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On-line two dimensional liquid chromatography/mass spectrometry for the analysis of triacylglycerides in peanut oil and mouse tissue

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ABSTRACT

Triacylglycerides (TAGs) are a large class of complex neutral lipids that naturally occur in both plants and animals. In the present work, an on-line comprehensive silver-ion liquid chromatography (silver-ion LC) × reversed-phase liquid chromatography (RPLC) system was constructed to analyze these compounds. A micro bore silver-ion modified column was employed in the first dimension with the commonly used hexane-based mobile phase. After a series of C18 columns were assessed, a wide bore column packed with 1.5 μm particles was selected as the second dimension column to reduce the negative effect caused by the large volume and strong solvent injection in the second dimension. The system coupled with mass spectrometry was applied to the analysis of an edible peanut oil and a mouse liver extract. Twenty-eight TAGs from the peanut oil and forty-four from the mouse liver were identified based on the TAGs' retention behaviors on the comprehensive two-dimensional LC system and their APCI MS fragments.

1. Introduction

Triacylglycerides (TAGs), consisting of the esterification of one glycerol molecule with three fatty acids, are a large class of complex neutral lipids that naturally occur in both plants and animals. While being the most common source of energy, fatty acids and some other nutrition for organisms [1], the TAG could cause metabolic syndromes such as obesity and diabetes when they were excessively accumulated [2–4]. A further investigation of TAGs' biological function needs more detailed information of individual TAG species and this calls for an effective separation method. However, the TAGs species are complex. The properties of TAGs depend on the number, position(s) and configuration (*cis*/*trans*) of the double bonds (DBs), the acyl chain lengths and stereochemical positions (sn-1, 2 and 3) of the fatty acids on the glycerol skeleton. Thus, the total number of TAG species is huge, making the determination of TAG composition in natural matrixes challenging [5].

Several strategies are applied to the investigation of TAG species. The colorimetric enzyme-linked methods using commercial available clinical kits are used to reveal the information about TAGs as a whole [4], while chromatographic methods including thin layer chromatography [6,7], supercritical fluid chromatography [8,9], gas chromatography [6,10] and high performance liquid chromatography (HPLC) [5,11–15] allow us to know more about the individual TAG species in matrix. The nonaqueous reversed-phase HPLC (RPLC) and the silver-ion HPLC (silver-ion LC) are most commonly used HPLC methods in the separation of TAG compounds. The considerable resolving power of HPLC in reversed-phase mode has been effectively applied to the separation of TAG species in natural oils and fats, the elution order is mainly based on partition numbers (PN) which equals to the total carbon number (CN) in the fatty acyl moieties minus two times of the double bond number (DB) in the same fatty acyl moieties (i.e. PN = CN − 2DB). The bottleneck of analyzing TAGs in complex mixtures by RPLC is the separation of those TAGs with the same PN (critical pair). On the other hand, the silver-ion LC is a quite effective option for such critical pairs. In the silver-ion chromatography, the silver ions can form reversible interactions with double bond electrons in organic compounds, such as TAGs [16,17]. The interaction varies according to the number, position and configuration of DBs. TAGs with more DB or DB in the *cis*-form which facilitate the form of the silver ion/double bond interaction are more retained [18,19]. Thus, the elution order is related to the degree of unsaturation of the compounds, and the separation of most of the TAG critical pairs is no longer an issue.

Although either of the two chromatographic techniques mentioned above can provide useful information on TAG profiles in lipid matrix, a more comprehensive analytical method is needed [20]. An elegant option is to combine the two chromatographic techniques together, either on-line or off-line [21–24]. Presently this multidimensional liquid chromatographic technique, especially the comprehensive two-dimensional liquid chromatography (LC × LC) is catching the attention of the modern analytical science [22,25–27]. Theoretically, the separation power of the LC × LC is

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closely connected with a nomenclature: "orthogonality". It means that two different mechanisms should be employed in the first and second dimension of a LC × LC system. Correspondingly, samples should bear at least two different properties which could be well separated by the two chromatographic techniques adopted by the LC × LC system respectively. TAGs are quite suitable for LC × LC analysis, since they hold two orthogonal properties. TAGs with different DB can be separated by the silver-ion column, while those with different PN are well resolved by the revered-phased LC [25,26]. Studies which employed the silver-ion × RPLC workflow to analyze TAGs from different sample sources have been reported. Dugo et al. [25,28] applied the silver-ion × RPLC system to investigate TAG compounds in soybean, linseed oils and the donkey milk fat. A micro-bore silver-ion column and a monolithic column were used in the first and second dimension, respectively. The mobile phases were the classical acetonitrile (ACN) – hexane system for silver-ion chromatography and ACN – isopropanol (IPA) for RPLC. 26 TAGs from the two plant oils and 57 TAGs from the milk fat (PN: 30–52, DB: 0–9) were obtained. van der Klift et al. [26] employed a different mobile phase system to convey the comprehensive analysis of TAGs. Gradient elution from pure methanol (MeOH) to 6% ACN in MeOH was employed in the first dimension, while for the second dimension the mobile phase was isocratic MeOH – methyl *tert*-butyl ether (70:30). In this case TAGs and oxygenated TAGs in corn oil were analyzed and 44 compounds were identified.

Mass spectrometry (MS) [29–32] was very suitable for studying TAGs because it provides structural information about the analytes and facilitates qualitative analysis. Atmospheric pressure chemical ionization (APCI) [29], electrospray ionization (ESI) [32], and atmospheric pressure photoionization (APPI) [33] were used to characterize triacylglycerides. Presently, the application of APCI is dominant as the TAGs are neutral compounds.

