Supplements

Separation and identification of diacylglycerols containing branched chain fatty acids by liquid chromatography-mass spectrometry

Andrea Palyzová and Tomáš Řezanka✉

2. Material and Methods

2.3. Isolation of lipids

The method of Bligh and Dyer [21] was used for lipid extraction. Briefly, chloroform/methanol solution (1/2, v/v; 1 mL) was added to the lyophilized pellet cultures and vortexed for 10-15 min. Then chloroform (1 mL) was added and the suspension of cells was shaken for 1 min, and 1 M NaCl solution (1 mL) was added. The sample was mixed for one minute and centrifuged. The lower phase was collected, the solvents were evaporated under nitrogen and further redissolved in chloroform/methanol solution (2/1, v/v; 10 μL) prior to analysis.

2.4. Analysis of fatty acids as FAMEs and 3-pyridylcarbonyl esters

Preparation and analysis of FAME was described previously [22,23]. Briefly, fatty acids after hydrolysis of total lipids were esterified by methanolic BF_3 and analyzed by GC-MS on polar capillary column (Supelcowax 10; 60 m x 0.25 mm i.d., 0.25 mm film thickness; Supelco, Prague). 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 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 3-pyridylcarbonyl ester mixture was analyzed on the instrument described above. Injection temperature (splitless injection) was 100 °C, and an Ultra-1 capillary column (Agilent Technology, CA, USA) 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. HILIC-LC/MS-ESI

The HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Hewlett Packard, USA), and Ascentis Express HILIC HPLC column (2.7 μm particle size, L × I.D. 3 cm × 2.1 mm) (Supelco, Prague, Czech republic). LC was

performed at a flow rate of 200 µL/min, with a linear gradient from mobile phase containing methanol/acetonitrile/aqueous 1 mM ammonium acetate (70:20:10, v/v/v) to methanol/acetonitrile/aqueous 1 mM ammonium acetate (5:75:20, v/v/v) with lithium acetate in methanol (1 µM/mL) for 20 min. To form lithiated adducts of molecular species, lithium acetate (1 µM/mL final concentration) was added to the solution of total lipids. An injection volume (10 µL) and a column temperature of 25 ◦C was used. The whole HPLC flow was introduced into the ESI source without any splitting. The system backpressure reached 1000 bars during the gradient analysis.

An LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a heated electrospray interface (HESI) was operated in positive and negative ionization mode. The MS scan range was performed in the FT cell and recorded within a window between 100–1000 *m/*z. The mass resolution was set to 105 000, and the ion spray voltage was set at 3.5 kV (in the positive ionization mode) and -2.5 kV (in the negative ionization mode). Both ionization modes used the following parameters: sheath gas flow, 18 arbitrary units (AU); auxiliary gas flow, 7 AU; ion source temperature, 250 °C; capillary temperature, 230 °C; capillary voltage, 50 V; and tube lens voltage, 170 V. Helium was used as a collision gas for collision-induced dissociation (CID) experiments. The CID normalization energy of 35% was used for the fragmentation of parent ions.

The calibration of the MS spectrometer was conducted with the use of a Pierce LTQ Orbitrap positive and/or negative ion calibration solution (Thermo Fisher Scientific, San Jose, CA, USA). The internal lock mass was used in mass spectra acquisition, i.e., 255.2330 *m/z* $[M-H]^-$ palmitic acid in the negative ESI. The mass accuracy was better than 2 ppm. The chemical structure of the compounds was confirmed with the help of the spectral database LIPID MAPS® Lipidomics Gateway (http://www.lipidmaps.org/).

2.7. AcTAG analysis using RP-HPLC/MS-ESI²

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 s Hichrom HIRPB - 250A columns with a 250 x 2.1 mm ID and 5 µm particle size connected in series. This setup was used as a high efficiency column, approximately 105,000 plates/m (see manufacturer's literature), with a flow rate of 0.95 mL/min, an injection volume of 10 µL, 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 800 min, and a hold for 10 min. The composition

was returned to the initial conditions over 10 min. The performance was measured with triheptadecenoin as an internal standard under the conditions described above.

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 high-resolution 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.

Table 1S. Accurate masses of fragments from unknown (U1-U6), see Fig. 1.

