Separation of regioisomers and enantiomers of triacylglycerols containing branched fatty acids (iso and/or anteiso)

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2.1 Chemicals and standards

Acetonitrile, 2-propanol, hexane, dichloromethane, Bacterial Acid Methyl Ester (BAME) Mix, 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine (15:0-15:0 PC), 1- palmitoyl-(13-methylmyristoyl)-sn-glycero-3-phosphocholine (16:0-i15:0 PC), 1-palmitoyl- (12S-methylmyristoyl)-sn-glycero-3-phosphocholine (16:0-ai15:0 PC), 1,2-dipalmitoyl-snglycero-3-phosphocholine (16:0-16:0 PC), 1-pentadecanoyl-2-hydroxy-sn-glycero-3- phosphocholine (15:0 lyso PC), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (16:0 Lyso PC), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDCI), N,Ndimethylpyridin- 4-amine (DMAP), lipase from porcine pancreas, Type VI-S, ≥20,000 units/mg protein, lyophilized powder,phospholipase C from *Clostridium perfringens* (*C. welchii*) Type XIV, lyophilized powder, 150 units/mg protein, Preparative TLC Glass Plates L × W 20 cm × 20 cm, silica gel 60 matrix, binder, Polymeric, fluorescent indicator, Discovery® Ag-Ion SPE Tube bed wt. 750 mg, volume 6 mL were purchased from Merck (previously Sigma-Aldrich, Prague, Czech Republic). 12-Methyltridecanoic acid (i-14:0), 12- methyltetradecanoic acid (ai-15:0), 13-methyltetradecanoic acid (i-15:0), 14- methylpentadecanoic acid (i-16:0), and 14-methylhexadecanoic acid (ai-17:0) were purchased from Larodan AB (Solna, Sweden). All other reagents used were of analytical grade and purchased from Merck (Prague, Czech Republic).

2.4. Preparation and analysis of methyl and 3-pyridylcarbonyl esters of fatty acids

2.4.1 Preparation of methyl esters

A ~10 mg sample was dissolved in 1 mL diethyl ether and 20 mL methyl acetate. 250 μL of 1 M sodium methoxide in methanol was added. The sample was worked up and left for 50 min at room temperature. Saturated oxalic acid solution (0.5 mL) was added, with brief agitation, to neutralize the solution. The solvent was removed under nitrogen, and an appropriate volume of hexane was added to bring the sample to the concentration required for analysis.

2.4.2 Preparation of 3-pyridylcarbonyl esters

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 FAME (~0.5 mg) in drydichloromethane (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.

2.4.3 GC-MS of FAMEs

Splitless injection was at 100 °C, and a fused silica capillary column (Supelcowax 10; 60 m x 0.25 mm i.d., 0.25 mm film thickness; Supelco, Prague) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C/min to 180 °C and at 2 °C/min to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. All spectra were scanned within the range of *m/z* 50–500. FAMEs were identified according to their mass spectra [25,26] and using a mixture of chemical standards obtained from Merck.

2.4.4. GC-MS of 3-pyridylcarbonyl ester of fatty acids

A fatty acid 3-pyridylcarbonyl ester mixture was analysed on the instrument described above. Injection temperature (splitless injection) was 100 °C, and an Ultra-1 capillary column (Supelco, Prague, 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 of *m/z* 50-500.

2.5 TAG analysis using RP-HPLC/MS-ESI

The LC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Agilent, Santa Clara, CA, USA) and three Luna Omega 1.6 µm, C18, 100 Å, LC Column 150 x 2.1 mm, connected in series. Although the efficiency stated by the manufacturer was not achieved, i.e. 350,000 plates/meter (see manufacturer's literature), yet the efficiency achieved in our case was ~327,000 plates/meter. Tripalmitine with tR 372.3 min was used as an internal standard, with a flow rate of 0.95 mL/min, an injection volume of 10 □L, and column temperature of 25 °C. TAGs were separated using a solvent gradient program with acetonitrile (ACN) containing 5 mM aqueous ammonium acetate, and 2- propanol (iPrOH) containing 5 mM aqueous ammonium acetate as follows: initial ACN/iPrOH (99:1, vol/vol), a linear change to 25:75 ACN/iPrOH (vol/vol) from 5 min to 510 min, and a hold for 10 min. The composition was returned to the initial conditions over 10 min.

An LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany) high-resolution hybrid mass spectrometer equipped with a heated electrospray interface (HESI) was used. The ESI-MS analysis was performed in the FT positive ion mode. MS spectra were acquired with a target mass resolution of R = 110,000 at *m/z* 800. The electrospray voltage was set to 3.5 kV, and the scan range of the instruments was set to *m/z* 200–1500. Nitrogen was used as a nebulizer

gas and set to 18 arbitrary units (sheath gas) and 7 arbitrary units (auxiliary gas). Helium was used as a collision gas for CID experiments. A CID normalization energy of 35% was used to fragment the precursor ions. The MS/MS product ions were detected in highresolution FT mode. Further source conditions in the positive mode included a vaporizer temperature of 250 °C, capillary temperature of 230 °C, capillary voltage of 50 V, and tube lens voltage of 170 V.