In the previous work of our group, a comprehensive two-dimensional hydrophilic interaction chromatography hyphenated with a quadrupole time-of-flight mass spectrometry (HILIC × HILIC-Q-TOF-MS) system was constructed and used to analyze an extract from *Quillaja saponaria* [34]. In the present work, a comprehensive silver-ion × RPLC system was developed to investigate TAGs. In order to get better separation efficiency and detection limitation, we optimized the second dimensional separation by comparing four C18-based columns of different dimensions. A wide bore column packed with 1.5 μm particles was selected as the second dimension column. Moreover, the orthogonality of the silver-ion LC and RPLC was evaluated. Finally, the developed system was applied to investigate TAGs in peanut oil and mouse liver.

2. Materials and methods

2.1. Reagents and samples

HPLC grade solvent hexane, acetonitrile (ACN), isopropanol (IPA), methanol (MeOH) and chloroform were purchased from Tedia (Fairfield, OH, USA). Pure standard compounds 1,2,3-trioctanoylglycerol (CCC0-24), tritetradecanoin (MMM0-42), trihexadecanoin (PPP0-48), triolein (OOO3-48), trihexadecenoin (PoPoPo3-42), tripetroselin (PePePe3-48), 1,2-dilinoleoyl-2-oleoyl-rac-glycerol (OLL5-44) and trioctadecadienoin (LLL6-42) were from J&K Chemical Limited (Beijing, China). All standards were dissolved in hexane.

The peanut oil was purchased from a local supermarket and was diluted by hexane at a ratio of 1:40 (for one-dimensional analysis) and 1:20 (for LC × LC analysis) before analysis. TAGs from mouse liver were extracted using the Bligh and Dyer's method [2,35] with some modifications. Briefly, the mouse liver stored at −80 °C was freeze-dried by liquid nitrogen and crushed to fine particles in a mortar. About 200 mg liver powder was weighted, sonicated with 6 mL chloroform/methanol (1:2) and incubated at room temperature for 1 h. Then 2 mL chloroform and 2 mL water were added. The mixture was sonicated for 1 min both before and after adding water. Finally, a fine phase separation was achieved after centrifugation (2600 × *g* for 10 min). The lower phase was recovered by a pipette and dried under gentle nitrogen. The residues were stored at −20 °C and reconstructed in 150 μL hexane before analysis.

2.2. Instruments and chromatographic conditions

Two sets of Shimadzu LC-20AB Prominence HPLC system (Shimadzu, Kyoto, Japan) were used to construct the two-dimensional LC system. The interface was a two-position ten-port electrical valve (7725i, Rheodyne, Rohnert Park, CA, USA), equipped with two 20-μL loops. Each Shimadzu LC-20AB Prominence HPLC system consists of a binary pump, a mixing chamber, a degasser and a column compartment. The two-dimensional LC system was controlled by a CBM-20 Alite controller (Shimadzu, Kyoto, Japan) and triggered by a manual injector (Rheodyne 7725i, Rheodyne, Rohnert Park, CA, USA). The schematic workflow was the same as our previous work [34]. Briefly, the interface collects the fractions from the first dimension and injects them onto the second column for rapid separations simultaneously and alternatively. For one position of the valve, one loop (20 μL) collects the effluents from the first column (10 μL/min) and the loop is filled in 2 min. Meanwhile, another loop loads its collection onto the second column for a rapid separation. Then the valve switches and the two loops change their roles in collecting and loading. Thus, the 2-position 10-port valve switches every 2 min and this process continues during the whole LC × LC analysis.

The first-dimensional separation was performed on a micro-bore TSK gel SP-2SW column (150 mm × 1 mm i.d., 5 μm particle size). The column was from Tosoh (Shanghai, China) and was converted to silver-ion form by flushing 1 mol/L $AgNO_3$ aqueous solution as proposed by Christie [36]. Hexane modified by minor ACN (adding 0–2% ACN in hexane) were used as mobile phase to conduct the gradient elution at a flow rate of 10 μL/min. ACN and IPA or IPA/hexane mixture were the mobile phases for the second dimensional analyses. The effluent was split and about a quarter was infused into the APCI MS for detection.

2.2.1. LC × LC analysis of standards

The chromatographic optimization of each dimension was performed in the conventional one dimensional form using one set of Shimadzu LC-20AB Prominence HPLC system. A mixture consisting of 8 TAG standards was used as test compounds. For the one dimensional silver-ion chromatography, hexane (A) and 0.6% ACN in hexane (B) were used as mobile phase and the flow rate was 50 μL/min. The gradient elution was initially kept at 40% B for 3 min, and then linearly increased to 85% B over a period of 5 min; it was maintained for 17 min. Next, the gradient was returned to 40% B in 2 min. As for the one dimensional RPLC analysis, ACN and IPA were used as eluents at the flow rate of 4 mL/min. The separation took 2 min and was performed in gradient: IPA was increased from 20% to 85% in the first 0.6 min, kept for 0.3 min, and then decreased to 20% at 1.0 min.

