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Preparative thin-layer chromatographic separation and subsequent gas chromatographic–mass spectrometric analysis of monoacylglycerols derived from butter oil by fungal degradation

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Abstract

A semi-micro method has been developed using preparative thin-layer chromatography (TLC) to separate acylglycerols for the subsequent analysis by gas chromatography–mass spectrometry (GC–MS). Monoacylglycerols (MAGs) were formed from butter oil by fungal degradation with *Penicillium roquefortii*. Total lipids were extracted with hexane–2-propanol (3:2, v/v) and separated on silica gel preparative TLC plates with fluorescence indicator (Merck). The plates were developed in hexane–diethyl ether–formic acid (80:20:2, v/v). Lipid bands were detected under UV light or with iodine vapour, removed and then extracted with hexane–2-propanol (3:2, v/v). The MAG band (R_F 0.03) was silylated into trimethylsilyl (TMS) ethers. Structures and composition of MAG-TMS ethers were analysed by GC and GC–MS. Formation of characteristic ions for the identification of *sn*-1(3)- and *sn*-2-MAG isomers was discussed. The method is simple, inexpensive and powerful for the separation and analysis of relatively small amounts of MAGs (0.2–5.0 mg) formed from fungal degradation. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *Penicillium roquefortii*; Oils; Monoacylglycerols; Acylglycerols; Glycerols; Lipids

1. Introduction

Lipids can be analysed by different chromatographic techniques. Column chromatography, including silica gel and ion exchange, is used to separate complex lipids into neutral and polar lipids [1]. Partition chromatography, such as high-performance liquid chromatography (HPLC) and gas chromatography (GC), has recently been a powerful method to separate homologous or isomeric series of fatty acids and acylglycerols [2,3]. Preparative HPLC is normally used to separate lipid classes and molecular species of triacylglycerols (TAGs) [4]. Clean fractions can be obtained and automatic collection is possible. However, the equipment is relatively expensive and these methods require large volumes and extremely pure solvent.

Thin-layer chromatography (TLC) is a variant of liquid chromatography and preceded HPLC [4]. It is the most widely used method for separation of lipid mixtures into classes of compounds [5,6]. Preparative TLC is normally used to separate lipid extracts from biological samples [7–9]. The method is sim-

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ple, inexpensive and needs smaller volumes of solvent than other methods.

GC has been extensively used for the analysis of mono- (MAGs) and diacylglycerols (DAGs) in the form of trimethylsilyl (TMS) ether derivatives [10,11]. Recent applications of preparative TLC in conjunction with GC or HPLC mainly involve characterising the pattern of molecular species of the TAGs [8,9]. Literature survey shows that relatively little work has been undertaken to analyse biochemically synthesised MAG isomers by preparative TLC, GC and GC–mass spectrometry (MS).

The aim of this study was to develop a simple and relatively inexpensive semi-micro method for the separation and analysis of acylglycerols with preparative TLC–GC–GC–MS techniques. The overall objective of the work was to compare MAG structural isomers hydrolysed from butter oil (a cheese model) by cell-bound and commercial lipases. Final objective was to use MAGs as natural anti-microbials in dairy products.

2. Experimental

2.1. Materials

Butter oil was obtained as a gift from Unilever Research (Colworth House, Bedford, UK) in February 1995 and was stored in sealed containers at 4°C. Standard fatty acids, mono-, di- and triacylglyerols, *N*,*O*-bis(trimethylsilyl)acetamide (BSA) as pre-prepared reagents in sealed ampoules (5 ml) were obtained from Sigma (Poole, UK) and were Sigma grade. Solvents were obtained from Romil (Shepshed, Loughborough, UK) and were double glass distilled. Chemical reagents were obtained from BDH (Merck House, UK) and were AnalaR grade. *Penicillium roquefortii* FRR 2456 was obtained as a gift from Dr. Ailsa D. Hocking (CSIRO, Australia) in 1995. It was isolated from a melon and had undergone fungal spoilage.