U1		U2		U3		U4		U5		U6	
m/z	abundance	m/z	abundance	m/z	abundance	m/z	abundance	m/z	abundance	m/z	abundance
241.2172	100	224.0328	32	223.1702	35	225.2212	18	241.2170	100	210.0174	23
315.0484	35	241.2172	100	241.2172	100	241.2168	100	282.2801	63	242.2135	14
477.1014	26	255.2333	45	255.2335	45	523.4726	54	487.3750	22	284.2240	5
701.3054	15	269.2486	47	269.2489	47	539.4676	62	509.4572	48	289.9845	31
719.3258	20	459.0907	26	459.0911	33	602.4317	11	523.4727	51	428.2656	45
775.3521	59	533.1277	28	533.1281	45	618.4266	75	582.3289	9	448.2110	26
793.3627	48	701.3054	16	846.3897	82	685.5255	42	596.3444	17	466.2214	12
943.5401	63	719.3261	23	925.5293	49	701.5204	19	618.4268	12	508.2322	18
1017.5775	42	757.3418	47	943.5401	37	764.4845	28	623.3680	59	526.2427	29
		775.3522	64	981.5551	24	780.4794	31	823.5219	42	654.4593	38
		925.5295	32	999.5663	31	821.5186	45	864.5848	27	734.4258	32
		943.5402	38	1017.5772	17	1078.7302	24	913.7144	31	766.9491	64
		999.5666	78	1070.6033	22	1303.9521	38	927.7301	18	832.4784	30
		1088.614	31	1115.6612	63			1105.8015	45	858.0326	12
				1159.6513	34					923.5617	6
										1041.6688	19
										1097.6872	47
										1177.6540	35
										1210.7348	33
										1323.8805	51
										1403.8469	87
										1454.9432	64
										1506.0477	17
										1520.8894	9
										1534.9096	15
										1556.8871	34
										1586.0140	46
										1637.1059	94
										1665.9635	100
										1687.9452	38
										1717.0724	21

Table 2S. Occurring product ions for compounds from Figs. 2, 3, 5, and 6.

PG (15:0/15:0) obtained by tandem MS.

m/z	**Ion Description**
693.4712	Precursor ion $[M-H]^-$
619.4344	Loss of glycerol from precursor ion
469.2572	Loss of sn1+sn2 acyl chain as ketene (RCH=C=O) from $[M-H]^-$
451.2466	Neutral loss of sn1+sn2 RCOOH group from $[M-H]^-$
395.2204	Loss of sn1+sn2 acyl chain as ketene (RCH=C=O) and glycerol from $[M-H]^-$
377.2099	Neutral loss of sn1+sn2 RCOOH group and glycerol from $[M-H]^-$
245.0432	Glycerophosphoglycerol ion
241.2173	sn1+sn2 $RCOO^-$ ion
227.0326	Glycerophosphoglycerol ion $-H_2O$
209.0221	Glycerophosphoglycerol ion $-2H_2O$
152.9958	Glycerol-3-phosphate ion with loss of H_2O

PC (16:0/14:0) obtained by tandem MS.

m/z	**Ion Description**
764.5447	Precursor ion $[M+acetate]^-$
690.5079	Loss of CH_3 and acetate from precursor ion
619.4344	Loss of choline and acetate from precursor ion
480.3096	Loss of sn2 acyl chain as ketene (RCH=C=O), CH_3 and acetate from precursor ion
462.2990	Neutral loss of sn2 RCOOH group, loss of CH_3 and acetate from precursor ion
452.2783	Loss of sn1 acyl chain as ketene (RCH=C=O), CH_3 and acetate from precursor ion
434.2677	Neutral loss of sn1 RCOOH group, loss of CH3 and acetate from precursor ion
255.2330	sn1 $RCOO^-$ ion
227.2017	sn2 $RCOO^-$ ion
224.0693	Glycerophosphocholine with loss of CH_3 and H_2O
168.0431	Phosphocholine with loss of CH_3
152.9958	Glycerol-3-phosphate ion with loss of H_2O

PC (15:0/15:0) obtained by tandem MS.

m/z	**Ion Description**
764.5447	Precursor ion $[M+acetate]^-$
690.5079	Loss of CH_3 and acetate from precursor ion
619.4344	Loss of choline and acetate from precursor ion
466.2939	Loss of sn1+sn2 acyl chain as ketene (RCH=C=O), CH_3 and acetate from precursor ion
448.2834	Neutral loss of sn1+sn2 RCOOH group, loss of CH_3 and acetate from precursor ion
241.2173	sn1+sn2 $RCOO^-$ ions
224.0693	Glycerophosphocholine with loss of CH_3 and H_2O
168.0431	Phosphocholine with loss of CH_3
152.9958	Glycerol-3-phosphate ion with loss of H_2O