2.8. Synthesis of triacylglycerols

In brief, phospholipase C from *C. welchii* (3 mg) in 0.5 M tris(hydroxymethyl)methylamine (tris) buffer (pH 7.5; 2 mL), containing 2 x 10^{-3} M calcium chloride, was added to phosphatidylcholine (5 mg) in diethyl ether (2 mL). The mixture was shaken at room temperature for 3 hours and extracted three times with diethyl ether (4 mL portions). The ether layer was dried over anhydrous sodium sulfate before being evaporated in a stream of nitrogen at ambient temperature. Pure 1,2-diacyl-*sn*-glycerols were obtained by preparative TLC on a layer of silica gel G impregnated with boric acid (10%, w/w), with hexane-diethyl ether (50:50, v/v) as solvent system. The appropriate band was located under UV light and was eluted from the adsorbent with diethyl ether [34]. 1-Monoacyl-sn-glycerols were similarly prepared.

A solution of 1,2-diacyl-*sn*-glycerols (10 µmol) or 1-monoacyl-*sn*-glycerols (5 µmol) and free acid (i-14:0, ai-15:0, i-15:0, i-16:0, and ai-17:0 (11 µmol) in dichloromethane (1.0 mL) was added to DMAP (10 µmol) and EDCI (12 µmol) in dichloromethane (1.0 mL) under an inert atmosphere (argon). 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.

Table 1Sa. Fatty acid content from four cultivations after isolation of triacylglycerols and after Ag^+ separation.

Fatty acid	control	propionate	isobutyrate	isovalerate
i-13:0	0.1	0.2	0.2	0.1
ai-13:0	0.3	0.4	0.2	0.2
i-14:0	8.0	5.4	28.3	5.9
n-14:0	1.2	0.3	0.4	0.5
i-15:0	7.7	5.8	4.6	27.5
ai-15:0	29.4	17.3	17.2	14.6
n-15:0	3.3	31.2	2.8	1.7
i-16:1 n-9	2.5	2.1	6.9	1.7
i-16:0	19.2	10.8	25.8	9.8
n-16:1	3.4	1.7	0.4	0.7
n-16:0	11.2	7.9	5.8	5.2
i-17:1	0.7	0.2	0.2	7.0
ai-17:1	1.0	0.1	0.2	0.1
i-17:0	0.7	0.4	0.3	18.3
ai-17:0	8.5	6.5	4.9	5.1
n-17:0	0.1	8.2	0.3	0.4
n-18:0	1.8	1.2	1.1	0.9
n-18:1	0.9	0.3	0.4	0.3

Table 1Sb. Fatty acid content from four cultivations after Ag^+ separation of triacylglycerols.

Fatty acid	control	propionate	isobutyrate	isovalerate
i-13:0	0.1	0.2	0.2	0.1
ai-13:0	0.3	0.4	0.2	0.2
i-14:0	8.7	5.6	30.8	6.5
n-14:0	1.3	0.3	0.4	0.6
i-15:0	8.4	6.1	5.0	30.5
ai-15:0	32.1	18.1	18.7	16.2
n-15:0	3.6	32.6	3.2	1.9
i-16:0	21.1	11.3	28.1	10.8
n-16:0	12.2	8.3	6.3	5.8
i-17:0	0.8	0.4	0.3	20.3
ai-17:0	9.3	6.8	5.3	5.7
n-17:0	0.1	8.6	0.3	0.4
n-18:0	2.0	1.3	1.2	1.0

Fig. 1S. Mass spectrum of 3-pyridylcarbinol ester of ai-15:0 acid.