2.2. Preparation of spore suspensions

Modified Czapek medium (pH 7.0) [12] with butter oil (4%, w/v) and casein enzymatic hydrolysate (1%, w/v) was used to grow cultures for spore production at 25°C for seven days. All glassware and materials used were sterile and the procedures were operated under aseptic controls. Phosphate buffer (20 ml, pH 7.0, 0.05 *M*) and 10 glass balls (3.5–4.5 mm diameter, BDH) were added into a slope showing heavy spore production. The slope was agitated with Rotamixer at full speed for 2 min. The suspension was filtered through two layers of buffered muslin (pH 7.0) into an Erlenmeyer flask (250 ml). The filtrate containing spores (20 ml) was then filtered under pressure through a 0.45-μm cellulose nitrate membrane overlaid by a 0.8-μm cellulose nitrate membrane in a Nalgene unit (250 ml) (New York, USA). The membrane with the spores was carefully removed from the filtration unit and was re-suspended in 20 ml phosphate buffer (pH 7.0, 0.05 *M*). The spore concentration was determined [13] and the suspensions were stored at 4°C for not more than 24 h prior to use.

2.3. Preparation of oil emulsions

Oil emulsion was prepared by homogenising 30 g autoclaved butter oil with 90 ml 2% gum arabic (membrane filtered). The mixture was emulsified with a sterile Polytron head at speed 2 for 2 min. The emulsion was then sonicated for 5 min in a Ultrasonicator (Bransonic 52, USA) at maximum current and ambient temperature.

2.4. Conversion of butter oil

Reaction mixtures were composed of butter oil emulsion (10 ml), phosphate buffer (pH 7.0, 0.1 *M*, 8 ml), $CaCl_2$ solution (0.01 *M*, 2 ml) and spore suspension (100–300 μl). Two types of controls were used: firstly, the mixtures without spores, and secondly, the mixtures with autoclaved spore suspensions (100–300 μl). Each sample was repeated in quadruplet.

Butter oil was degraded by *P. roquefortii* (FRR 2456) at pH 7.0 and 25°C. Reaction was carried out in Erlenmeyer flasks (250 ml) in a shaking water bath. The shaking water bath was set at 72 strokes per minute and the stroke length was 23.5 mm. The reactions were started by adding the spore suspensions after equilibrating the mixtures at 25°C for 30 min. Reactions were carried out at 25°C for 2 h.

2.5. Solvent extraction of acylglycerols from suspension cultures

Reaction samples (4 ml) were moved into pear-shaped flasks (25 ml) with 4 ml 2-propanol. The flasks were put into boiled water immediately for 5 min. The samples were cooled, acidified with 0.4 ml 0.01 *M* hydrochloric acid and extracted with 2×6 ml hexane–2-propanol (3:2, v/v). The upper hexane layers were collected and dried with anhydrous Na_2SO_4. Samples were stored under nitrogen atmosphere in sealed glass vials at −18°C until analysed.

2.6. Qualitative analysis of acylglycerols by TLC

Butter oil and extracted acylglycerols (2–5 μl, 1–2%, w/v) were applied on activated (105°C, 50 min) silica gel 60 TLC plates (20×5 cm, 0.25 mm layer thickness, with a fluorescent indicator at 254 nm) (Whatman, UK). The plates were developed with hexane–diethyl ether–formic acid (80:20:2, v/v/v) in a sealed glass TLC chamber (20×20×5 cm, Merck, Dorset, UK) at ambient temperature. The plates were then air-dried and visualised under UV light (254 nm) or with iodine vapour in a sealed tank. Methyl ketones were detected by spraying with 1% $KMnO_4$ containing 2% Na_2CO_3.

