METHOD IMPROVED FOR THE SEPARATION OF NEUTRAL LIPIDS VIA HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING EVAPORATIVE LIGHT-SCATTERING DETECTION: ANALYSIS OF PLANT OILS AND FLOURS

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ABSTRACT

A method aimed at the analysis of Neutral Lipids (NL) classes by HPLC-ELSD was improved in this research effort. In preliminary attempts, separation of the same Sterol Ester (SE) into more than one peak was obtained; however, when a washing procedure between runs was introduced and a more polar mobile phase was used, SE has eluted as a single peak.

Two silica columns (100 x 2.0 mm, 3 µm) and a similar guard column, mounted in series, were employed. The mobile phase was prepared as a binary gradient, and consisted of hexane and hexane:MTBE:AcOH (20:80:1 v/v/v). All lipids but MAG were eluted at the (optimized) flow-rate of 0.1 ml/min; total run time was 76 min, at room temperature. Elution with hexane for 58 min between runs was critical to equilibrate the column.

The method thus developed is highly reproducible, and produces a stable baseline separation in the following order: SE, high-molecular weight triacylglycerols (HMW-TAG), free fatty acids (FFA), low-molecular weight triacylglycerols (LMW-TAG), diacylglycerols (DAG), free sterols (S) and monoacylglycerols (MAG). Good performance was demonstrated for a wide concentration range of edible oils, as well as maize and rye flours. Furthermore, additional studies indicated relatively good separation of TAG with the same degree of unsaturation but different number of acyl carbons in the acyl carbon number (ACN) range 12-42, and partial separation of TAG with the same chain length but different degree of unsaturation; positional isomers of DAG were separated to the baseline; and DAG, FFA and MAG with distinct chain lengths were only partially separated.

The technique described here is being employed for the qualitative and quantitative investigation of the NL classes in flours and their baking products, after previous separation of the glyco- and phospholipids and with proper calibration curves.

EXPERIMENTAL METHODS

HPLC system

LC 1090, Hewlett Packard

Columns

- 2 Phenomenex Luna 3u silica gel columns (100x2.0 mm, 3 µm) in series
- Guard column (4x2.0mm)

ELSD system

SEDEX 55,T: 52ºC, Voltage: 600 V, Gas pressure (air): 1 bar

Elution Program for the binary gradient system

Time (min)	% *B*	Flow (ml/min)
0	0	0.1
2	7.5	
16	7.5	
22	9	
30	9	
31	45	
52	45	
62	90	
63	99	
64	99	0.5
75	99	
76	0	

Solvent *A* = Hexane
Solvent *B* = Hexane:MTBE:AcOH (60:40:1, V/V/V)

LC-MS SYSTEM

LC
Hewlett Packard LC 1100
External pump
Reagent solvent: chloroform/methanol/ammonia water (25%) 20:10:3 (v/v)
Pump: Waters 510 HPLC Pump
Flow rate: 6 ml/min via 1:100 split device to the effluent flow
MS
Bruker Esquire LC-MS, ESI Pos mode, Capillary voltage: 3000 V
Capillary exit offset: 10 V, Skimmer potential: 20 V
Trap drive values: 70 for SE, TAG, DAG and S; 40 for FFA and MAG
ESI mass spectra range: 50-1000 m/z, summation: 15 spectra
Nebulizer gas: N_2, P = 40 psi, Dry gas: N_2, 8 l/min, P = 40 psi, T = 300ºC
MS/MS
Collision gas: He (99.996%)

ABBREVIATIONS

AcOH, acetic acid; **ACN**, acyl carbon number; **C**, cholesterol; **CE**, cholesterol ester; **DAG**, diacylglycerol; **EIC**, extracted ion chromatogram; **ESI**, electrospray ionization; **FA,** fatty acid **FFA**, free fatty acid; **HPLC-ELSD,** high-performance liquid chromatography with evaporative light scattering detector; **MAG**, monoacylglycerol; **MS**, mass spectometry; **MTBE**, methyl-tert-butyl ether; **NL**, neutral lipids; **S**, sterol; **SE**, sterol ester; **TAG**, triacylglycerol

