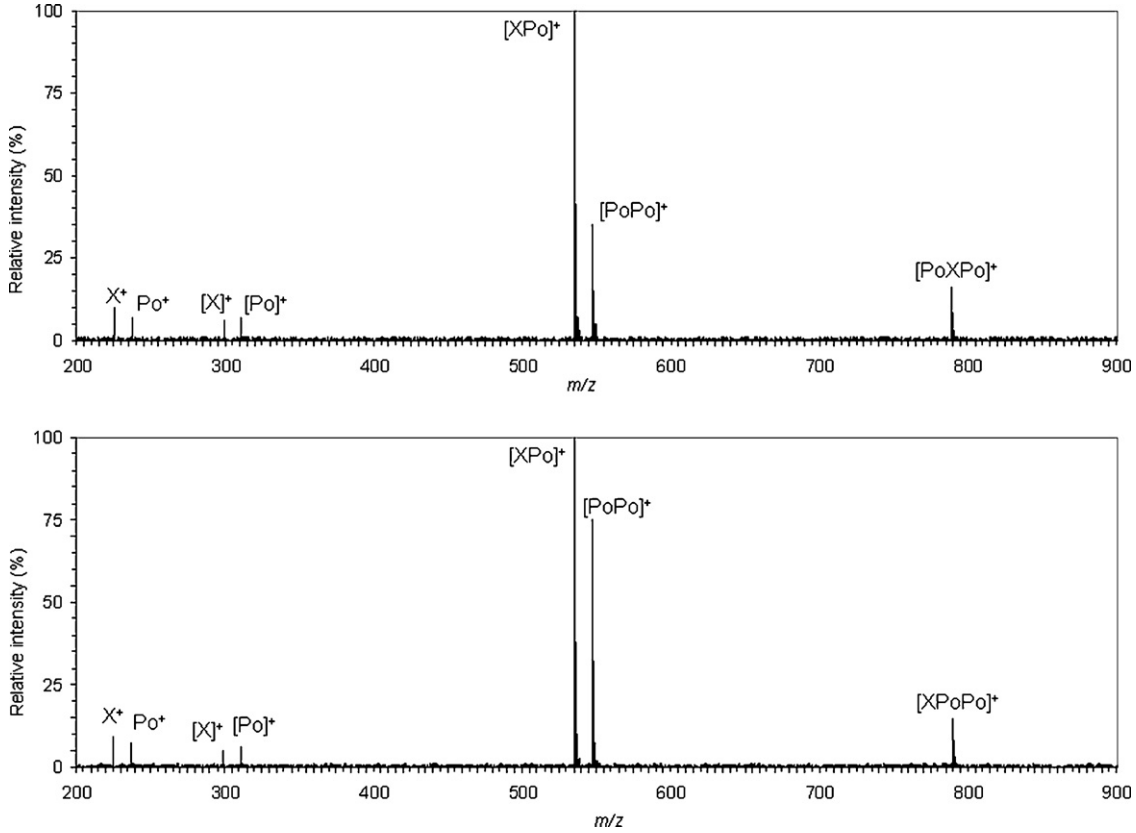

Fig. 6. Mass spectra (APCI) of two molecular species of TAGs, i.e., PoXPo and PoPoX.

Table 4
Fatty acid compositions (%) of four cultivations of *Rhodococcus* at different cultivation temperatures (at 20 and 30 °C) and on different sources of carbon (phenol and succinic acid) determined by GC–MS.

FA	Phenol at 20 °C	Phenol at 30 °C	Succinic acid at 20 °C	Succinic acid at 30 °C
M	4.5	9.8	5.1	10.4
X	2.6	8.2	3.2	9.1
P	26.3	19.3	25.7	16.2
Po	12.5	18.5	11.4	19.8
Ma	6.1	4.9	5.8	4.8
Mo	4.3	9.2	5.2	10.3
S	11.7	3.1	12.3	2.7
O	32.0	27.0	31.3	26.7

the model. Inspection of Table 2 shows that, to achieve a still better agreement, one would have to perform analysis of additional standard samples of TAG mixtures containing odd-chain TAGs. Still, our predicted data, though not entirely accurate, have proved very useful in MS-APCI identification of TAG containing odd-chain FAs.