In the LC × LC analysis of the TAG standard mixture, the first dimension employed the same mobile phases as mentioned above at a flow rate of 10 μL/min. The mobile phase was 40% B for the first 20 min and then increased to 85% B at 119 min, and was kept to the end of the analysis. The second dimension separation was identical

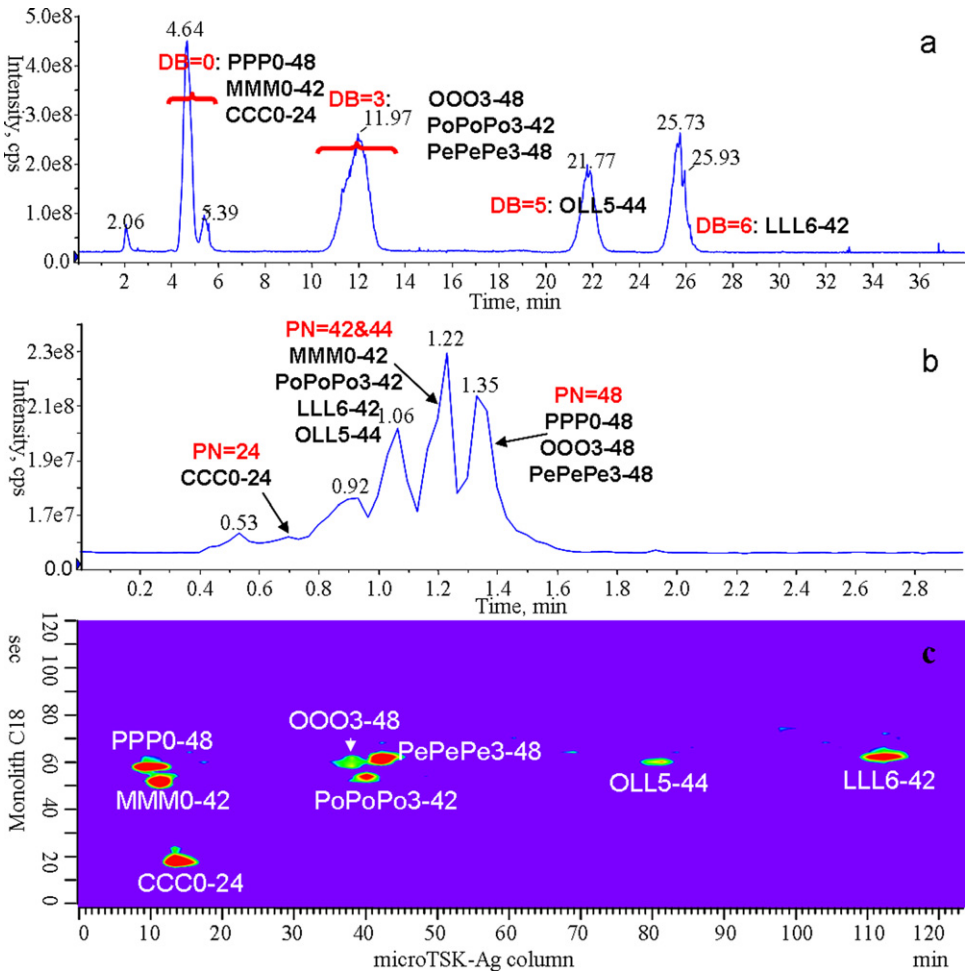

Fig. 1. Total ion chromatograms of TAG standards on the individual microTSK silver-ion column (a) and the individual monolithic RP C18 column (b), respectively. (c) Contour plot of silver-ion × RPLC separation of TAG standards. For detailed experimental conditions, see Section 2.2.1.

to the aforementioned one dimensional reversed-phase analysis. The injection volume was 2 μL.

2.2.2. LC × LC analysis of real samples

Several reversed-phase columns were tested for the second dimensional analysis of the real sample: A Chromolith™ Performance RP-18e monolithic column (100 mm × 4.6 mm i.d., Merck, Darmstadt, Germany), a Hypersil ODS2 column (50 mm × 4.6 mm, 5 μm, Elite, Dalian, China), a fused-core particles packed column Halo™ C18 (50 mm × 4.6 mm i.d., 2.7 μm, Advanced Materials Technology Inc., USA) and a Platinum™ EPS C18 (33 mm × 7 mm, i.d., 1.5 μm, Alltech Associates Inc., USA). And the EPS C18 column was used to carry out the second separation in the comprehensive 2D LC analysis of the real samples. Since the real samples were more complex than the standards, the chromatographic conditions were slightly different from that of the standards to get a better separation.

For the peanut oil, the mobile phases used in the first dimension were 0.3% ACN in hexane (A) and 1.3% ACN in hexane (B). And for the second dimension, the solvents were ACN (C) and IPA: hexane (v/v, 2:1) (D). The injection volume was 2 μL. The gradient elution of the first dimension was as follows: the elution B was 10% at the beginning, and then increased to 35% at 40 min. Next, it was increased to 70% 5 min later and held to the end. The second dimension separation was isocratic elution with 25% D at a flow rate of 4 mL/min.

When analyzing the mouse liver, the first dimension separation was an isocratic elution using 0.8% ACN in hexane as effluent and the optimized condition for the second dimension was a series of 2-min isocratic segments, 30% IPA in ACN eluted at 3 mL/min. The injection volume was 10 μL.

2.3. Data acquisition and treatment

A Q-TRAP LC/MS/MS system (Applied Biosystems/MDS SCIEX, USA) and a Waters Q-TOF micro (Waters MS Technologies, Manchester, UK) were used for mass spectrometric detection, both equipped with an APCI ion source. For Q-TRAP, the current of the corona discharge needle was set at 3 μA and the source temperature was 425 °C. Nitrogen was used as curtain gas (40 psi), auxiliary gas (50 psi) and nebulizer gas (75 psi). The ion source and ion optic parameters were optimized by standard TAGs. The other parameters were as follows: scan type, EMS (enhanced mass scan); mass range (m/z), 350–1000; scan mode, positive; and scan rate, 4000 amu/s. For Q-TOF micro, the current of the corona discharge needle was 5 μA, the source temperature and the APCI probe temperature were 130 °C and 450 °C, respectively. Nitrogen was used as the desolvation gas (350 L/h) and cone gas (50 L/h). The collision energy was 3 eV. The MCP detector voltage was set to 2500 V. The Q-TOF micro MS was operated in positive scanning mass range of 200–1000 m/z and the acquisition rate was set to 0.48 s with a 0.1 interscan delay.