RESULTS

Improvement of SE elution

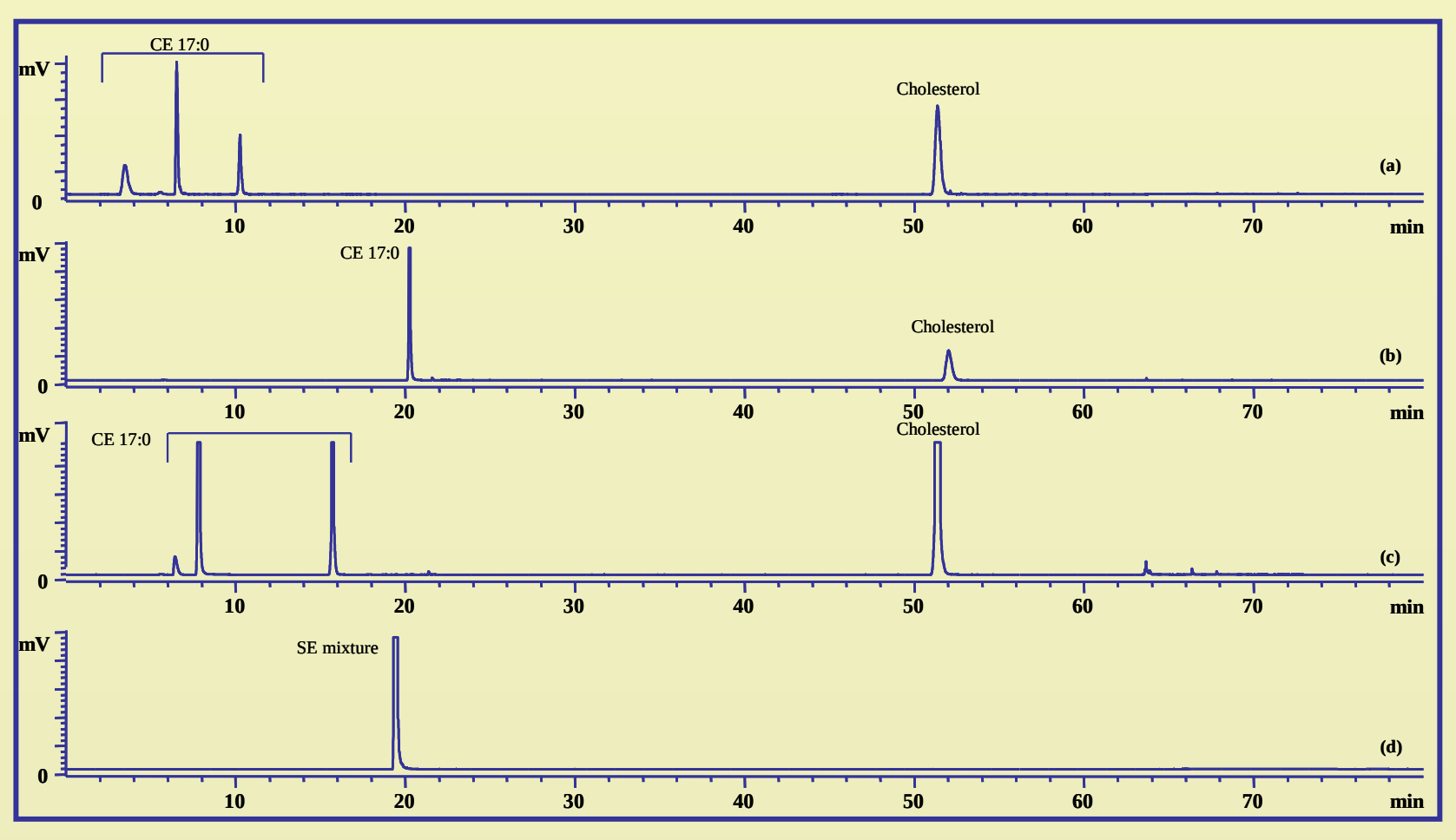

Fig1. Development of a suitable elution program in order to obtain a single peak elution for the SE and identification thereof. **(a)** Run with a preliminary programme and before the wash procedure; **(b)** Run with a preliminary programme and after a wash procedure; **(c)** Run with a preliminary programm; and **(d)** Run with the final method. Mix (SE): CE 4:0 + CE 8:0 + CE 12:0 + CE 13:0 + CE 16:0 + CE 16:1 + CE 17:0 + CE 18:0 + CE 18:1 + CE 18:2 + CE 18:3.

HPLC-ELSD & LC-MS of NL Standards, Maize Flour and Plant Oils

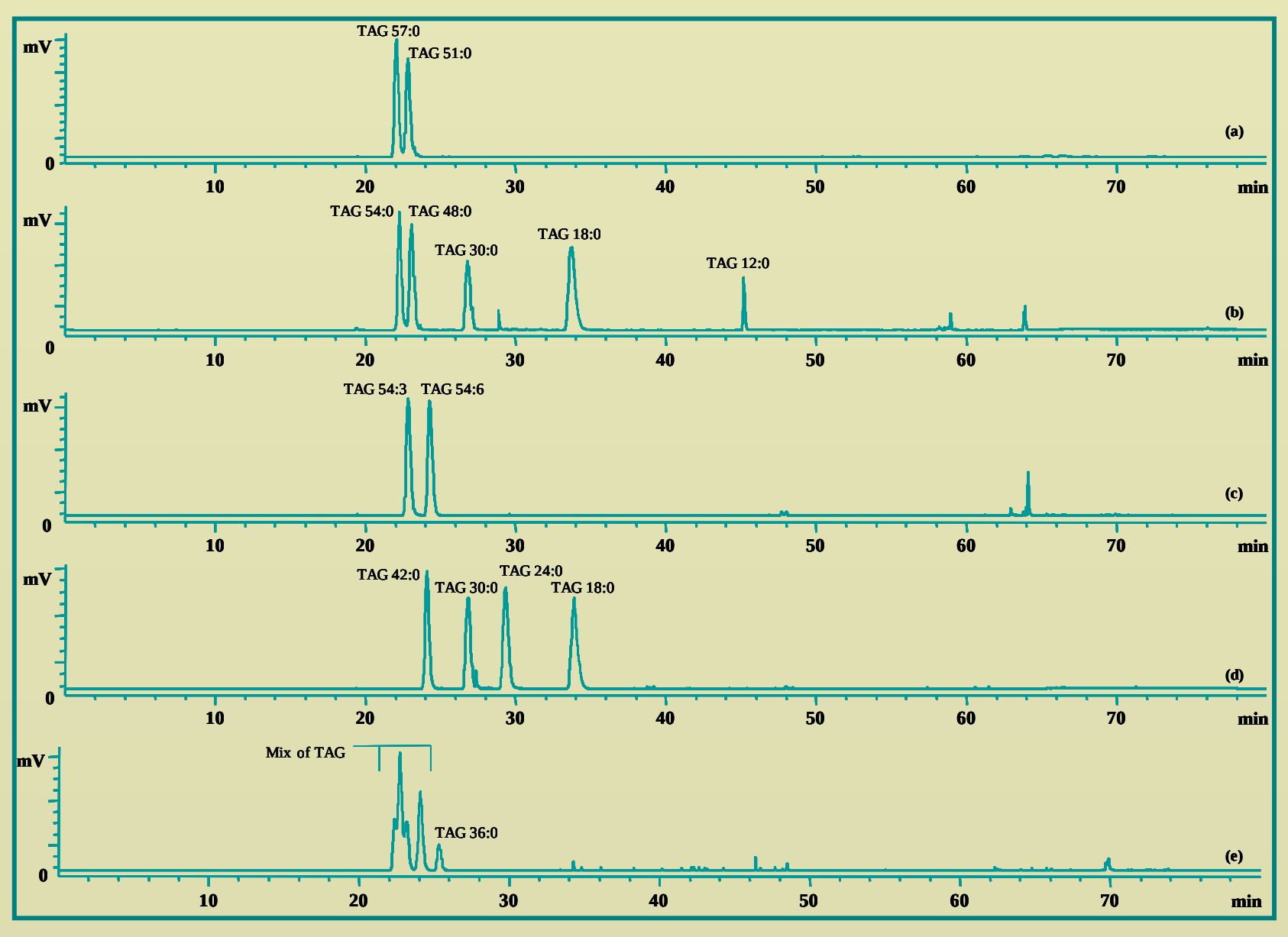

Fig. 2. HPLC-ELSD chromatograms for identification of TAG. TAG composition in figure **(e)**: TAG 54:0 + TAG 54:3 + TAG 54:6 + TAG 51:0 + TAG 48:0 + TAG 48:3 + TAG 42:0 + TAG 36:0.