As stated in the introduction, many studies have focused on the biosynthesis and cultivation of TAGs in various species of the genus *Rhodococcus* (Alvarez et al., 1996, 1997; Waltermann et al., 2005). Although FAs from the TAG fraction were analyzed, only a single paper (Waltermann et al., 2000) aimed at providing an at least partial reply to the question which molecular species of TAGs are present. All other authors were satisfied with merely analyzing the fatty acids since, as indicated by some studies (Gouda et al., 2008; Waltermann et al., 2000), the FA composition, appears to be the key problem in the further use of TAGs as, e.g., single cell oil. Experiments on mammals have namely shown that, e.g., odd-numbered-chain TAGs administered to mice accumulated mainly in the epididymal fat. This result suggests that odd-chain TAGs (i.e., labeled by pentadecanoic and heptadecanoic acids) might not be favorable substrates for β-oxidation-related enzymes (Gotoh et al., 2008).

Table 4 shows the content of FAs in bacterial cells cultivated at 20 and 30 °C on phenol and succinate; the effect of the carbon source on the content of FAs is seen to be all but negligible. This can be fully explained by looking at the biodegradation pathway of phenol (Martinkova et al., 2009), which includes the transformation of phenol to succinate, which is a component of the citric acid cycle. The subsequent biosynthesis of FAs proceeds without any preference for odd-chains. The effect of cultivation temperature on the content of FAs was far greater. Our data on the content of FAs are fully in keeping with the previous often described finding (Russell, 1984) that increasing temperature brings about increasing unsaturation of FAs and shortening of their acyl chains. Though this finding was originally made in phospholipids that are constituents of cell membranes, the effect of temperature on the structure of FAs in TAGs is not surprising because it is hardly possible (see the microscopic photos in the papers of Alvarez et al., 1996; Waltermann and Steinbuchel, 2005) that TAG inclusions (lipid bodies) would be in a semiliquid and not in a crystalline state.

As seen in Table 4, our strain also evinces the two features mentioned above. With decreasing temperature the content of stearic acid dropped by some 75% and the content of palmitic acid dropped by tens of relative per cent. The content of oleic acid, which remains the major FA at both temperatures and in cultivations with both carbon sources, has not changed, and neither did the content of margaric acid. On the other hand, higher temperature brings about an increase in the proportion of myristic and margaroleic acids (twice), pentadecanoic (up to four times) and also palmitoleic acid (tens of relative per cent).

Similar findings were published in two papers on *Rhodococcus* (Takaichi and Ishidsu, 1993; Whyte et al., 1999). The former paper

Table 5
Relative quantities (%) of TAGs identified in *Rhodococcus erythropolis* at different cultivation temperatures (20 and 30 °C) and at different sources of carbon (phenol and succinic acid) determined by RP-HPLC/MS-APCI.

TAG	Phenol at 30 °C	Phenol at 20 °C	Succinic acid at 30 °C	Succinic acid at 20 °C
PoPoO	3.2	3.1	2.5	3.3
PoPoMo	0.4	2.1	0.0	2.3
XPoPo	2.1	2.0	1.7	2.5
MPoO	1.7	2.6	2.0	2.8
PPoPo	1.8	2.6	2.1	2.7
MPPo	1.4	2.1	1.6	2.1
MMO	1.7	3.0	1.5	3.2
MMP	1.2	2.6	1.1	2.7
PoMoO	0.0	2.5	0.0	2.7
PPoMo	0.1	2.1	0.0	2.1
PoOO	3.5	3.6	3.8	3.7
MOO	3.4	3.1	3.3	3.2
PPoO	3.2	3.1	3.4	3.1
PoPoS	0.9	1.7	1.2	1.9
MPO	3.0	2.6	2.9	2.5
PPPo	3.1	2.7	3.0	2.5
MPP	2.6	2.2	2.5	1.9
MoMoO	1.6	2.8	1.4	2.7
MPoS	0.8	1.2	0.7	1.3
MoOO	3.1	3.2	3.2	3.2
XOO	3.0	3.0	3.1	3.4
PMoO	2.7	2.6	2.9	2.5
XPO	3.0	2.5	2.8	2.5
MMS	0.3	0.6	0.1	0.7
MPP	2.3	2.2	2.5	1.9
OOO	5.3	4.1	5.1	4.1
POO	4.5	3.6	4.7	3.5
PoSO	2.3	2.2	2.5	2.3
PPO	4.4	3.2	4.3	2.9
PoPS	2.0	1.7	2.1	1.7
MOS	1.8	1.7	1.9	1.7
MaOO	3.1	2.8	3.4	2.8
XOS	0.0	1.6	0.0	1.7
PPP	3.7	2.7	3.9	2.3
MPS	1.4	1.2	1.6	1.1
PMoS	0.0	1.2	0.0	1.1
MaOP	3.2	2.3	3.0	2.2
PoSS	0.9	0.3	1.1	0.2
OOS	3.6	2.7	3.7	2.7
POS	3.2	2.2	3.4	2.1
PPS	3.1	1.8	3.0	1.5
MSS	0.5	0.0	0.6	0.0
MaSO	2.3	1.4	2.0	1.4
MoSS	0.0	0.3	0.0	0.3
PMaS	0.1	0.9	0.0	0.8
SSO	2.3	0.3	2.4	0.2
SSP	1.9	0.0	2.0	0.0
MaSS	0.3	0.0	0.0	0.0