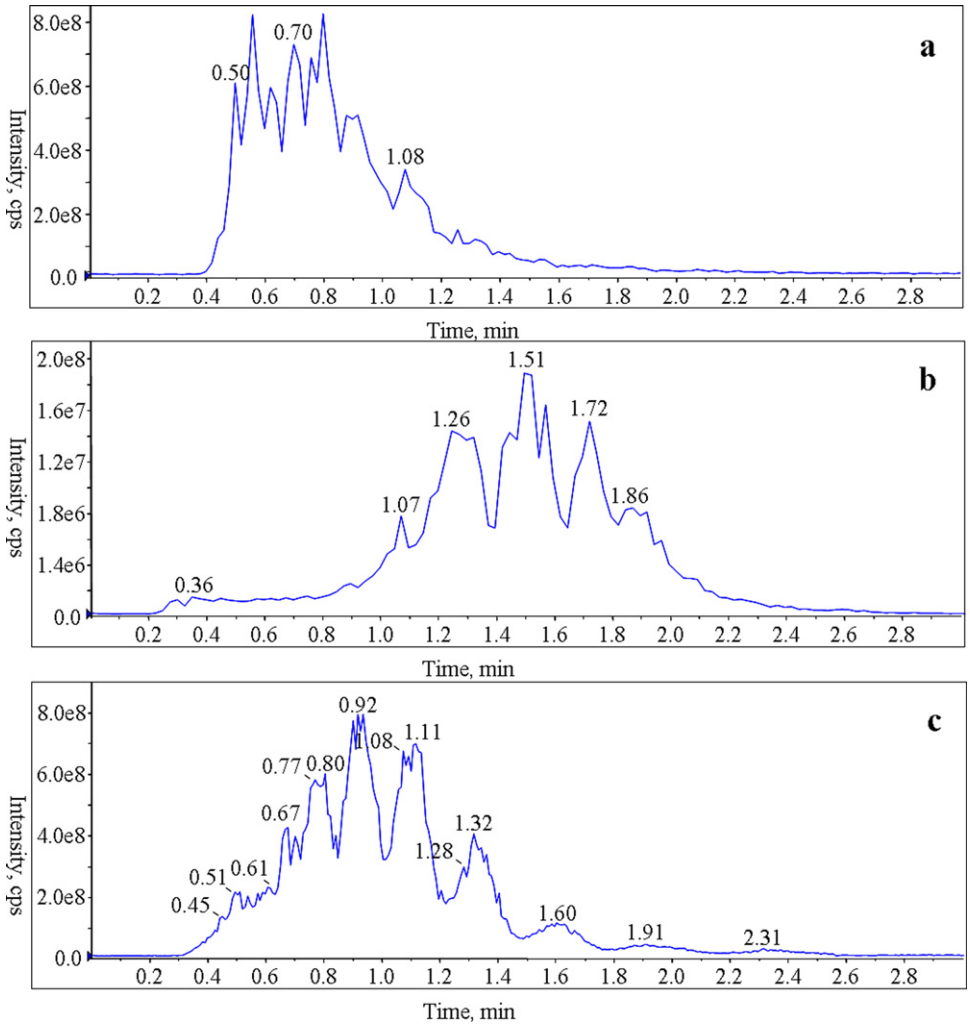

Fig. 2. Fast RP analysis of TAG species in peanut oil on different columns: (a) monolith column RP18e, the elution was 25% ACN in IPA; (b) Elite ODS2, the elution was from 30% ACN in IPA at the beginning to 70% ACN at 1.4 min, then decreased to 30% at 1.8 min and kept to the end (3 min); and (c) Alltech EPS C18, the elution was 25% ACN in IPA/Hexane (2/1). For other parameters, see Table 1.

The software Transform (Ver. 3.4, Noesys Software Package, Research Systems International, Crowthorne, UK) was used for data processing.

3. Results and discussion

3.1. Orthogonality of the silver-ion and RPLC system

In LC × LC system, the orthogonality of the two dimensions is an important issue. As the orthogonality increases, the peak capacity of the LC × LC system is improved and the separation power of the system approaches to the utmost. To achieve a higher orthogonality, the argentation and the reversed-phase chromatographic techniques with distinct mechanisms are usually employed in the comprehensive two dimensional LC. In our work, a silver-ion modified micro-bore TSK column and a monolithic column (C18-based) were selected to evaluate the orthogonality of silver-ion LC and RPLC. Retention times of eight TAG standards on the two columns were measured, respectively. It was found that the relative orthogonality of this system was 52.9% calculated by the reported method [37]. Considered that the relative orthogonality of an ideal orthogonal 2D system was only 84.13% [37], 52.9% indicated that the silver-ion LC and the RP C18-LC were of moderate orthogonality.

The resolution of the silver-ion × RPLC system was also evaluated by TAG standards. The resulting two dimensional contour plot and one-dimensional chromatograms are shown in Fig. 1. As depicted in the contour plot, the standards were well separated horizontally according to the number of DBs on the first silver-ion column and vertically according to that of PNs on the second RPLC column.

3.2. Optimization of the second dimension

To achieve a better comprehensive separation, four RPLC columns including a monolithic column, a Hypersil ODS2 column, a Halo^{TM} C18 and a Platinum^{TM} EPS C18, were tested as the second column. The peak capacity and the dilution to the sample of each column were calculated and used to evaluate their separation efficiency. The dilution factors were calculated according to the method proposed by Schure [38] and some parameters recommended therein were also adopted. The number of column plates (N_{sep}) was calculated using the data in Fig. 2. All the parameters and the results were summarized in Table 1.