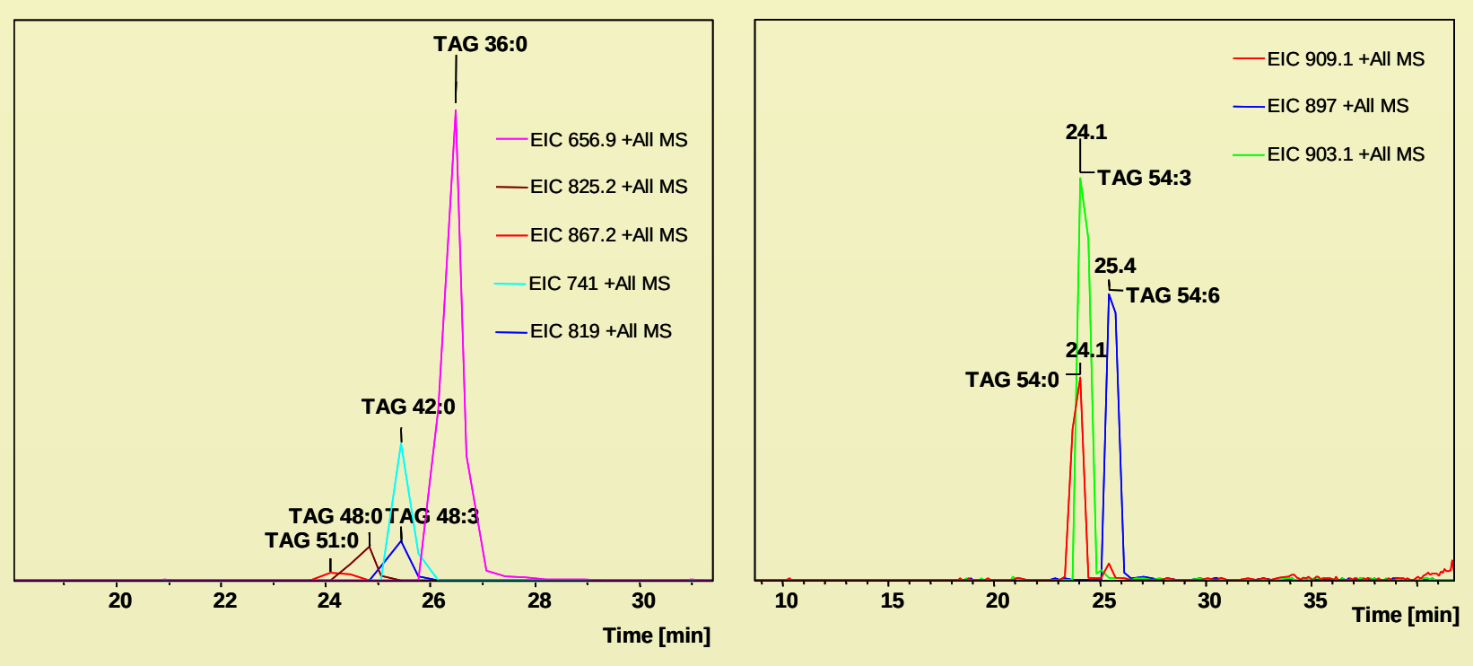

Fig. 3. Extracted ion chromatograms of TAG-ammonium adducts in the standard mixture, cf. chromatogram in ***Fig. 2*** **(e)**.