states that the content of odd-chain FA changes with changes in temperature and increasing temperature is seen to cause a drop in the content of these FA but the fact is not further commented. A similar phenomenon is illustrated in Table 4, which clearly shows a significant (nearly two-fold) increase in the content of odd-chain FAs in cultivations at lower temperatures, and it was also observed in *Sphingomonas* sp. by Mannisto and Puhakka (2002), where the fatty acid composition shifted from predominantly even-carbon fatty acids to odd-chain FAs as temperature decreased from 25 to 8 °C. By contrast, in an Antarctic bacterium (Nichols and Russell, 1996) the content of odd-chain FAs increased at elevated temperatures. In *Schizochytrium limacinum* (of genus marine fungi) a 4.37-fold increase was observed for the odd-chain FAs caused by the significant increases of 15:0 and 17:0 when temperature changed from 30 to 37 °C (Zhu et al., 2007).

Table 5 implies that a change in the content of FAs was found also in TAGs, although it was less perceptible owing to the 10-fold higher number of molecular species. For instance, in the cultivation on succinate the content of all distearoyl-acyl TAGs dropped from 6.1 (at

30 °C) to 0.7% (at 20 °C) of total TAGs. On the other hand, the per cent proportion of MoMoO rose from 1.4 to 2.7%, i.e., about twice, whereas the content of oleic acid remained nearly unchanged (see Table 4). When the cultivation temperature was lowered from 30 to 20 °C, the concentration of TAGs featuring an increased content of two out of three FAs, such as, e.g., PoPoMo, PoMoO and PPoMo (i.e., Po and Mo acids) in cultivation on succinate increased from 0.0 to 7.1% and in cultivation on phenol from 0.5 to 6.7% total TAGs.

We found a total of 17 TAGs having at least one odd-chain FA, the content of these TAGs increasing approximately by half (from 23.5 to 34.2%) in the cultivation on succinate when the temperature was changed from 30 to 20 °C.

3. Conclusion

To our knowledge, this is the first time when odd-chain TAGs were identified by RP-HLPC/MS-APCI on such a large scale since so far their identification has been more or less marginal. By using multiple linear regression analyses we predicted the RT and succeeded in identifying by APCI altogether 17 molecular species of odd-chain TAGs (i.e., TAGs having at least one odd-chain FA) out of the total number of 48 TAGs. Using RP-HPLC we separated regioisomers PoXPo–XPoPo and also OMaO–MaOO and determined thus directly their proportions without having to perform complicated calibration by standards, which are in this case not commercially available.

By using different cultivation conditions we found that the carbon source (phenol and succinate) has an insignificant effect on the content of FAs and thereby also TAGs in *R. erythropolis* CCM 2595. On the other hand, a change in cultivation temperature from 30 to 20 °C) altered the content of FAs, causing an increase in the content of odd-chain FAs and also shorter-chain FAs. The change in the content of TAGs more or less copied that of FAs. In the cultivation on succinate, a change in cultivation temperature from 30 to 20 °C brought about a drop in TAGs having two S acids from 6.1 to 0.7% of total TAGs and a rise in the content of TAGs with at least one odd-chain FA from 23.5 to 34.2%. A detailed RP-HLPC/MS-APCI analysis thus enabled us to identify TAGs from *Rhodococcus* as a suitable source for biotechnological production of bacterial single cell oil.

4. Experimental

4.1. Microbial material

R. erythropolis CCM 2595 was obtained from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic). The strain was subjected to a 6-month physiological adaptation to phenol.