Of the four candidate columns, the monolithic column was a good option for fast separation and was used more often as the second column in LC × LC analyses in previous publications [25,28]. However, this column gave a lower peak capacity as shown in

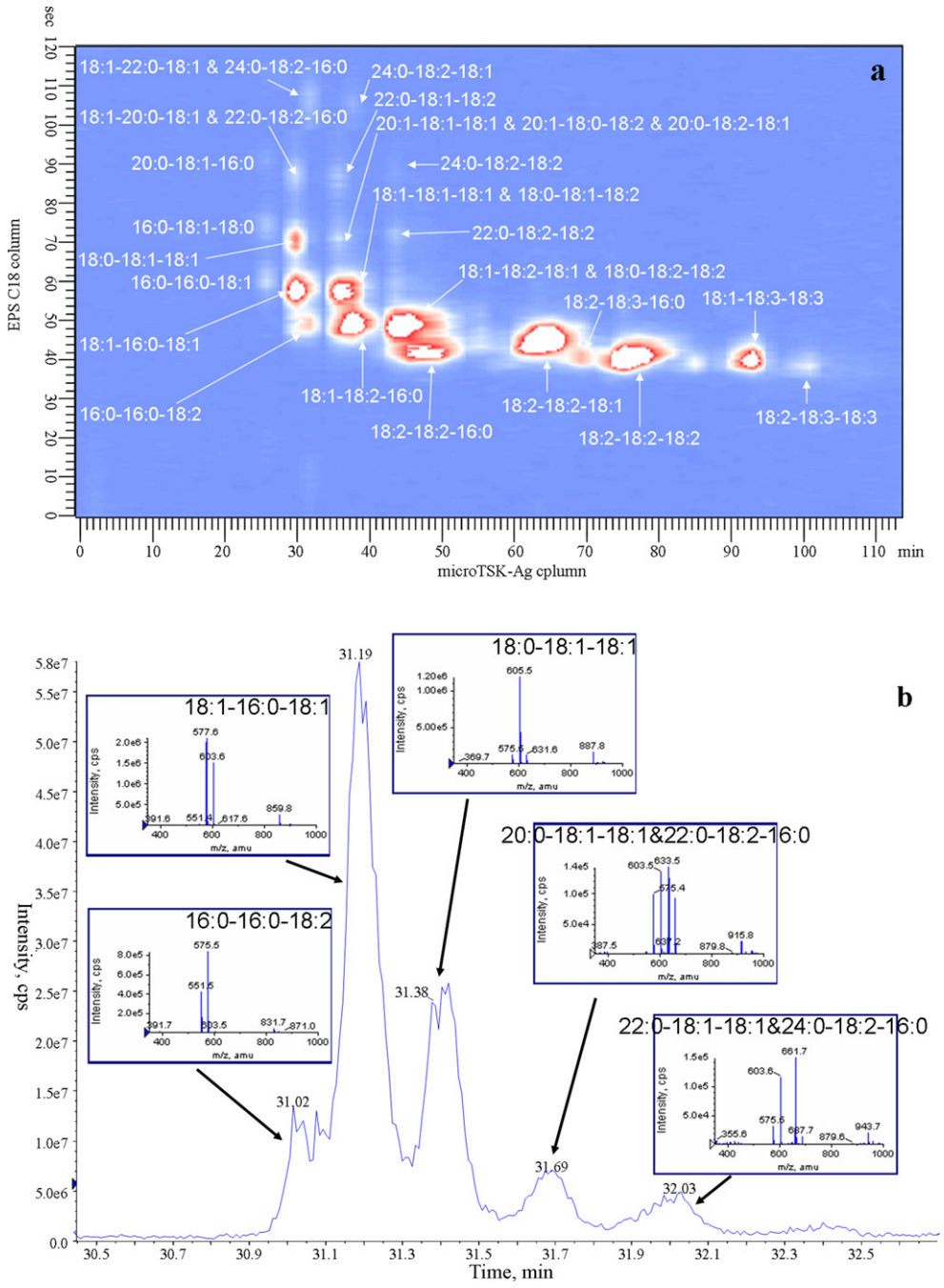

Fig. 3. (a) Contour plot of comprehensive two-dimensional silver-ion × RPLC separation of peanut oil. (b) A slice of expanded raw chromatogram of the comprehensive two-dimensional LC analysis of peanut oil. TAGs were denoted by the three fatty acids linked to the glycerol backbone and their positions did not represent the stereochemical positions.

Table 1
Comparison of different C18 columns.

	Monolith C18	Hypersil ODS2	Halo C18	EPS C18
Column length (cm)	10	5	5	3.3
Column diameter (mm)	4.6	4.6	4.6	7
Particle size (μm)	–	5	2.7 (shell)	1.5
Flow rate (mL/min)	4	4	2.5	4
Injection volume (μL)	20	30	1–2	20
Analysis time (min)	2	3	2	2
Retention volume (mL)	3.99	1.99	–	3.05
N_{sep}	167	639	–	937
Peak capacity	4	4	–	8
Dilution factor[a]	38.69	6.57	–	12.48

[a] The dilution factor $f = V_{dil}/V_{inj} = \varepsilon_T \pi d_c^2 (1 + k')L\sqrt{2\pi}/4V_{inj}\sqrt{N}$, where V_{dil} and V_{inj} are the final diluted volume and the inject volume of the sample, k' is the capacity factor of the analyte, N, ε_T, d_c and L are the plate number, total interstitial, internal diameter and length of the column [36].