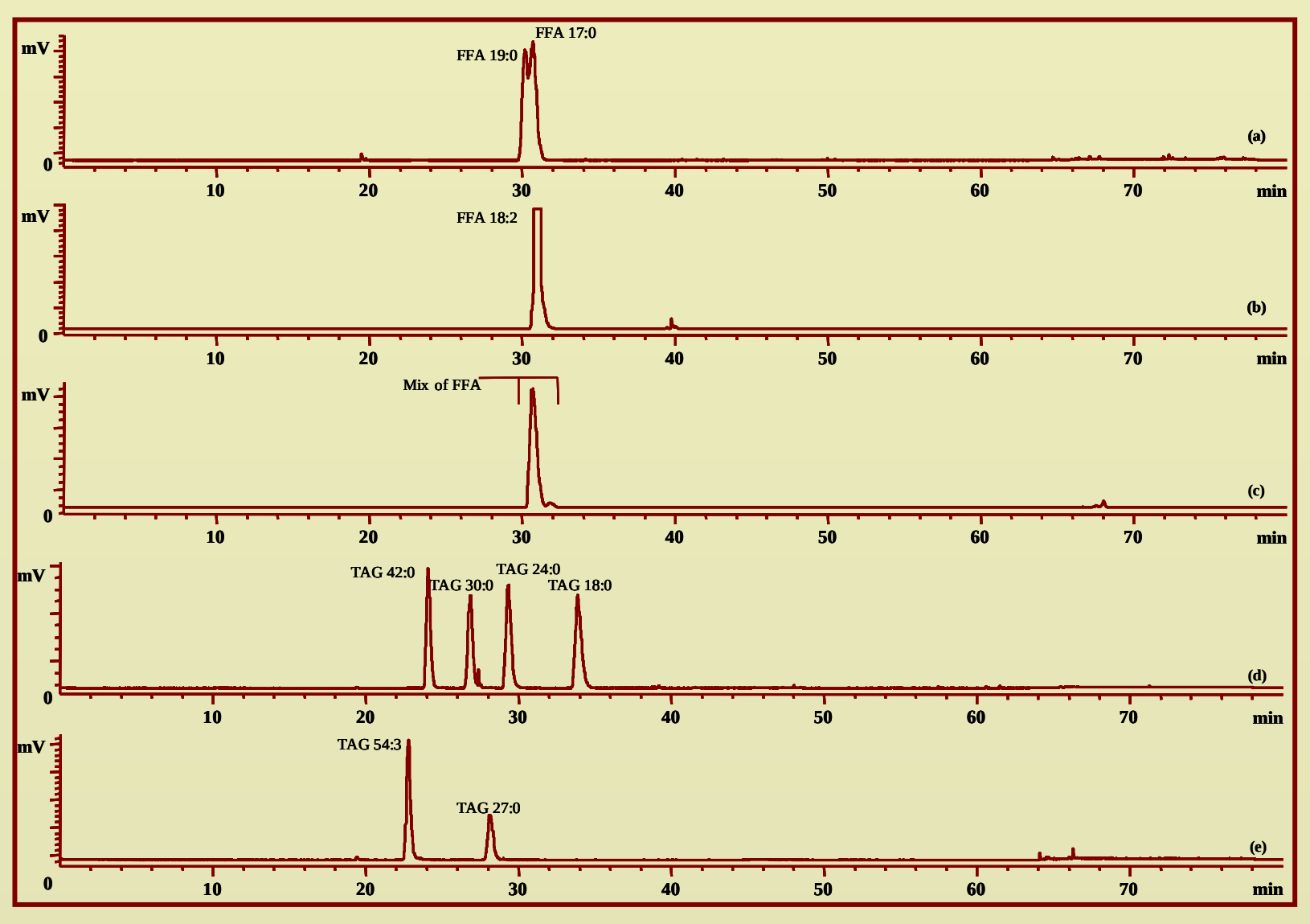

Fig. 4. HPLC-ELSD chromatograms for identification of FFA and comparison with retention times of some short chain-length TAG. FFA composition in figure **(c)**: FFA 18:0 + FFA 14:0 + FFA 10:0 + FFA 6:0; FFA composition in figure.

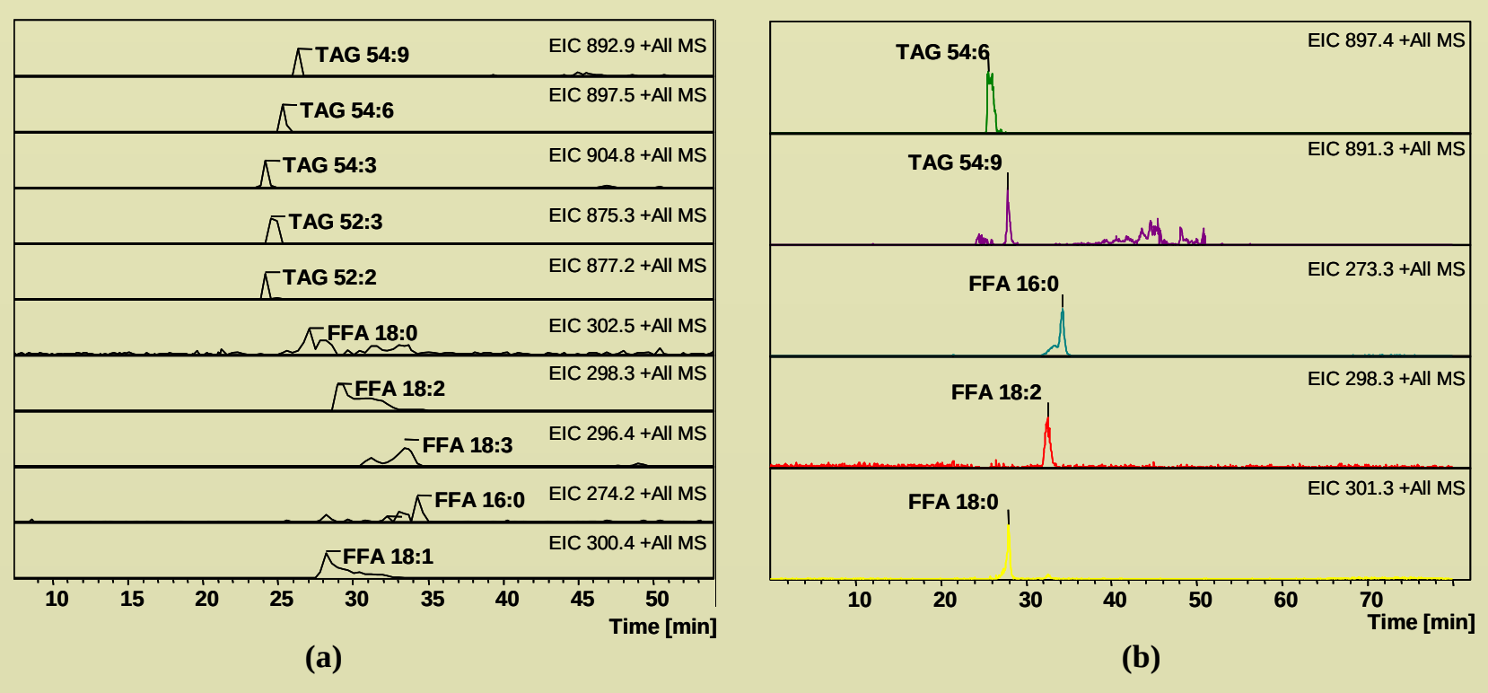

Fig. 5. Extracted ion chromatograms of TAG- and FFA-ammonium adducts in: **(a)** starch lipids from maize flour and **(b)** sunflower oil (deprotonated ammonium adducts for FFA 16:0 and 18:0) .