2.7. Separation of acylglycerols by preparative TLC

Silica gel 60 F_{254} preparative TLC plates (20×20 cm with 4×20 cm concentration zone, 0.5 mm layer thickness and fluorescence at 254 nm) (Merck) were pre-heated at 105°C for 50 min. A relatively large amount of sample (80 mg ml^{-1}) was applied on the plates with a glass pipette (2 ml). Standard MAGs (1–10:0, 1–12:0, 1–14:0, 1–16:0 and 1-*cis*-9–18:1, 16 mg ml^{-1} each) were dissolved in hexane and 2 ml standard was applied on the preparative TLC plates (×3). The plates were developed with the same solvent mixture under the same conditions as in the TLC analysis. Acylglycerols were visualised under UV light (254 nm) or by exposure to iodine vapours for less than 20 s. The positions of the MAG bands were lightly marked with a glass rod.

2.8. Extraction of monoacylglycerols from preparative TLC plates

Individual bands of MAGs were scraped from the preparative TLC plates and extracted with 3×2.5 ml mixture of hexane–2-propanol (3:2, v/v). The samples were centrifuged at 1500 rpm for 2 min in a Lab Centrifuge (WIFUG 500E, Bradford, UK). The supernatants were collected in 5-ml pre-weighed reaction vessels. Solvent was removed by blowing off with nitrogen gas. Monoacylglycerols were weighed and stored at 4°C under nitrogen gas for less than 18 h prior to GC and GC–MS analyses.

The recovery of MAGs from preparative TLC plates was evaluated with the standard mixture. The recovery rate (%, w/w) was defined as follows.

$$\frac{\text{extracted MAGs from the TLC plates}}{\text{original MAGs applied on the plates}} \cdot 100\%$$

It was calculated that the recovery was in the range of 92–95% for the standard MAGs.

2.9. Analysis of monoacylglycerols by GC

Monoacylglycerols were silylised with BSA to give MAG-TMS ethers [1]. The TMS ethers were separated on a 20 m×0.22 mm fused-silica capillary column (30QC2/BP1 0.25) (SGE, UK) in a Varian 3400 gas chromatograph. The GC system was equipped with a flame ionisation detection (FID) system and coupled to a 486 computer with Varian Star Chromatography Workstation (Version 4.0) (Varian, USA). Nitrogen was used as the carrier gas with flow-rate at 1.5 ml min^{-1}. Column temperature was programmed from 65°C to 100°C at 35°C min^{-1}, from 100°C to 240°C at 20°C min^{-1} and then from 240 to 300°C at 12°C min^{-1}. The injector was held at 320°C. The FID system was set at 370°C and run at 10^{-11} with attenuation of ×1. Split or splitless injection was used. The split ratio was 10:1. Samples (0.5–1.0 μl) were injected manually.

Quantitative analysis of MAG structural isomers were achieved by adding an internal standard (1-monotetradecanoyl-glycerol, 1–14:0) and considering the response factors of major MAGs. The MAG composition was calculated as molar percent of individual MAGs with respect to total MAGs.

2.10. Identification of MAG-TMS ethers by GC–MS

GC–MS analysis was carried out on the same column as GC analysis in a gas chromatograph (Hewlett-Packard 5890A) coupled with a quadrupole mass spectrometer (VG Trio-1) (Finnigan, Manchester, UK). Helium was used as the carrier gas. The temperature program was set up from 35 to 240°C at 20°C min^{-1}, from 240 to 300°C at 12°C min^{-1} and from 300 to 320°C at 2.5°C min^{-1}. The head pressure was 7 p.s.i. (1 p.s.i. = 6894.76 Pa). The ion source (electron impact, 70 eV) was set at 200°C and the interface temperature was at 250°C. Splitless injection was used and samples (1.0 μl) were injected manually.

The structural isomers of MAGs were identified by comparison of the sample spectra to the standard and NBS (National Bureau of Standards) library spectra. Furthermore, the fragmentation patterns were compared and the mechanism for formation of the characteristic ions was discussed.

