Separation of Neutral Lipids by High-Performance Liquid Chromatography Using ELSD and ESI/MS: Analysis of Plant Oils and Maize Flour

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OBJECTIVES

Silica gel HPLC-columns have been frequently used for the separation of lipid classes. Our major goal was to study the use of narrow-bore, small particle-size silica gel columns and evaporative light scattering detection (ELSD) in the analysis of lipid classes. Another goal was to study the applicability of electrospray mass spectrometry (ESI-MS) for detection and identification of separated lipids.

A method for the analysis of neutral lipid classes by HPLC with ELSD was developed. This method was highly reproducible, and produced a stable baseline separation of all neutral lipid classes, in the following order: sterol esters, high-molecular weight triacylglycerols, free fatty acids, low-molecular weight triacylglycerols, diacylglycerols, free sterols and monoacylglycerols. The performance of the method was demonstrated for a wide concentration range of edible oil samples, as well as free, non-starch and starch lipids of maize flour.

This technique is useful for qualitative and quantitative research encompassing neutral lipid classes, especially in plant tissues, after previous separation of the glyco- and phospholipid fractions, in the presence of appropriate calibration curves.

EXPERIMENTAL METHODS

EXTRACTION

Maize flour

↓

Thermal processing to inactivate hydrolytic enzymes

↓

Sequential extraction:

Extraction 1			
Solvent	*Time*	*Temperature*	*Cereal lipids*
Hexane	(1 + 1) hr	Room temperature	FL
WSB	(30 + 30) min	Room temperature	BL
1-propanol:H_2O (3:1)	(2 + 2 +1)hr	100°C	SL

Extraction 2			
Solvent	*Time*	*Temperature*	*Cereal lipids*
WSB	(30 + 30) min	Room temperature	NSL
1-propanol:H_2O (3:1)	(2 + 2 + 1) hr	100°C	SL

↓

Remove non-lipid impurities: *Blight & Dyer* cleanning procedure

↓

Flash column chromatography: NL were eluted with hexane

↓

HPLC/LC-MS

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)

Elution Program for the binary gradient system

Time (min)	*% B*	*Flow (ml/min)*
0	0	
2	15	
16	15	
22	18	0.1
30	18	
31	90	
49	90	
50	99	
64	99	0.5
65	0	
75	0	

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

ELSD SYSTEM
SEDEX 55,T: 52°C, Voltage: 600 V, Gas pressure (air): 1 bar

LC-MS SYSTEM

LC
Hewlett Packard LC 1100

External pump
Reagent solvent: $CHCl_3$:MeOH (2:1 by vol.) + 10% of 25% NH_3 in water
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, TG, DG and S; 40 for FFA and MG
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 = 300°C

MS/MS
Collision gas: He (99.996%)

ABBREVIATIONS

AcOH, acetic acid; **C**, cholesterol; **CE**, cholesterol esters; **DG**, diacylglycerol; **ESI**, electrospray ionization; **EIC**, extracted ion chromatogram; **FA,** fatty acid **FFA**, free fatty acid; **HPLC-ELSD,** high-performance liquid chromatography with evaporative light scattering detector;**MG**, monoacylglycerol; **MS**, mass spectometry; **MTBE**, methyl-tert-butyl ether; **NL**, neutral lipids; **S**, sterol; **SE**, sterol ester; **TG**, triacylglycerol; **WSB**, water-saturated 1-butanol

RESULTS and DISCUSSION

SEPARATION OF LIPID CLASSES

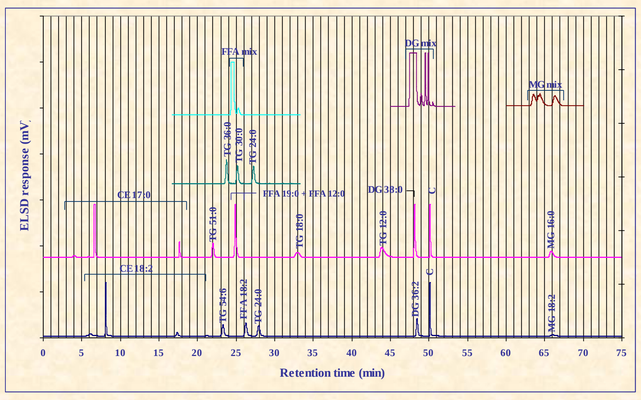

Figure 1 - HPLC-ELSD chromatograms of 2 standard mixtures of NL; TG mix: TG 24:0, 30:0, 36:0; FFA mix: FFA 4:0, 8:0, 12:0, 16:0; DG mix: DG 16:0, 24:0, 24:2, 32:0, 32:2, 36:0, 36:2, 36:4; and MG mix: MG 12:0, 16:0, 18:0.