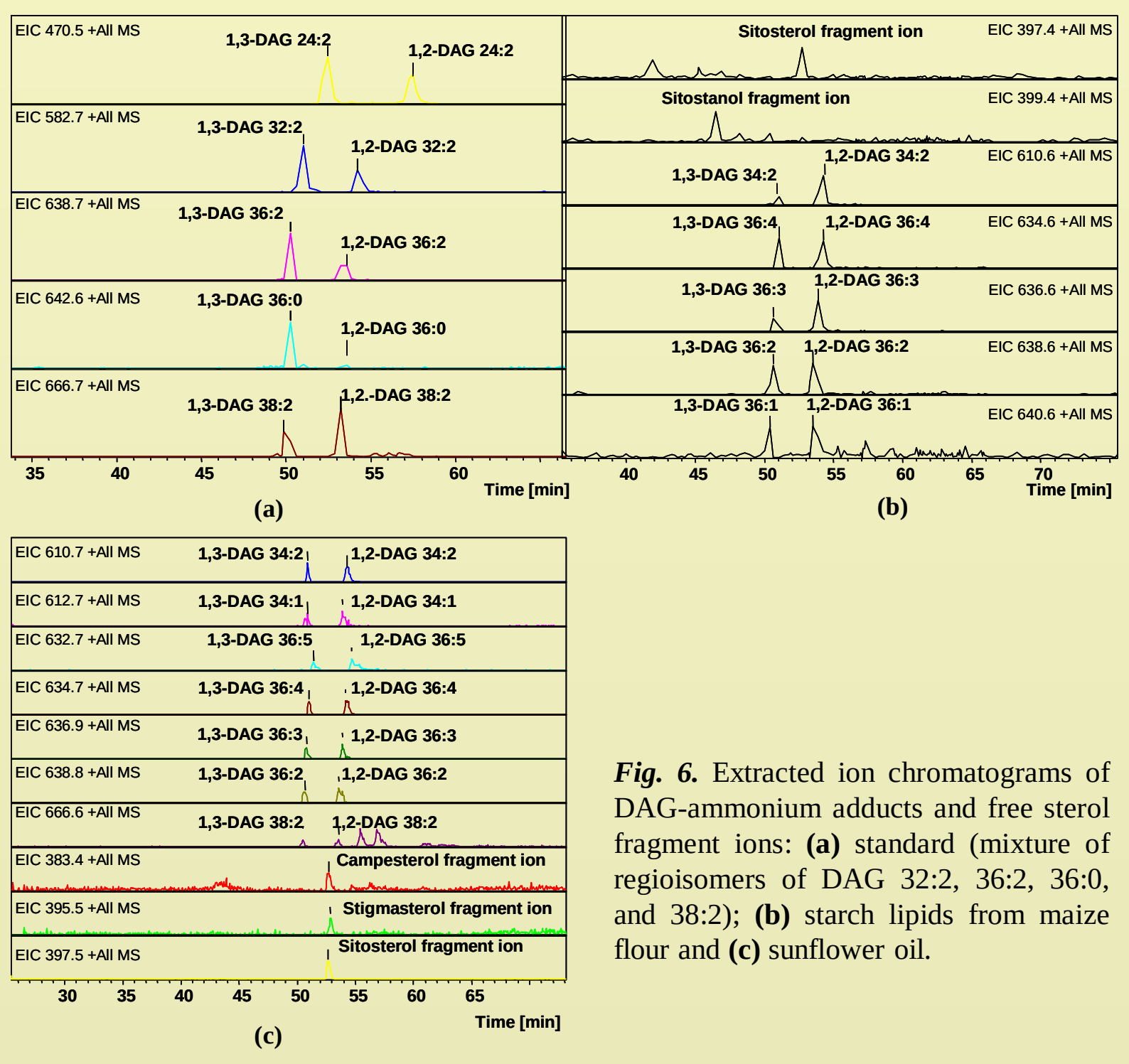

Fig. 6. Extracted ion chromatograms of DAG-ammonium adducts and free sterol fragment ions: **(a)** standard (mixture of regioisomers of DAG 32:2, 36:2, 36:0, and 38:2); **(b)** starch lipids from maize flour and **(c)** sunflower oil.

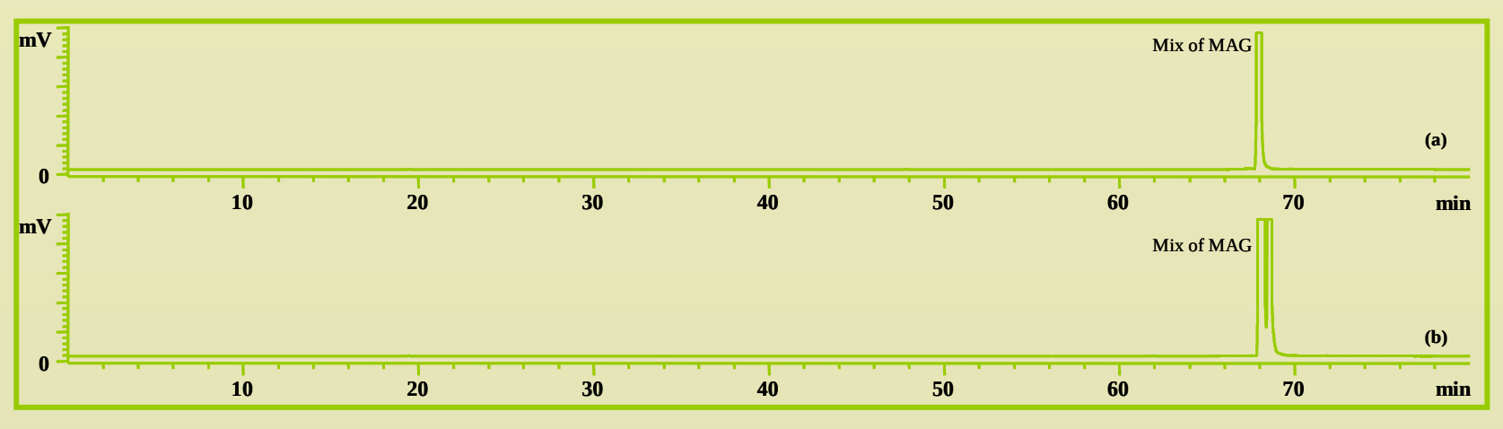

Fig. 7. HPLC-ELSD chromatograms for identification of MAG. MAG composition in figure **(a)**: MAG 19:0 + MAG 18:2 + MAG 17:0; MAG composition in figure **(b)**: MAG 18:0 + MAG 16:0 + MAG 12:0.