Table 2
TAGs in mouse liver analyzed by comprehensive silver-ion × RP LC chromatography.

No.	DB-PN	$[M+H]^+/[M+NH_4]^+$	TAG	DAG ions		
1	1–46	805.83/822.87	14:0–16:0–18:1	14:0–16:0 523.54	14:0–18:1 549.55	16:0–18:1 577.59
2	1–48	833.89/850.92	16:0–16:0–18:1	16:0–16:0 551.57	16:0–18:1 577.59	
3	1–50	861.93/878.96	16:0–18:0–18:1	16:0–18:0 579.60	16:0–18:1 577.59	18:0–18:1 605.62
4			16:0–16:0–20:1	16:0–16:0 551.57	16:0–20:1 605.62	
5	2–44	803.85/820.97	14:0–16:0–18:2	14:0–16:0 523.55	14:0–18:2 547.55	16:0–18:2 575.58
6	2–46	831.89/848.92	16:0–16:0–18:2	16:0–16:0 551.58	16:0–18:2 575.58	
7			16:0–16:1–18:1	16:0–16:1 549.56	16:1–18:1 575.58	16:0–18:1 577.58
8	2–48	859.92/876.95	16:1–18:0–18:1	16:1–18:0 577.58	16:0–18:2 575.58	18:0–18:2 603.61
9			16:0–18:1–18:1	16:0–18:1 577.58	18:1–18:1 603.61	
10			16:0–18:0–18:2	16:0–18:0 579.61	16:0–18:2 575.58	18:0–18:2 603.61
11	2–50	887.96/904.99	18:0–18:1–18:1	18:0–18:1 605.62	18:1–18:1 603.61	
12	3–44	829.86/846.89	16:1–16:1–18:1	16:1–16:1 547.54	16:1–18:1 575.57	
13			16:0–16:1–18:2	16:0–16:1 549.56	16:0–18:2 575.57	16:1–18:2 573.56
14			14:0–18:1–18:2	14:0–18:1 549.56	14:0–18:2 547.54	18:1–18:2 601.59
15	3–46	857.90/874.93	16:1–18:1–18:1	16:1–18:1 575.57	18:1–18:1 603.60	
16			16:0–18:1–18:2	16:0–18:1 577.59	16:0–18:2 575.57	18:1–18:2 601.59
17	3–48	885.94/902.97	18:0–18:1–18:2	18:0–18:1 605.62	18:0–18:2 603.60	18:1–18:2 601.59
18			18:1–18:1–18:1	18:1–18:1 603.60		
19			16:0–18:1–20:2	16:0–18:1 577.59	16:0–20:2 603.60	18:1–20:2 629.63
20	4–42	827.97/844.97	14:0–18:2–18:2	14:0–18:2 547.56	18:2–18:2 599.59	
21	4–44	855.95/872.97	16:0–18:2–18:2	16:0–18:2 575.58	18:2–18:2 599.59	
22	4–46	884.05/901.10	18:0–18:2–18:2	18:0–18:2 603.62	18:2–18:2 599.59	
23			18:1–18:1–18:2	18:1–18:1 603.62	18:1–18:2 601.60	
24	4–48	911.97/928.99	18:1–18:1–20:2	18:1–18:1 603.62	18:1–20:2 629.63	
25	5–42	853.98/871.07	16:0–18:2–18:3	16:0–18:2 575.64	16:0–18:3 573.69	18:2–18:3 597.65
26			16:1–18:2–18:2	16:1–18:2 573.62	18:2–18:2 599.64	
27	5–44	881.98/899.00	18:1–18:2–18:2	18:1–18:2 601.66	18:2–18:2 599.67	
28			16:0–18:1–20:4	16:0–18:1 577.64	16:0–20:4 599.62	18:1–20:4 625.63
29	5–46	909.96/926.99	16:0–18:1–22:4	16:0–18:1 577.60	16:0–22:4 627.61	18:1–22:4 653.65
30	6–42	879.91/-	16:0–18:2–20:4	16:0–18:2 575.59	16:0–20:4 599.59	18:2–20:4 623.59
31			18:2–18:2–18:2	18:2–18:2 599.59		
32	6–44	907.98/925.02	16:0–18:2–22:4	16:0–18:2 575.59	18:2–22:4 651.63	
33			16:0–18:1–22:5	16:0–18:1 577.60	16:0–22:5 625.60	18:1–22:5 651.63
34	7–40	877.99/894.98	16:0–18:2–20:5	16:0–18:2 575.64	16:0–20:5 597.60	18:2–20:5 621.65
35			18:2–18:2–18:3	18:2–18:2 599.62	18:2–18:3 597.60	
36	7–42	905.93/922.96	16:0–18:2–22:5	16:0–18:2 575.65	18:2–22:5 649.66	
37			18:1–18:2–20:4	18:1–18:2 601.68		
38			16:0–18:1–22:6	16:0–18:1 577.65		
39	8–40	903.99/921.01	16:0–18:2–22:6	16:0–18:2 575.64	16:0–22:6 623.65	18:2–22:6 647.66
40			18:2–18:2–20:4	18:2–18:2 599.61	18:2–20:4 623.67	
41	8–42	931.97/-	18:1–18:1–22:6	18:1–18:1 603.69	18:1–22:6 649.64	
42	9–38	901.99/-	18:2–18:2–20:5	18:2–18:2 599.60	18:2–20:5 621.56	
43			16:1–18:2–22:6	16:1–18:2 573.58	16:1–22:6 621.56	18:2–22:6 647.66
44	9–40	929.98/947.07	18:1–18:2–22:6	18:1–18:2 601.65	18:1–22:6 649.67	18:2–22:6 647.65

Table 1. Besides, we have found that a large volume of injection (i.e. 20 μL fraction transferred from the first column in the case of LC × LC) on the column introduced noticeable peak distortion as shown in Fig. 2a.