PC (16:0/15:0) obtained by tandem MS.

m/z	**Ion Description**
778.5604	Precursor ion $[M+acetate]^-$
704.5236	Loss of CH_3 and acetate from precursor ion
633.4501	Loss of choline and acetate from precursor ion
480.3096	Loss of sn2 acyl chain as ketene (RCH=C=O), CH_3 and acetate from precursor ion
466.2939	Loss of sn1 acyl chain as ketene (RCH=C=O), CH_3 and acetate from precursor ion
462.2990	Neutral loss of sn2 RCOOH group, loss of CH_3 and acetate from precursor ion
448.2834	Neutral loss of sn1 RCOOH group, loss of CH_3 and acetate from precursor ion
255.2330	sn1 $RCOO^-$ ion
241.2173	sn2 $RCOO^-$ ion
224.0693	Glycerophosphocholine with loss of CH_3 and H_2O
168.0431	Phosphocholine with loss of CH_3
152.9958	Glycerol-3-phosphate ion with loss of H_2O

PC (16:0/18:1) obtained by tandem MS.

m/z	**Ion Description**
818.5917	Precursor ion $[M+acetate]^-$
744.5549	Loss of CH_3 and acetate from precursor ion
673.4814	Loss of choline and acetate from precursor ion
506.3252	Loss of sn1 acyl chain as ketene (RCH=C=O), CH_3 and acetate from precursor ion
488.3147	Neutral loss of sn1 RCOOH group, loss of CH_3 and acetate from precursor ion
480.3096	Loss of sn2 acyl chain as ketene (RCH=C=O), CH_3 and acetate from precursor ion
462.2990	Neutral loss of sn2 RCOOH group, loss of CH_3 and acetate from precursor ion
281.2486	sn2 $RCOO^-$ ion
255.2330	sn1 $RCOO^-$ ion
224.0693	Glycerophosphocholine with loss of CH_3 and H_2O
168.0431	Phosphocholine with loss of CH_3
152.9958	Glycerol-3-phosphate ion with loss of H_2O

TAG (15:0/15:0/2:0) obtained by tandem MS.

m/z	**Ion Description**
600.5200	Precursor ion $[M+NH_4]^+$
583.4934	Precursor ion $[M+H]^+$
523.4721	Neutral loss of sn3 RCOOH + NH_3 from $[M+NH_4]^+$
341.2686	Neutral loss of sn1+sn2 RCOOH + NH_3 from $[M+NH_4]^+$
299.2581	sn1+sn2 acyl chain ($[RC=O +74]^+$)
281.2475	sn1+sn2 acyl chain ($[RC=O +74]^+$ with loss of H_2O)
225.2213	sn1+sn2 acyl chain ($[RC=O]^+$)
207.2107	sn1+sn2 acyl chain ($[RC=O]^+$ with loss of H_2O)

TAG (16:0/14:0/2:0) obtained by tandem MS.

m/z	**Ion Description**
600.5198	Precursor ion $[M+NH_4]^+$
583.4932	Precursor ion $[M+H]^+$
523.4722	Neutral loss of sn3 RCOOH + NH_3 from $[M+NH_4]^+$
355.2844	Neutral loss of sn2 RCOOH + NH_3 from $[M+NH_4]^+$
327.2530	Neutral loss of sn1 RCOOH + NH_3 from $[M+NH_4]^+$
313.2739	sn1 acyl chain ($[RC=O+74]^+$)
295.2632	sn1 acyl chain ($[RC=O+74]^+$ with loss of H_2O)
285.2422	sn2 acyl chain ($[RC=O+74]^+$)
267.2320	sn2 acyl chain ($[RC=O+74]^+$ with loss of H_2O)
239.2371	sn1 acyl chain ($[RC=O]^+$)
221.2263	sn1 acyl chain ($[RC=O]^+$ with loss of H_2O)
211.2056	sn2 acyl chain ($[RC=O]^+$)

TAG (16:0/15:0/2:0) obtained by tandem MS.