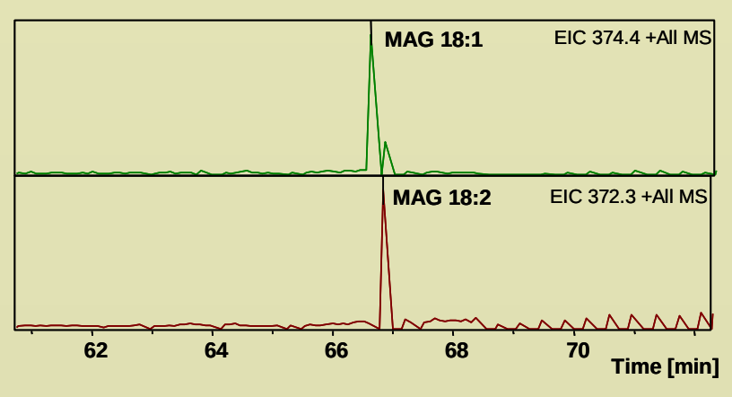

Fig. 8. Extracted ion chromatograms of starch lipids from maize flour.

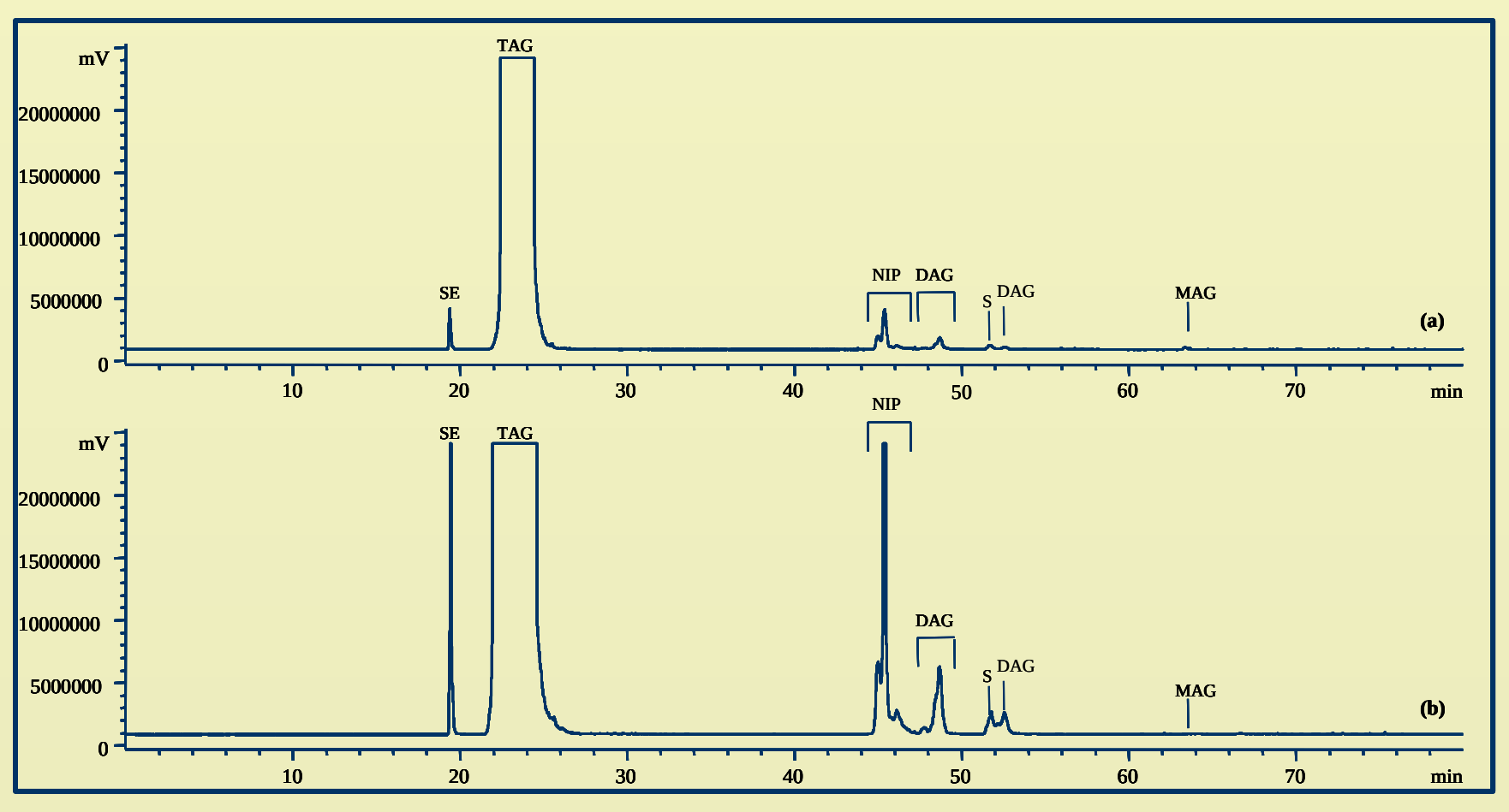

Fig. 9. HPLC-ELSD chromatograms of NL classes of sunflower oil (SO), diluted (in chloroform to 10% (v/v)), as 2 different injection volumes: **(a)** 2 µl and **(b)** 4 µl.