Cells were cultivated in 500 ml Erlenmeyer flasks containing 200 ml of basic salt medium. Cultivation was performed on a rotary shaker (100 rpm, 20 or 30 °C). Medium composition in 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. Carbon source (initial concentration: phenol 0.3 g/l or succinate 10 g/l) was added after sterilisation (120 °C, 20 min).

The cells were collected at the end of exponential phase (succinate 20 °C–70 h; succinate 30 °C–24 h; phenol 20 °C–80 h; phenol 30 °C–24 h) by centrifugation (10 min, 9050 × *g*), washed twice with physiological solution and lyophilized (biomass yield: succinate 20 °C–560 mg/l, succinate 30 °C–890 mg/l, phenol 20 °C–240 mg/l, phenol 30 °C–450 mg/l).

4.2. Standards and isolation

Acetonitrile, 2-propanol, hexane and standards of glyceryl tripentadecanoate, glyceryl tripalmitate, glyceryl triheptadecanoate and glyceryl tristearate were purchased from Sigma–Aldrich (Prague, CR).

The lyophilized cells were mixed with 10 ml of hexane and the mixture was stirred for 15 min. The cells were filtered out, 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 to the column.

4.3. FAMEs analysis

The TAGs (10 mg) were saponified in 10% KOH-MeOH at room temperature overnight. 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. GC–MS of a fatty acid methyl esters (FAMEs) 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 *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).

4.4. 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 (ACN) and 2-propanol (iPrOH) as follows: initial ACN/iPrOH (99:1, vol/vol); linear from 5 to 120 min ACN/iPrOH 30:70, vol/vol); held until 30 min; the composition was returned to the initial conditions over 10 min. Isocratic separation by the mixture ACN/iPrOH 65:35, vol/vol) was used for resolution of the regioisomers. 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.

4.5. Multiple linear regression analyses

To establish a predictive correlation for the RT of the TAGs in our samples, we used the RT observed for the pure TAG standards. Multiple linear regression analysis of the data leads to the coefficients of the following expression for the RT:

$$RT = b_0 + b_1[CN] + b_2[DB] + b_3[MUFA][1],$$

where [CN] is the sum of the carbon numbers of the FA residues attached to the glycerol backbone, [DB] is the number of double bonds contained in these residues, and [MUFA] is the number of monounsaturated FA present in the TAG molecules. All regression analyses were carried out using the commercial software Excel 2007.

Acknowledgement

This work was supported by the Institutional Research Concepts AV0Z50200510, MSMT projects 1M0571, MSM6046137305 and AROMAGEN 2B08062.

References

Alvarez, H.M., Kalscheuer, R., Steinbuchel, A., 1997. Accumulation of storage lipids in species of *Rhodococcus* and *Nocardia* and effect of inhibitors and polyethylene glycol. Fett/Lipid 99, 239–246.

Alvarez, H.M., Mayer, F., Fabritius, D., Steinbuchel, A., 1996. Formation of intracytoplasmatic lipid inclusions by *Rhodococcus opacus* strain PD630. Arch. Microbiol. 165, 377–386.

Barksdale, H., Kim, K.S., 1977. Mycobacterium. Bacteriol. Rev. 41, 217–372.

Byrdwell, W.C., 2004. APCI-MS in lipid analysis. In: Adlof, R.O. (Ed.), Advances in Lipid Methodology-Five. The Oily Press, Bridgwater, England, pp. 171–253.

Byrdwell, W.C., 2005. Qualitative and quantitative analysis of triacylglycerols by atmospheric pressure ionization (APCI & ESI) techniques. In: Byrdwell, W.C. (Ed.), Modern Methods for Lipid Analysis by Liquid Chromatography/Mass Spectrometry and Related Techniques. AOCS Press, Champaign, IL, pp. 298–412.

Castilho, P.C., Costa, M.D.C., Rodrigues, A., Branco, P.C., Costa, M., 2004. Characterization of triacylglycerols in madeira laurel oil by HPLC-atmospheric pressure chemical ionization-MS. J. Am. Oil Chem. Soc. 81, 913–919.

Fauconnot, L., Hau, J., Aeschlimann, J.M., Fay, L.B., Dionisi, F., 2004. Quantitative analysis of triacylglycerol regioisomers in fats and oils using reversed-phase high-performance liquid chromatography and atmospheric pressure chemical ionization mass spectrometry. Rapid Commun. Mass Spectrom. 18, 218–224.

Finnerty, W.R., 1992. The biology and genetics of the genus *Rhodococcus*. Annu. Rev. Microbiol. 46, 193–218.