3. Results and discussion

3.1. TLC and preparative TLC separation of acylglycerols

Fig. 1 shows the separation of acylglycerols by TLC and preparative TLC. Monoacylglycerols, lactones, 1,2- and 2,3-diacylglycerols (DAGs), long-chain DAGs and free fatty acids were separated completely under the solvent elution conditions. Carbonyl compounds such as methyl ketones gave a transient yellow spot against purple background (Fig. 1a). Triacylglycerols and methyl ketones overlapped and the TAGs were not separated completely. The wide bands represent butter oil TAGs with different chain lengths (C_4–C_{20}). Monoacylglycerols were not

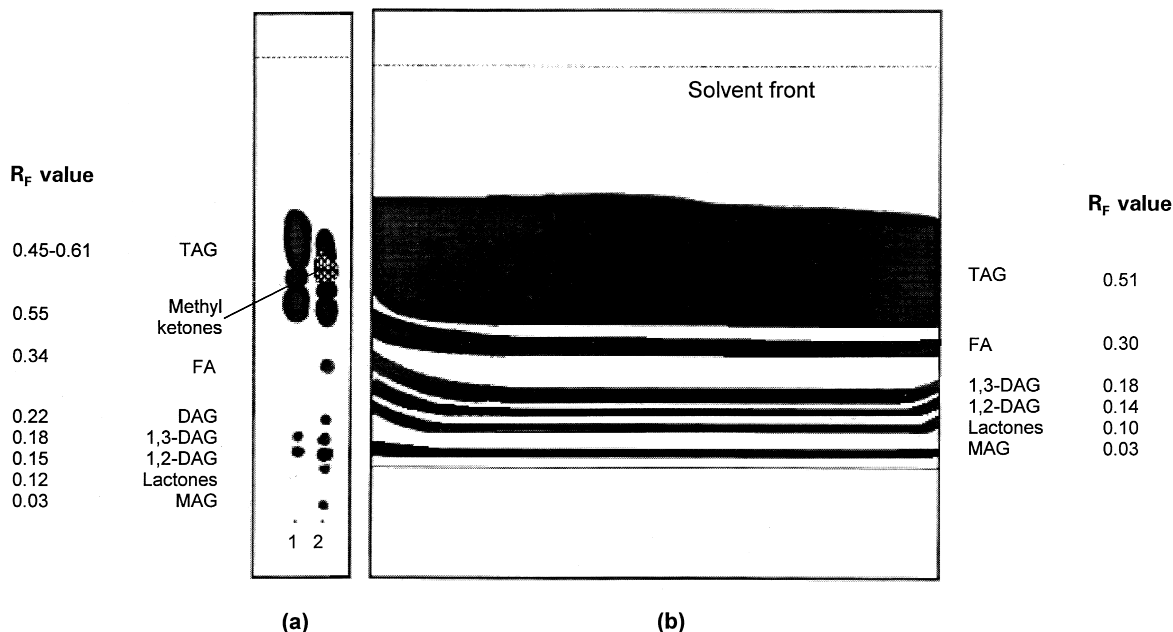

Fig. 1. Separation of acylglycerols on (a) silica gel 60 Å TLC plates (20×5 cm, 0.25 mm layer thickness, Whatman) and (b) silica gel 60 F_{254} preparative TLC plate (20×20 cm, 0.5 mm thickness with 20×4 cm concentrating zone, Merck). Acylglycerols were produced from butter oil by *P. roquefortii* on modified Czapek medium at pH 7.0 and 25°C for 2 h. Plates were eluted with hexane–diethyl ether–formic acid (80:20:2, v/v). (a) TLC analysis: 1 = unreacted butter oil; 2 = acylglycerols produced by *P. roquefortii* FRR 2456. (b) Preparative TLC separation: acylglycerols produced by *P. roquefortii* FRR 2456. MAG: monoacylglycerols, DAG: diacylglycerols, FA: free fatty acids, TAG: triacylglycerols.

detected in control samples with inactivated *P. roquefortii* spores. It suggested that the MAGs were derived from butter oil. However, 1,2- and 1,3-DAGs were found in original butter oil as well. It is uncertain whether the MAGs were derived from butter oil TAGs or DAGs or from both.