Next, three particle packed columns were tested as they could produce higher peak capacity theoretically. The analysis using the conventional column packed with hypersil ODS2 could not accomplish the separation within 2 min (Fig. 2b). Thus, the modulation time was prolonged to 3 min and the injection volume into the second dimension was increased to 30 μL. As a rule of thumb, in the LC × LC analysis every first dimensional peak should be sampled to the second dimension at least three times to avoid the incomplete sampling of the first dimension [39]. So in the case of this hypersil ODS2 column, incomplete sampling easily occurred and it would reduce the resolution of multidimensional analysis. Alternatively, to increase the sampling rate of the first dimension, we could decrease its flow rate but it would increase the overall analysis time which we were reluctant to do. Furthermore, the larger injection volume (30 μL) and higher flow rate (4 mL/min) applied to the column made the separation worse and led to lower peak capacity (Table 1). For the core-fused Halo^{TM} C18, a fast separation was feasible within 2 min with a lower flow rate (2.5 mL/min). But the separation was acceptable only with a small injection volume (1–2 μL). The peaks were severely deteriorated and crushed to one peak when the injection volume increased to 20 μL. It might be due to the low column capacity of this core-fused column, and the large volume injection in strong solvent ever exacerbates this issue. Though the use of the core-shell column in the second dimension has been reported in several LC × LC studies [40–42], it did not work in our case. Table 1 and Fig. 2c demonstrated that EPS C18 column gave a superior result with the higher peak capacity and better column efficiency compared with the other three columns. It was because the column had a larger inner diameter (I.D. 7 mm) and was packed with 1.5 μm particles, which made it more suitable for sustaining a high speed (3–4 mL/min) flush and a large injection volume (20 μL). However, its dilution to the fractions from the first column was a little higher as shown in Table 1. As a compromise, the EPS C18 column was selected as the second column in present LC × LC setup.

3.3. Applications of the LC × LC system in real sample analyses

The comprehensive silver-ion × RPLC/APCI MS was applied to an edible peanut oil. Twenty-eight TAGs have been identified based on the TAGs' retention behaviors on both columns and their APCI MS fragments, which were labeled on the contour plot (Fig. 3a).

It is easily understandable that the compounds aligned vertically or horizontally on the 2D contour plot would be co-eluted if the conventional one-dimensional analysis were performed. For example, 16:0–16:0–18:2, 18:1–16:0–18:1, 18:0–18:1–18:1,

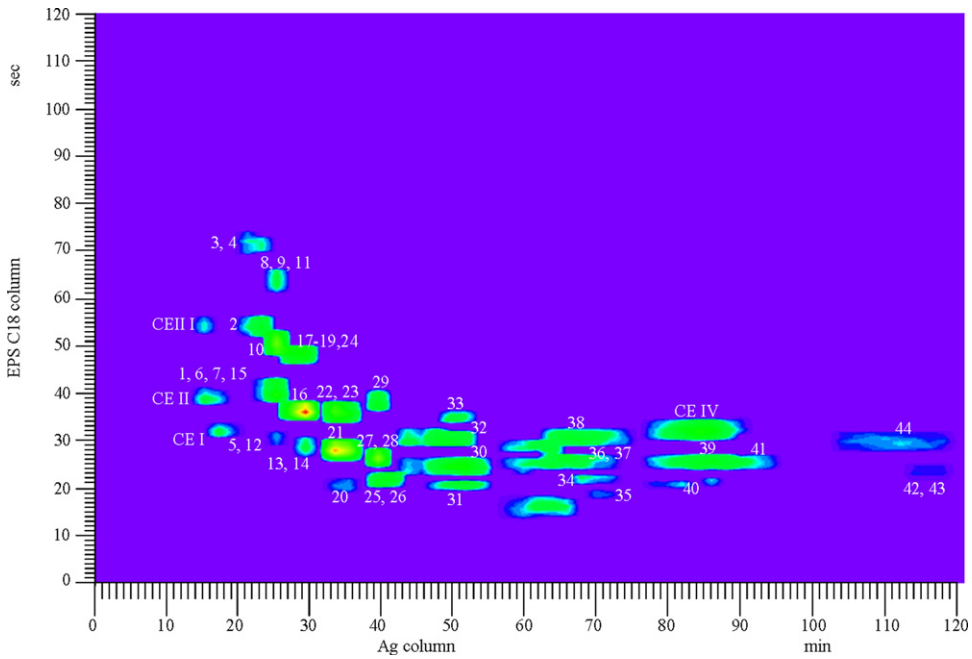

Fig. 4. Reconstructed contour plot of comprehensive two-dimensional silver-ion × RPLC separation of mouse liver extract. For TAG species of the denoted numbers, see Table 2.

20:0–18:1–18:1, 22:0–18:2–16:0, 22:0–18:1–18:1 and 24:0–18:2–16:0 have two unsaturated centers in their molecules, so they tend to be co-eluted in the silver-ion column. When applying the LC × LC separation system, the co-eluted fractions were subjected to a second dimension, and then were well separated based on the individual PNs of the coeluents. As a result, five peaks which were almost baseline separated were obtained (Fig. 3b).