Gotoh, N., Moroda, K., Watanabe, H., Yoshinaga, K., Tanaka, M., Mizobe, H., Ichioka, K., Tokairin, S., Wada, S., 2008. Metabolism of odd-numbered fatty acids and even-numbered fatty acids in mouse. J. Oleo Sci. 57, 293–299.

Gouda, M.K., Omar, S.H., Aouad, L.M., 2008. Single cell oil production by *Gordonia* sp. DG using agro-industrial wastes. World J. Microbiol. Biotechnol. 24, 1703–1711.

Holcapek, M., Jandera, P., Zderadicka, P., Hruba, L., 2003. Characterization of triacylglycerol and diacylglycerol composition of plant oils using high-performance liquid chromatography atmospheric pressure chemical ionization mass spectrometry. J. Chromatogr. A 1010, 195–215.

Kalo, P., Kemppinen, A., Ollilainen, A., 2009. Determination of triacylglycerols in butterfat by normal-phase HPLC and electrospray-tandem mass spectrometry. Lipids 44, 169–195.

Kuksis, A., Itabashi, Y., 2005. Regio- and stereospecific analysis of glycerolipids. Methods 36, 172–185.

Leskinen, H., Suomela, J.-P., Kallio, H., 2007. Quantification of triacylglycerol regioisomers in oils and fat using different mass spectrometric and liquid chromatographic methods. Rapid Commun. Mass Spectrom. 21, 2361–2373.

Lin, J.-T., Snyder, L.R., McKeon, T.A., 1998. Prediction of relative retention times of triacylglycerols in nonaqueous reversed-phase high-performance liquid chromatography. J. Chromatogr. A 808, 43–49.

Lin, J.-T., Woodruff, C.L., McKeon, T.A., 1997. Non-aqueous reversed-phase high-performance liquid chromatography of synthetic triacylglycerols and diacylglycerols. J. Chromatogr. A 782, 41–48.

Lisa, M., Holcapek, M., Rezanka, T., Kabatova, N., 2007. HPLC/APCI-MS and GC/FID characterization of Δ5-olefinic fatty acids in triacylglycerols from conifer seed oils. J. Chromatogr. A 1146, 67–77.

Lopez-Hernandez, A., Torres, C.F., Garcia, H.S., Hill Jr., C.G., 2004. Method for analysis of TAG formed by reaction of fish oil with hydrogenated soybean oil. J. Am. Oil Chem. Soc. 81, 743–747.

Makula, R.A., Lockwood, P.J., Finnerty, W.R., 1975. Comparative analysis of lipids of *Acinetobacter* species grown on hexadecane. J. Bacteriol. 121, 250–258.

Mannisto, M.K., Puhakka, J.A., 2002. Temperature- and growth-phase-regulated changes in lipid fatty acid structures of psychrotolerant groundwater Proteobacteria. Arch. Microbiol. 177, 41–46.

Martinkova, L., Uhnakova, B., Patek, M., Nesvera, J., Kren, V., 2009. Biodegradation potential of the genus *Rhodococcus*. Environ. Int. 35, 162–177.

Momchilova, S., Itabashi, Y., Nikolova-Damyanova, B., Kuksis, A., 2006. Regioselective separation of isomeric triacylglycerols by reversed-phase high-performance liquid chromatography: stationary phase and mobile phase effects. J. Sep. Sci. 29, 2578–2583.

Momchilova, S., Tsuji, K., Itabashi, Y., Nikolova-Damyanova, B., Kuskis, A., 2004. Resolution of triacylglycerol positional isomers by reversed-phase high-performance liquid chromatography. J. Sep. Sci. 27, 1033–1036.

Mottram, H.R., Crossman, Z.M., Evershed, R.P., 2001. Regiospecific characterisation of the triacylglycerols in animal fats using high performance liquid chromatography–atmospheric pressure chemical ionisation mass spectrometry. Analyst 126, 1018–1024.

Mottram, H.R., 2005. Regiospecific analysis of triacylglycerols using high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. In: Byrdwell, W.C. (Ed.), Modern Methods for Lipid Analysis by Liquid Chromatography/Mass Spectrometry and Related Techniques. AOCS Press, Champaign, IL, pp. 276–297.

Najera, A.I., Barcina, Y., De Renobales, M., Barron, L.J.R., 1998. Determination of triacylglycerol composition of Idiazabal cheese. Chromatographia 47, 579–586.