A relatively large amount of sample (0.30 g) was applied on the preparative TLC plates. R_F values varied slightly compared to the separation on TLC plates. However, mono-, diacylglycerols and fatty acids were separated completely to give the same pattern of separation as in the TLC analysis (Fig. 1b).

3.2. GC chromatogram of MAG-TMS ethers

Fig. 2 shows the GC chromatogram of MAG-TMS ethers derived from butter oil by *P. roquefortii* on modified Czapek medium at pH 7.0 and 25°C. The structural isomers of MAG-TMS ethers with chain lengths from 10 to 18 were separated in pairs within 16 min. Monooctadecanoyl glycerol (monostearin) and *cis*-9-monooctadecenoyl glycerol (monoolein) were separated according to their degree of unsaturation.

3.3. Identification of structural isomers of monoacylglycerol-TMS ethers

Every peak separated on GC column was identified by GC–MS. Mass spectra of 1(3)- and 2-monohexadecanoyl glycerol TMS ethers are shown in Fig. 3.

The most abundant ions at $[M-103]^+$ were found in all 1- or 3-MAG-TMS ethers (Figs. 3 and 4). These ions were $[M–(CH_3)_3SiOCH_2]^+$ and were due to the cleavage between carbons 2 and 3, or 1 and 2. The ion at m/z 205 was characteristic for all

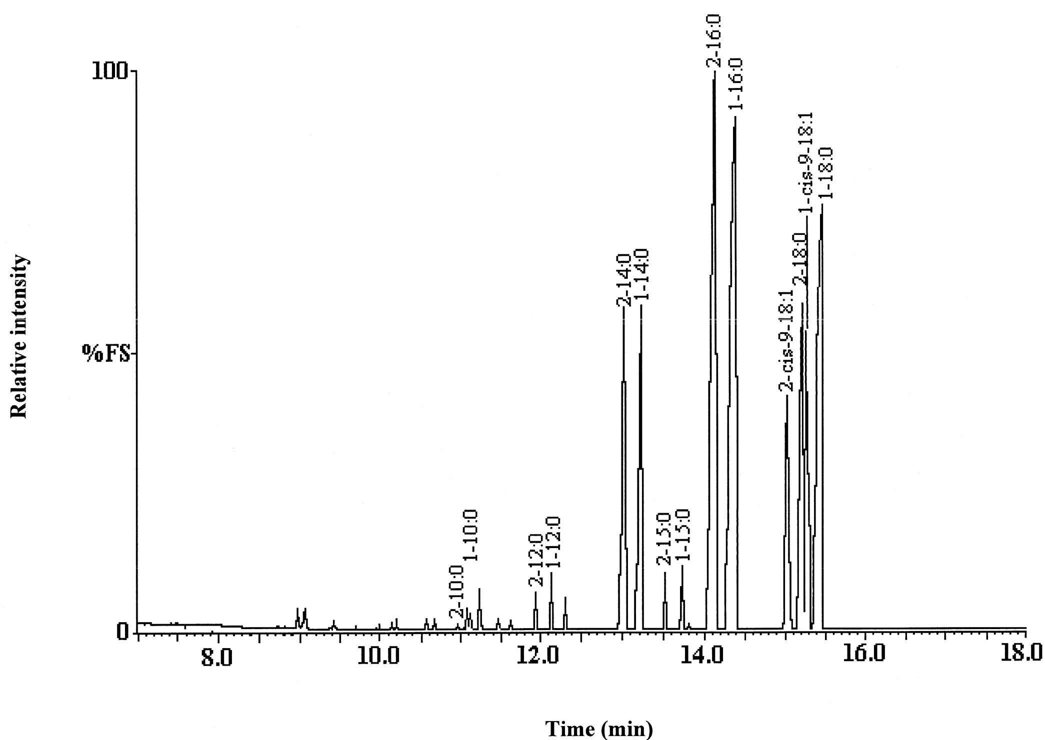

Fig. 2. Total ion chromatogram of MAG-TMS ethers produced from butter oil by *P. roquefortii* at pH 7.0 and 25°C. Every peak was identified by GC–MS. Peaks not labelled are contaminants of silanes and fatty acid methyl esters. The GC system was programmed from 35°C (with initial stay of 0.5 min) to 240°C at 20°C min^{-1} and from 240°C to 300°C at 12°C min^{-1}. Electron impact quadrupole MS was coupled to GC. Ion source was at 200°C.