m/z	**Ion Description**
614.5354	Precursor ion $[M+NH_4]^+$
597.5089	Precursor ion $[M+H]^+$
537.4877	Neutral loss of sn3 RCOOH + NH_3 from $[M+NH_4]^+$
355.2842	Neutral loss of sn2 RCOOH + NH_3 from $[M+NH_4]^+$
341.2686	Neutral loss of sn1 RCOOH + NH_3 from $[M+NH_4]^+$
313.2739	sn1 acyl chain ($[RC=O+74]^+$)
299.2580	sn2 acyl chain ($[RC=O+74]^+$)
295.2631	sn1 acyl chain ($[RC=O+74]^+$ with loss of H_2O)
281.2475	sn2 acyl chain ($[RC=O+74]^+$ with loss of H_2O)
239.2370	sn1 acyl chain ($[RC=O]^+$)
225.2212	sn2 acyl chain ($[RC=O]^+$)
221.2264	sn1 acyl chain ($[RC=O]^+$ with loss of H_2O)
207.2106	sn2 acyl chain ($[RC=O]^+$ with loss of H_2O)

TAG (16:0/18:1/2:0) obtained by tandem MS.

m/z	**Ion Description**
654.5667	Precursor ion $[M+NH_4]^+$
637.5402	Precursor ion $[M+H]^+$
577.5190	Neutral loss of sn3 RCOOH + NH_3 from $[M+NH_4]^+$
381.2999	Neutral loss of sn1 RCOOH + NH_3 from $[M+NH_4]^+$
355.2843	Neutral loss of sn2 RCOOH + NH_3 from $[M+NH_4]^+$
339.2894	sn2 acyl chain ($[RC=O+74]^+$)
321.2788	sn2 acyl chain ($[RC=O+74]^+$ with loss of H_2O)
313.2737	sn1 acyl chain ($[RC=O+74]^+$)
295.2631	sn1 acyl chain ($[RC=O+74]^+$ with loss of H_2O)
265.2526	sn2 acyl chain ($[RC=O]^+$)
247.2420	sn2 acyl chain ($[RC=O]^+$ with loss of H_2O)
239.2369	sn1 acyl chain ($[RC=O]^+$)
221.2264	sn1 acyl chain ($[RC=O]^+$ with loss of H_2O)

Fig. S1. Mass spectrum of 3-pyridylcarbinol esters of 13-methyltetradecanoate.