Nichols, D.S., Russell, N.J., 1996. Fatty acid adaptation in an Antarctic bacterium—changes in primer utilization. Microbiology 142, 747–754.

Olukoshi, E.R., Packter, N.M., 1994. Importance of stored triacylglycerols in *Streptomyces*: possible carbon source for antibiotics. Microbiology 140, 931–943.

Packter, N.M., Olukoshi, E.R., 1995. Ultrastructural studies of neutral lipid localization in *Streptomyces*. Arch. Microbiol. 164, 420–427.

Perona, J.S., Ruiz-Gutierrez, V., 1999. Characterization of the triacylglycerol molecular species of fish oil by reversed-phase high performance liquid chromatography. J. Liq. Chromatogr. Related. Technol. 22, 1699–1714.

Rezanka, T., 1990. Identification of very long polyenoic acids as picolinyl esters by Ag ion-exchange high-performance liquid chromatography, reversed-phase high-performance liquid chromatography. J. Chromatogr. 513, 344–348.

Rezanka, T., Mares, P., Husek, P., Podojil, M., 1986. Gas-chromatography mass-spectrometry and desorption chemical ionization mass-spectrometry of triacylglycerols from the green-alga *Chlorella kessleri*. J. Chromatogr. 355, 265–271.

Rezanka, T., Nedbalova, L., Sigler, K., 2008a. Identification of very-long-chain polyunsaturated fatty acids from *Amphidinium carterae* by LC–MS/APCI. Phytochemistry 69, 2391–2399.

Rezanka, T., Nedbalova, L., Sigler, K., 2008b. Odd-numbered very-long-chain polyunsaturated fatty acids from the dinoflagellate *Amphidinium carterae* identified by APCI-LC–MS. Phytochemistry 69, 2849–2855.

Rezanka, T., Podojil, M., 1985. Gradient separation of fatty acids (C14–C30) by reversed-phase high-performance liquid chromatography. J. Chromatogr. 346, 453–455.

Rezanka, T., Sigler, K., 2007. The use of atmospheric pressure chemical ionization mass spectrometry with high performance liquid chromatography and other separation techniques for identification of triacylglycerols. Curr. Anal. Chem. 3, 252–271.

Russell, N.L., 1984. The regulation of membrane fluidity in bacteria by acyl chain length changes. In: Kates, M., Manson, L.A. (Eds.), Membrane Fluidity. Plenum, New York, pp. 329–347.

Scott, C.C.L., Finnerty, W.R., 1976. Characterization of intracytoplasmic hydrocarbon inclusions from the hydrocarbon-oxidizing *Acinetobacter* species HO1-N. J. Bacteriol. 127, 481–489.

Takaichi, S., Ishidsu, J., 1993. Influence of growth temperature on compositions of carotenoids and fatty acids from carotenoid glucoside ester and from cellular lipids in *Rhodococcus rhodochrous* RNMS1. Biosci. Biotechnol. Biochem. 57, 1886–1889.

Waltermann, M., Hinz, A., Robenek, H., Troyer, D., Reichelt, R., Malkus, U., Galla, H.-J., Kalscheuer, R., Stoveken, T., Von Landenberg, P., Steinbuchel, A., 2005. Mechanism of lipid-body formation in prokaryotes: how bacteria fatten up. Mol. Microbiol. 55, 750–763.

Waltermann, M., Luftmann, H., Baumeister, D., Kalscheuer, R., Steinbuchel, A., 2000. *Rhodococcus opacus* strain PD630 as a new source of high-value single-cell oil? Isolation and characterization of triacylglycerols and other storage lipids. Microbiology 146, 1143–1149.

Waltermann, M., Steinbuchel, A., 2005. Neutral lipid bodies in prokaryotes: recent insights into structure, formation, and relationship to eukaryotic lipid depots. J. Bacteriol. 187, 3607–3619.

Whyte, L.G., Slagman, S.J., Pietrantonio, F., Bourbonniere, L., Koval, S.F., Lawrence, J.R., Inniss, W.E., Greer, C.W., 1999. Physiological adaptations involved in alkane assimilation at a low temperature by *Rhodococcus* sp. strain Q15. Appl. Environ. Microbiol. 65, 2961–2968.

Zhu, L., Zhang, X., Ji, L., Song, X., Kuang, C., 2007. Changes of lipid content and fatty acid composition of *Schizochytrium limacinum* in response to different temperatures and salinities. Process Biochem. 42, 210–214.