Figure 2 - ESI-MS spectra of 18:2 sterol esters-ammonium adducts of maize flour NSL.

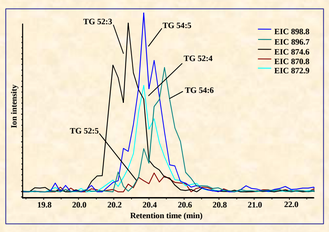

Figure 3 - ESI-MS spectra of TG-ammonium adducts of maize flour NSL.

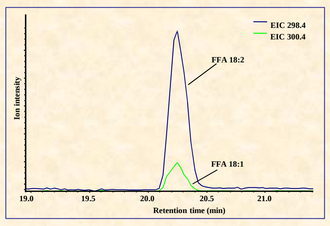

Figure 4 - ESI-MS spectra of FFA-ammonium adducts of maize flour NSL.

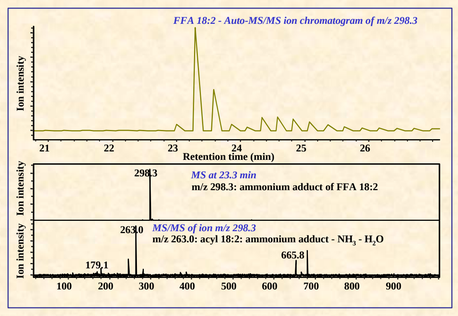

Figure 5 - ESI-MS/MS fragmentation of FFA 18:2.

➡ **Separation of the same sterol esters into more than one peak was observed (*Fig. 1*).**

➡ **Sitosterol, sitostanol, campesterol, and campestanol linoleates coeluted (*Fig. 2*).**

➡ **TG with the same level of saturation and different number of acyl carbons separated from each other (*Fig.1*).**

➡ **TG with the same number of acyl carbons but different number of double bonds separated partially (*Fig. 3*).**

➡ **Retention times of FFA with different chain length and level of unsaturation varied to some extent (*Fig. 1* and *4*).**

➡ **FFA eluted within the same retention time window than TG 26:0-30:0 (thus the proposed method is not applicable in the analysis of milk lipids and palm kernel oils.**

➡ **DG with different chain-length separated only partially (*Fig. 1*), but 1,3- and 1,2(2,3)-DG separated distinctly (*Fig. 6*).**

➡ **DG with same acyl carbon number but with different degree of unsaturation separated partially (data not shown here). DG eluted close to sterols (*Fig. 1*).**

➡ **All sterols eluted within very short retention time range: plant sterol mixture composed by β-sitosterol, stigmasterol, campesterol and brassicasterol was eluted with the same retention time (data not shown).**

➡ **MG with different acyl chain separated at least partially (*Fig. 1*).**

IDENTIFICATION BY MS and MS/MS

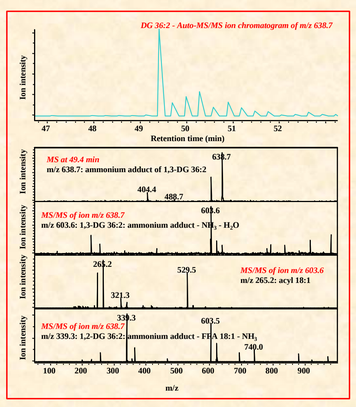

Figure 6 - ESI-MS/MS fragmentation of DG 36:2 positional isomers by MS/MS.