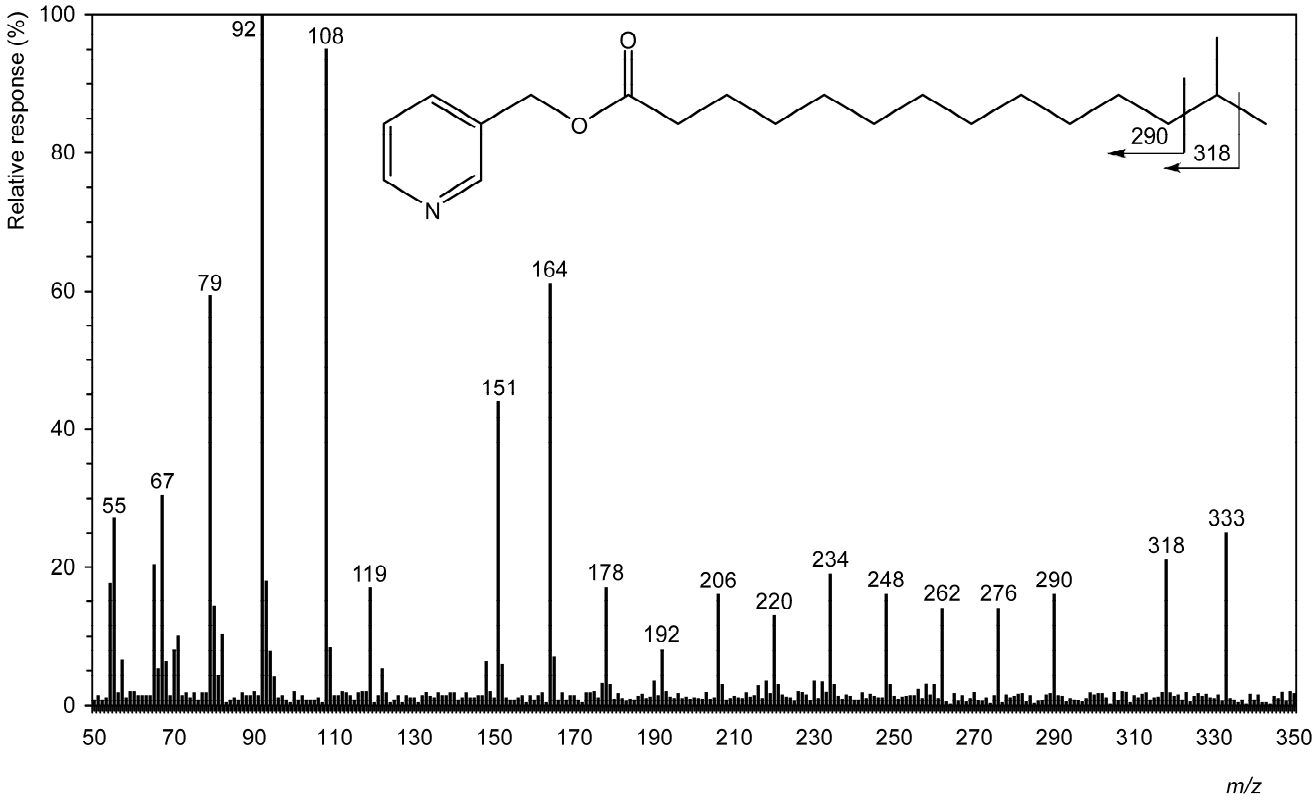

Fig. S2. Mass spectrum of 3-pyridylcarbinol esters of 12-methyltetradecanoate.

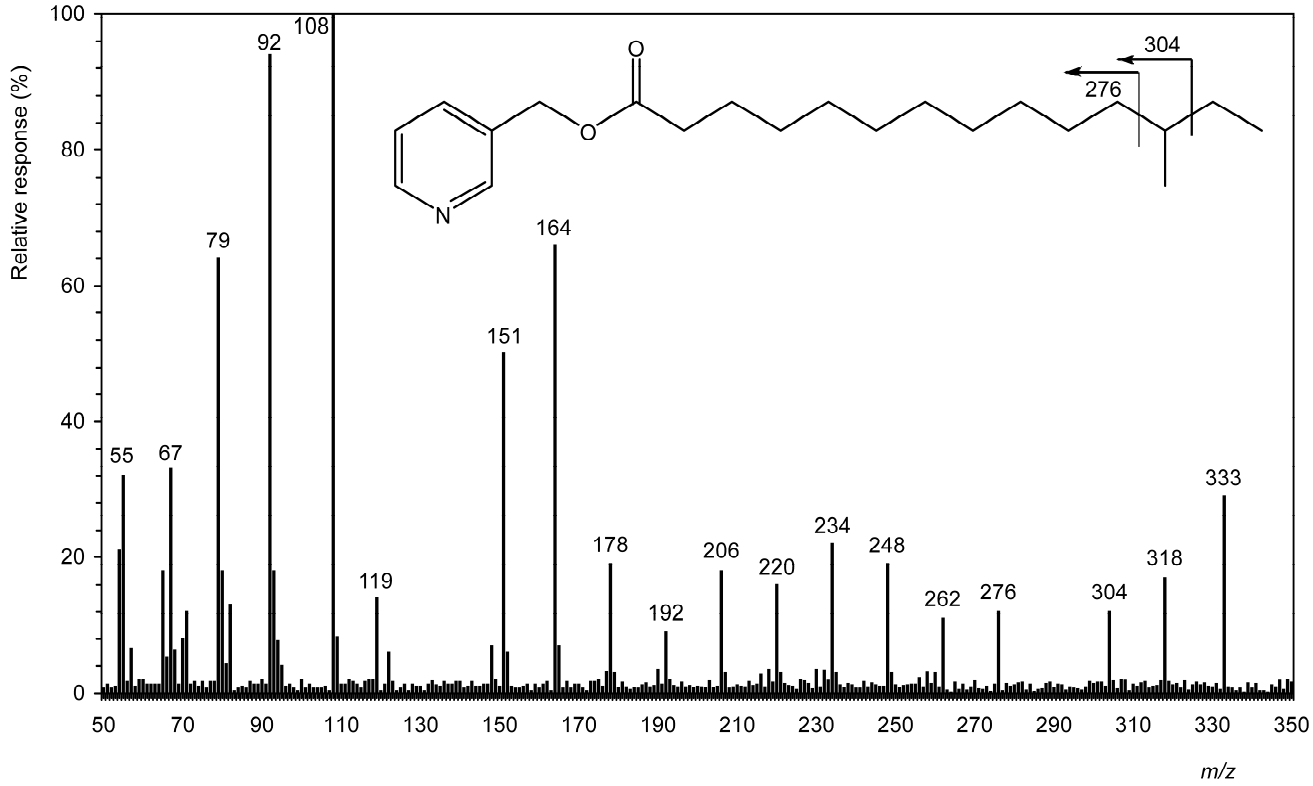

Fig. S3. Mass spectrum of unknown (U6), see Fig. 1.