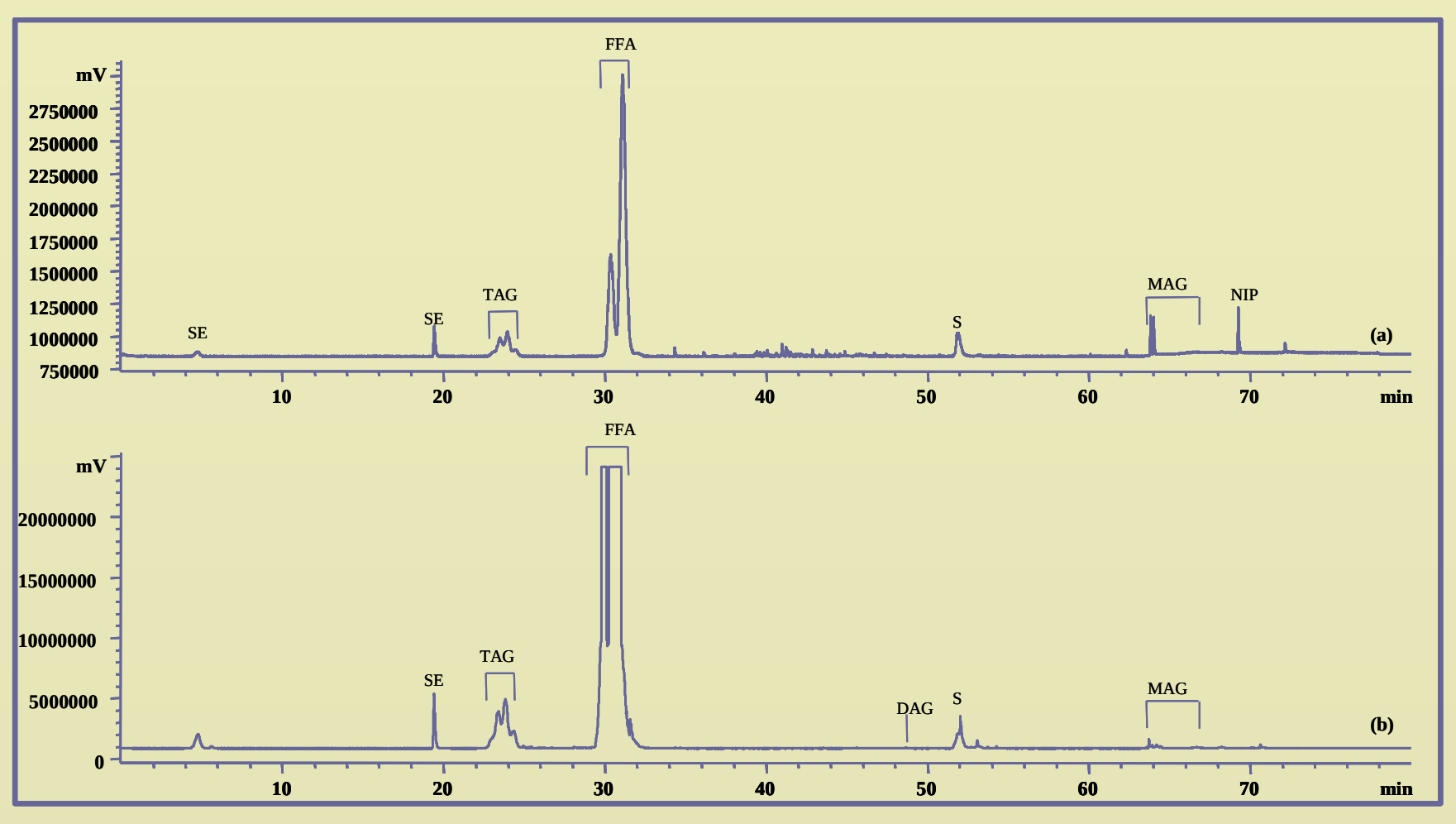

Fig. 10. HPLC-ELSD chromatograms of NL classes of bound lipids (BL) from 2 different amounts of maize flour: **(a)** 502.87 µg (2 µl injection volume) and **(b)** 2011.48 µg (8 µl injection volume).