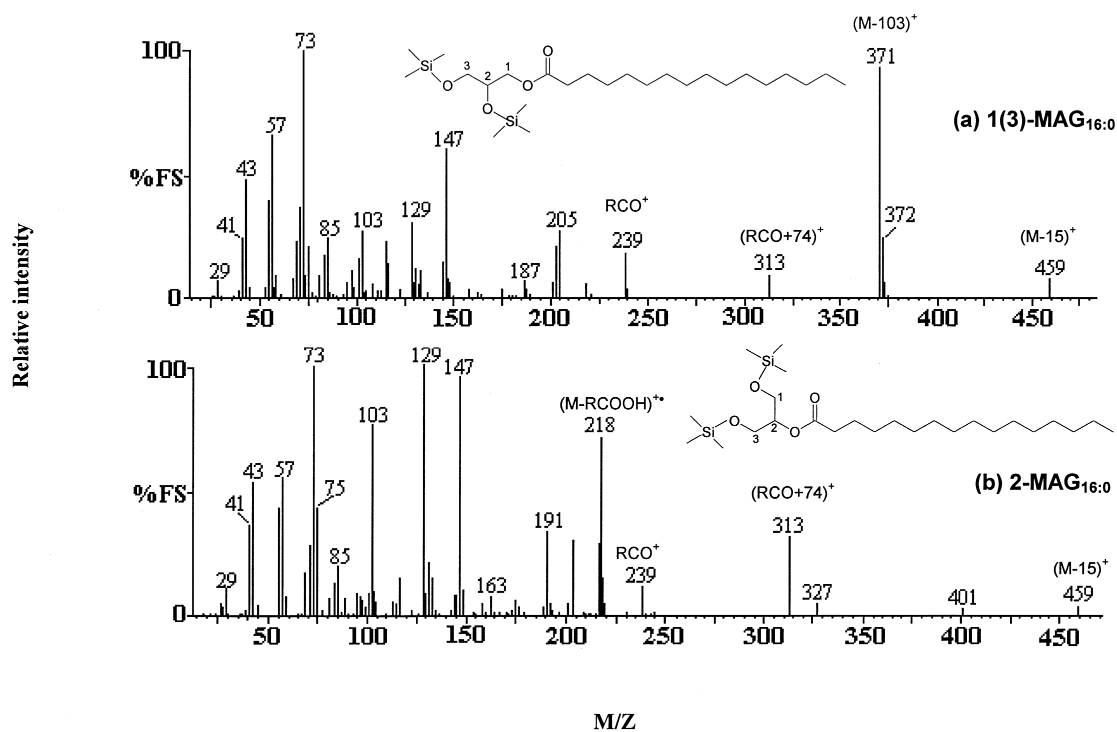

Fig. 3. Mass spectra of 1(3)- and 2-monohexadecanoyl-*sn*-glycerol-TMS ethers derived from butter oil by *P. roquefortii* at pH 7.0 and 25°C. GC–MS conditions as in Fig. 2.