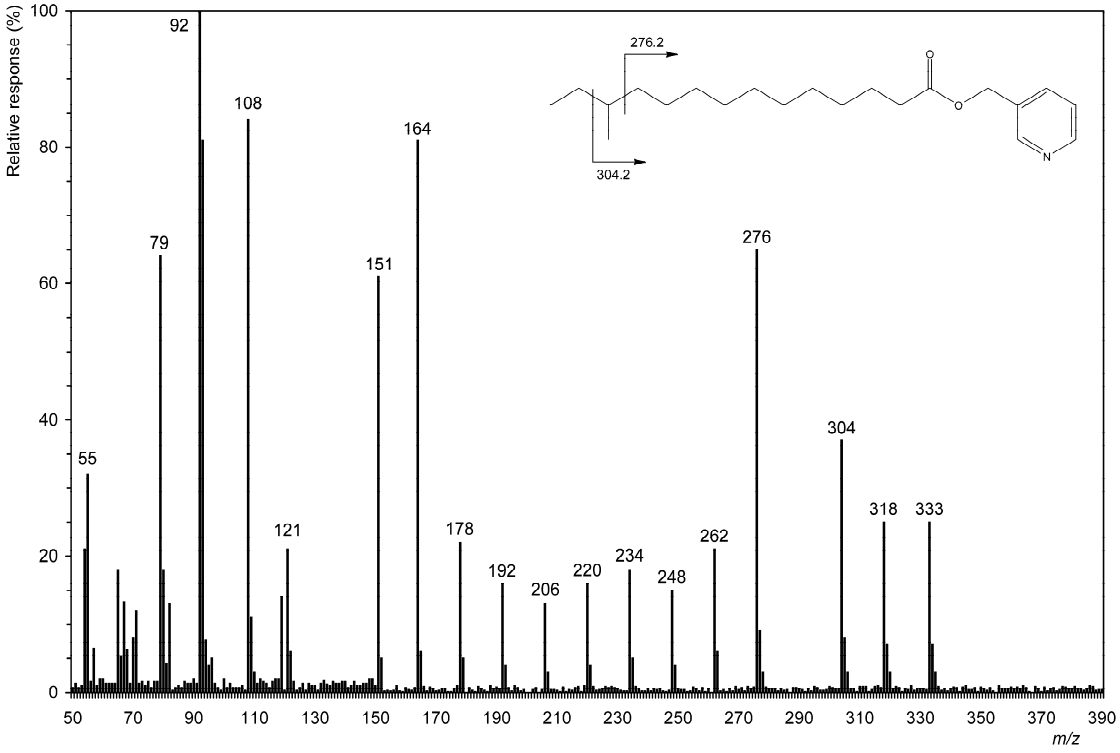

Fig. 2S. Mass spectrum of 3-pyridylcarbinol ester of i-15:0 acid.

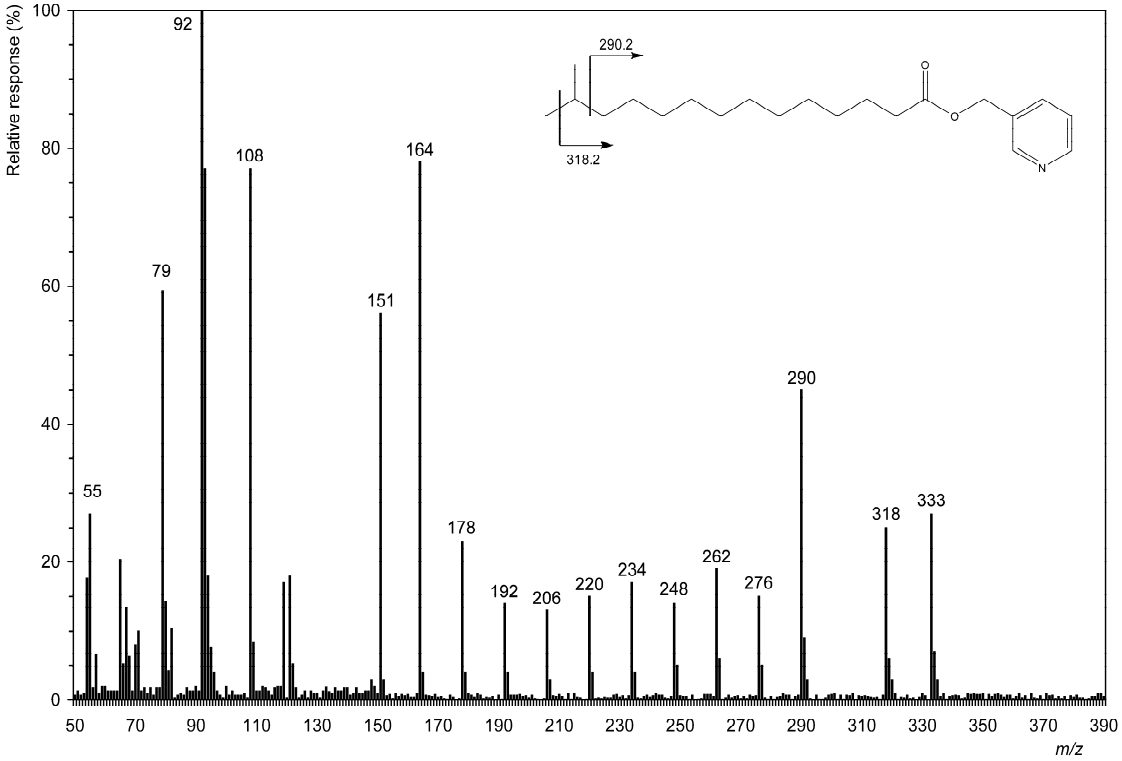

Fig. 3S. Mass spectrum of 3-pyridylcarbinol ester of n-15:0 acid.