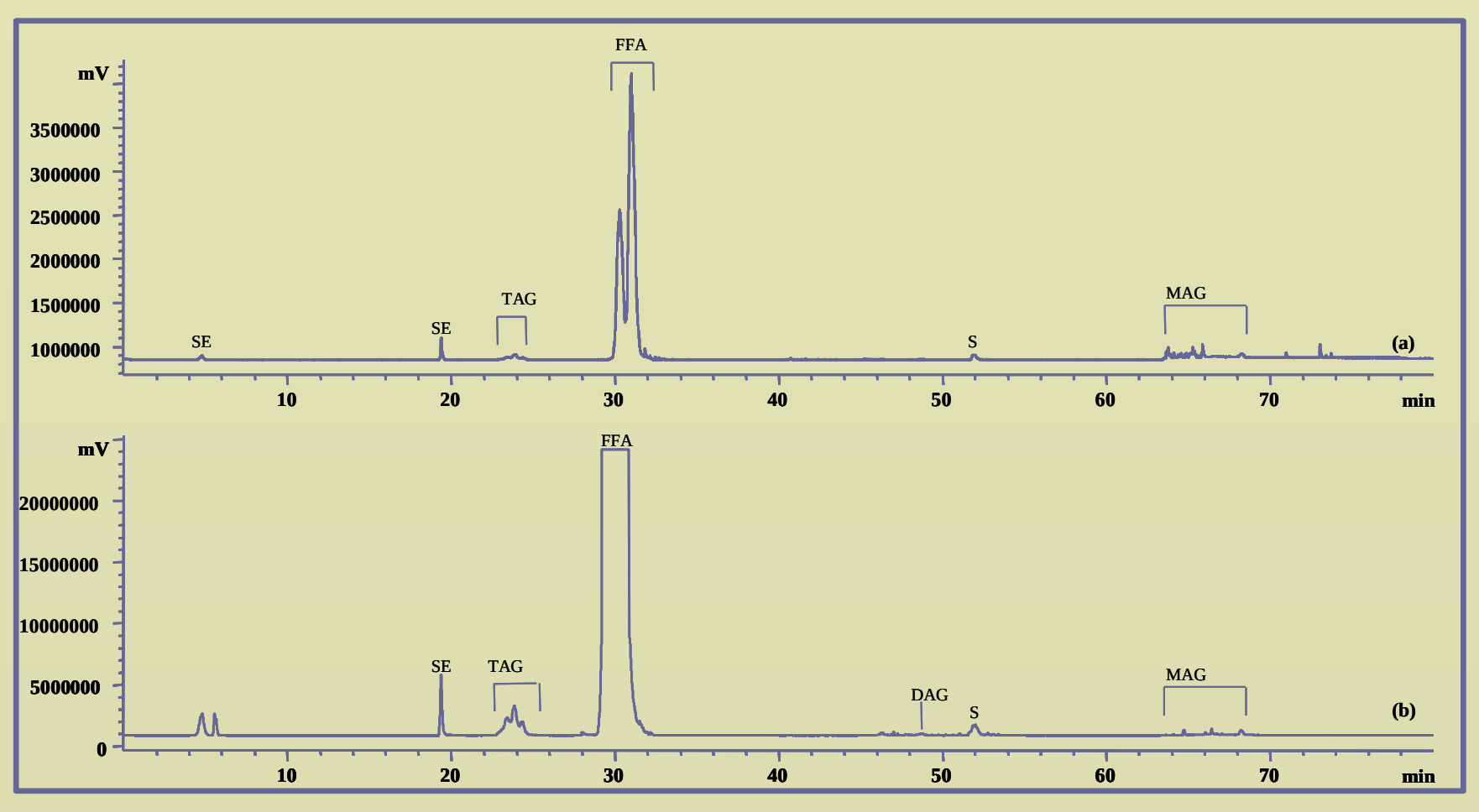

Fig. 11. HPLC-ELSD chromatograms of NL classes of starch lipids (SL) from 2 different amounts of maize flour: **(a)** 502.87 µg (2 µl injection volume) and **(b)** 2011.48 µg (8 µl injection volume).

SE ELUTION IMPROVEMENT

- ➢ The current method, as virtually all lipid separation methods by HPLC, requires polarity gradient elution. Therefore, the given time for column regeneration between sample analysis is critical.
- ➢ Separation of the same SE into more than one peak was observed. In order to obtain the same sterol ester eluting in a single peak, the preliminary method developed has been improved. For that purpose, a mixture of CE 17:0 with Cholesterol and a mixture composed by all classes of NL were injected before and after the system being run with a wash program between samples. It was observed (***Fig. 1*** **(a)** and **(b)**) that the wash procedure between runs has a great effect in SE elution and that a post-run time procedure was required. The effect of the presence of the guard column was tested as well and the results showed that there was no influence upon the SE elution. After the preceding results, the post-time was increased and the method was developed to the actual one. However, ***Fig. 15*** and ***16*** shows that unretained compounds may elute (RT ≈ 5 min) and especially after a long set of continuous runs.

SEPARATION OF NL CLASSES

- ➢ TAG possessing different number of acyl carbons were separated and eluted within 24 min (***Fig. 2***). Furthermore, studies also indicated: relatively good separation of TAG with the same unsaturation but different number of acyl carbons in the ACN range 12-42; and partial separation of TAG with the same number of acyl carbons but different degree of unsaturation (***Fig. 3*** and ***5***). Mass spectrometric identification (***Fig. 3***) of the sample from ***Fig. 2*** (**e**) was as follows: First shoulder - TAG 54:0 and TAG 51:0; Next peak - TAG 54:3; Next shoulder - TAG 48:0; Next peak - TAG 42:0 + TAG 48:3 + TAG 54:6; and Next peak - TAG 36:0.
- ➢ FFA 18:0 and TAG 54:9 may overlap (***Fig. 5***).
- ➢ FFA, DAG and MAG with different chain-lenghts were only partially separated (***Fig. 4 - 8***).
- ➢ Retention times of FFA with different chain length and level of unsaturation varied to some extent (***Fig. 4*** and ***5***).
- ➢ DAG with same acyl carbon number but with different degree of unsaturation separated partially (***Fig. 6***). 1,3- and 1,2-DAG were separated to the baseline. DAG isomers eluted close to sterols. ***Fig. 6*** shows that free sterols elute between 1,3- and 1,2-DAG. 1,3-DAGs and sterols are resolved to the baseline and 1,2-DAGs and sterols close to the baseline.
- ➢ All sterols eluted within very short retention time range: plant sterol mixture composed by β-sitosterol, stigmasterol, campesterol and brassicasterol was eluted in a single peak (***Fig. 6*** **(b)**).
- ➢ The chromatograms presented here showed that MAG can elute from 68 to 70 min (***Fig. 7*** and ***8***).

SEPARATION OF NL CEREAL LIPIDS and NL PLANT OILS

- ➢ All edible oils were similar in terms of lipid classes, with TAG as the major NL class (***Fig. 9***).
- ➢ Different lipid classes were resolved in maize lipids, extracted with different selective solvents (***Fig. 10 - 11***):
  - FL - high concentrations of TAG;
  - BL & SL - high concentrations of FFA and S.

GENERAL CONCLUSIONS

The column chosen, packed with 3 µm particles, offers considerable advantages in resolution when compared with the widely used 5 µm particles. Considering that the more complex the gradient the more the problems, the simple binary gradient chosen for the developed method is attractive and desirable. The injected volumes generally used during the method development did not exceed 30 µl, as is advisable for silica columns. The use of the guard column is advantageous to the column life time.

- ❑ The different lipid classes were completely separated. However, most unsaturated TAG and saturated FFA may overlap. 1,3-DAGs and sterols are resolved to the baseline and 1,2-DAGs and sterols close to the baseline. DAG regioisomers were well separated. The TAG with different chain length were separated effectively, and those with the same chain length and different unsaturation were separated partially. The FFA, DAG and MAG with different chain-length were separated only partially.
- ❑ Extracted ion chromatograms, mass spectra and especially tandem mass spectra are indispensable to identify accurately the HPLC-ELSD chromatograms. The same operation conditions can be applied to analyze all different lipids found in the samples. Tandem mass spectrometry is extremely useful for determination of molecular species composition of several NL lipid classes.
- ❑ Procedures for the NL determination are widely described in the literature. Nevertheless, they are frequently laborious for routine analysis of food, or even when a large number of samples are to be analysed. The methodology developed here is greatly promising as a rapid and powerfull tool for further studies in the analysis of flours and baking specialities.

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