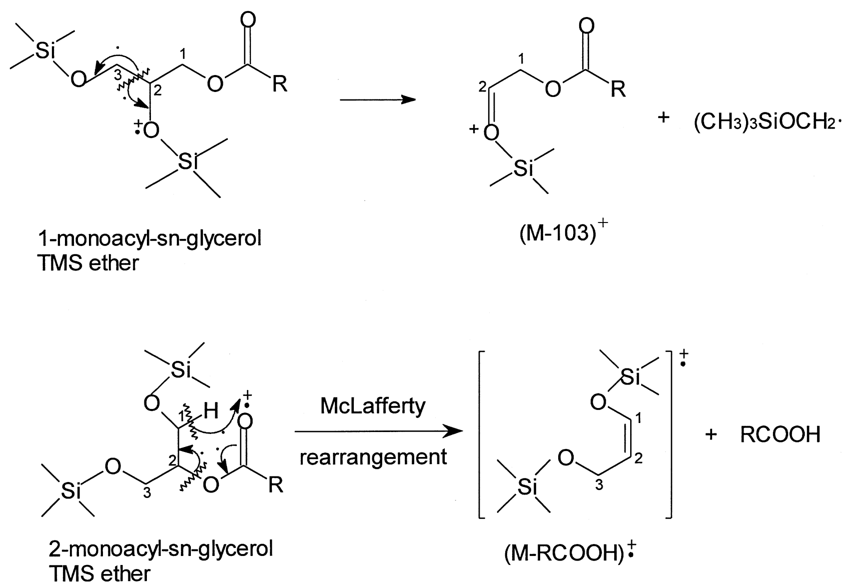

Fig. 4. Mechanism for the formation of characteristic ions for identification of *sn*-1(3)- and 2-MAG-TMS ethers in GC–MS analysis. $R=CH_3(CH_2)_n^-$.

Table 1
Structural isomers of MAGs produced from butter oil by *P. roquefortii* FRR 2456 at pH 7.0 and 25°C[a]

MAG	Composition (mol%)		Molar ratio[b]	
	sn-1(3)	*sn*-2	*sn*-1(3)	*sn*-2
10:0	0.64±0.09	0.37±0.06	1.72±0.20	1.00±0.00
12:0	0.56±0.08	0.63±0.08	1.42±0.09	1.68±0.13
14:0	3.96±0.19	3.20±0.14	10.69±0.34	8.66±0.31
15:0	2.31±0.10	2.47±0.07	6.29±0.21	6.75±0.17
16:0	26.71±0.14	15.59±0.15	73.59±1.30	42.84±0.85
18:0	22.80±0.68	6.39±0.20	63.75±0.89	17.69±0.44
18:1 (9c)	7.33±0.14	7.05±0.13	20.31±0.64	19.52±0.62
Total	64.31	35.70	197.28	78.62

[a] Results are the mean of six GC analyses±standard deviation. Monoacylglycerols were derived from one fermentation and analysed as TMS ethers by GC–MS. Total ion chromatogram is shown in Fig. 2. ND: not detected.
[b] Calculated relative to 2-monodecanoylglycerol (2–10:0).

sn-1(3)-isomers. This fragment was $[(CH_3)_3SiO]_2C_2H_3^+$ and was due to the cleavage of TMS groups and part of the glycerol backbone of the *sn*-1(3)-isomers. The ion at m/z 218 was characteristic of the spectra of all *sn*-2-isomers (Fig. 3). This ion was $[M–RCOOH]^{+\cdot}$ and was due to the cleavage of TMS groups and part of the glycerol backbone of the *sn*-2-isomers and was derived by a McLafferty rearrangement (Fig. 4). Common ions at m/z 73, 75, 129 and 147 were found for all MAG-TMS ethers as well as repeated ions of $[M-15]^+$, RCO^+ and $[RCO+74]^+$.

3.4. Composition of monoacylglycerols produced from butter oil by P. roquefortii

Table 1 shows the composition of MAG structural isomers produced from butter oil by *P. roquefortii* at pH 7.0 and 25°C. It demonstrates that with the preparative TLC–GC–GC–MS system, quantitative analysis of MAG structural isomers produced from complex butter oil by fungal degradation was achieved.

4. Conclusions

In general, preparative TLC is a simple and inexpensive method for the separation of acylglycerols. Complete separation of monoacylglycerols, diacylglycerols and fatty acids produced from butter oil by fungal catalysis was achieved. Analytical TLC is used to screen MAGs produced from butter oil. Preparative TLC is used to isolate MAGs for the subsequent characterisation. Followed by GC and GC–MS analyses, the structures and composition of MAGs were determined.

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