The liver tissue is an important complex sample containing a large class of TAG compounds. To the best of our knowledge, TAGs in animal tissues analyzed by LC × LC have not been reported yet. Here we employed the developed LC × LC system in the investigation of TAG species in mouse liver using the Q-TOF APCI MS as the detector. As there were not enough commercially available standards for TAGs, exact match of the retention times between standards and the sample was not used for identification of TAGs. In our work, the identification was based on TAGs' chromatographic and mass spectrometric behaviors, and the same approach was also used for identifying TAGs in peanut oil. On the LC × LC chromatogram, TAGs were separated with increasing DBs on Ag^+-LC, and those coeluted on the Ag^+-LC were further separated according to their PNs on RP LC. This was very useful in pinning down the individual TAG with specific DB and PN. Besides, MS data of TAGs played an important role in the identification. Generally speaking, the APCI-MS spectra of TAGs mainly displayed three sections of fragmentation/molecular *m*/*z* in the low ($[RCO]^+$), medium ($[M+H-RCOOH]^+$) and high ($[M+X]^+$, X = H, NH_4, etc.) mass regions. The intensities of ions in the high *m*/*z* region were related to DBs in the molecule, which was in accordance with previous publications [43,44]. TAGs were identified based on the diacylglycerol fragment ions ($[M+H-RCOOH]^+$), ammonium adduct ions $[M+NH_4]^+$ or pseudomolecule ions $[M+H]^+$ and the corresponding chromatographic behaviors on both dimensions. The identified results are depicted in Table 2 and Fig. 4. Forty-four TAGs with the DB ranged from 1 to 9 and PN from 38 to 50, as well as 4 cholesteryl esters (CEs), were identified from the mouse liver. The four CEs (denoted as CEI-IV in Fig. 4) were assigned by their characteristic ion (*m*/*z* 369.43) [45] but their specific structures were not identified as there was not sufficient MS information. The TAG molecules were denoted by the fatty acids (FAs) and the orders of the FAs here did not represent their stereochemical position as this required an exact match of standards or the $[M+H-RCOOH]^+$ ion intensity ratios of standards' [46].

As the LC × LC technique provides higher resolution, it can facilitate not only credible identification but also precise quantification in further work. As shown in Fig. 5, a pair of TAG isomers was separated by the present LC × LC system. Two peaks in the

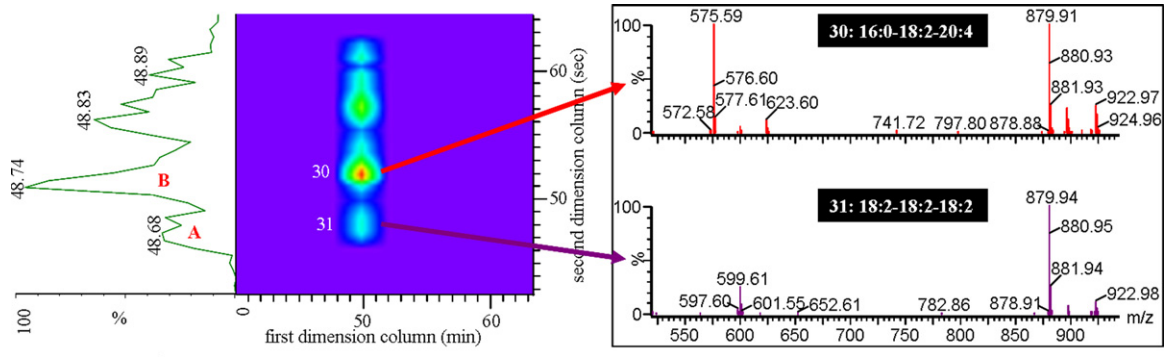

Fig. 5. Contour plot of two TAG isomers from the mouse liver and the corresponding MS spectra.

chromatogram show the same $[M+H]^+$ ions (m/z 879) in their MS spectra. In the MS spectra of peak A, there was a single $[M+H\text{-}RCOOH]^+$ ion with m/z 599 in the medium m/z region. The explanation for this was that the fatty acids of this TAG were probably the same. It was not difficult to deduce that the diacylglycerol fragment ion was $[18:2–18:2]^+$ based on the value of the m/z, and finally A was assigned as 18:2–18:2–18:2. The same procedure was done with peak B, and it was identified as 16:0–18:2–20:4 according to three diacylglycerol fragment ions: $[16:0–18:2]^+$ (m/z: 575), $[18:2–20:4]^+$ (m/z: 623) and $[16:0–20:4]^+$ (m/z: 599).

In the online LC × LC analysis, the reconstructed average peak width on the first dimension was about 5 min and then the peak capacity of a 100-min first dimensional analysis time was 20. In the second dimension, the average peak width was about 0.15 min and the peak capacity of this dimension was 13 in a 2 min separation. Thus the theoretical peak capacity of the present two-dimensional LC system was 260. Since hyphenation with MS could improve the peak capacity by at least 5 fold [47], this system could have a total peak capacity up to 1300. This is very useful for complex sample separation and analysis.

4. Conclusions

In this study, a comprehensive silver-ion × RPLC system was established for the separation of TAGs in complex samples. To satisfy the requirements of a fast separation and a large volume injection of the second dimension, a new wide bore and short C18 column packed with 1.5 μm particles was used as the second column to construct the LC × LC system. As a result, the resolution of the second dimension and the peak capacity of the whole LC × LC system were improved. The high resolution mass spectrometer as the detector significantly improved the total peak capacity by adding a third dimension of separation. TAG species from plant oil and animal tissue were well resolved by the present system. According to their diacylglycerol fragment ions ($[M+H\text{-}RCOOH]^+$), ammonium adduct ions $[M+NH_4]^+$ or pseudomolecule ions $[M+H]^+$ and retention behaviors on two-dimensions, 28 TAGs from the peanut oil and 44 TAGs from the mouse liver have been identified.

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