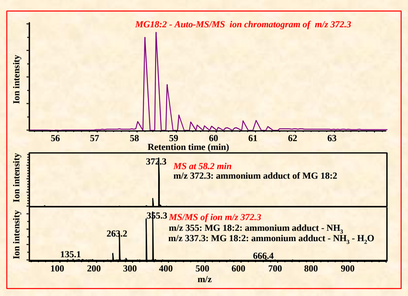

Figure 7 - ESI-MS/MS fragmentation of MG 18:2.

➡ **All lipid classes formed ammonium adducts. These could be identified by MS, and from their product ion tandem mass spectra. MS reveal molecular weight and unsaturation, and MS/MS esterified fatty acids of acylglycerols and sterol esters.**

➡ **Ammonium adduct of FFA 18:2 is ion m/z 298.3. MS/MS shows ion m/z 263.0, which is acyl 18:2, produced by loss of ammonia and water from ammonium adduct (*Fig. 5*).**

➡ ***Fig. 6* shows ammonium adduct of 1,3-DG 36:2: m/z 638.7, which produces in MS/MS m/z 603.6 by loss of ammonia and water. This produces further m/z 265.2, which is acyl ion 18:1. The 1,2(2,3)-DG 36:2 fragments differently producing m/z 339.3 by loss of FFA 18:1 and ammonia.**

➡ **Ammonium adduct MG 18:2 forms 3 fragment ions, corresponding to the loss of NH_3 (m/z 355), NH_3 and H_2O (m/z 337.3) and acyl ion 18:2 (m/z 263.2) (*Fig. 7*).**

HPLC-ELSD OF CEREAL LIPIDS and PLANT OILS

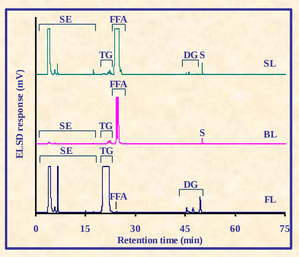

Figure 8 - HPLC-ELSD chromatograms of NL classes of free lipids (FL), bound lipids (BL) and starch lipids (SL) from maize flour.

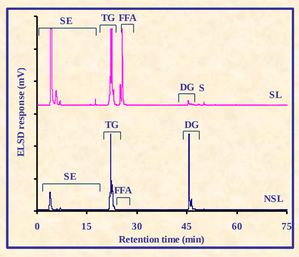

Figure 9 - HPLC-ELSD chromatograms of NL classes of non-starch lipids (NSL) and starch lipids (SL) from maize flour.

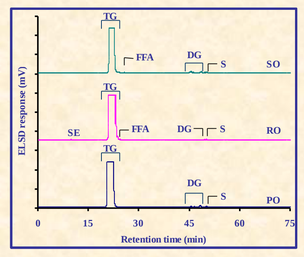

Figure 10 - HPLC-ELSD chromatograms of NL classes of edible oils: sunflower (SO), rapeseed (RO) and peanut (PO).

➡ **Different lipid classes were resolved in maize flour lipids extracted with selective solvents (*Fig. 8* and *9*):**

- **FL & NSL - high concentrations of TG;**
- **BL & SL - high concentrations of FFA and S;**

➡ **All edible oils were similar in terms of lipid classes (*Fig. 10*).**

CONCLUSIONS

- In general, the different lipid classes were completely separated. The TG with different chain length were separated effectively, and those with the same chain length and different unsaturation were separated partially. The FFA, DG and MG with different chain-length and unsaturation were separated only partially. The 1,3- and 1,2(2,3)-DG were separated. The method is applicable for a wide variety of fats and oils, but not for milk lipids and palm kernel oils (due to overlap of TG 26-34 and FFA).

- Components of all lipid classes formed ammonium adducts under the same experimental conditions. These could be identified from MS and product ion tandem MS. MS data provides information on the molecular weight, the level of unsaturation, and MS/MS data on the esterified FA of acylglycerols and sterol esters. The 1,3- and 1,2(2,3)-DG could be differentiated on the basis of MS/MS data.

- Maize flour lipids, extracted with different solvents and under different conditions, showed different, and vegetable oils showed similar lipid class compositions.

- MS spectra may be 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 can be also extremelly useful for determination of the fatty acid composition in several different classes of NL.

- The methods described in this work provide excellent sensivity for separation and identification of NL in plant tissues and edible oils, even if present at low concentrations.

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