Maximizing the Chromatographic Resolution and Detection Content of Complex Plant Lipid Analyses with Optimized UHPLC Systems

Jerry Zweigenbaum, Michael Woodman, Agilent Technologies, Inc., Wilmington, Delaware, USA

RAFA 2009 poster A-46

Abstract

Recent UHPLC experiments on columns packed with materials in the 1.5-1.9um particle size range have shown great utility in the analysis of complex lipid mixtures. Maximized resolution requires the use of high column efficiency and, typically, shallow gradient profiles. Achieving high resolution while minimizing overall operating pressure and analysis time is also a critical part of method design. The highest resolution separation is a combination of column and mobile phase physics and chemistry with system dispersion (bandspreading) also optimized.
Refined and unrefined triglyceride mixtures and phytosterols were acquired and subjected to a diversity of solvent and column configurations to explore high peak capacity separations. We typically use UV, ELSD and MS detection to enhance detection information.

Introduction

In this work we have exploited a broad range of resolution parameters on systems optimized for very high chromatographic resolution. We have also developed tools for method design and method translation to make these new technologies easier to use, and to significantly shorten the time required for method optimization.

Triglycerides play an important role for food research, biofuel, plant and animal physiology and lipidomics. High chromatographic resolution of intact triglycerides requires substantial effort to optimize the column and mobile phase choices and column operating temperature. The inherent sample complexity requires high peak capacity with relatively shallow gradients, typically under largely non-aqueous reversed phase conditions.

Phytosterols, as naturally occurring anticholesteremic materials, have received considerable attention from nutritional supplement producers and consumers. MS detection was used to propose peak identity or confirmation, which was especially beneficial with these highly complex mixtures where many standards have limited availability and/or are very expensive.

Experimental

Agilent 1290 Infinity LC, consisting of:
G4220A binary pump with integral vacuum degasser
G4212A high performance autosampler
G1316C thermo. column compartment SL
G4226A UV/VIS Diode Array Detector with 10 mm, 1 µL flow cell
G4218A ELSD with standard nebulizer
G6140A MSD with ESI/APCI Multi-Mode source

ChemStation 32-bit version B.04.02

Figure 1. Photo of (1290 Infinity LC) with ELSD

Figures 1 shows the Agilent 1290 Infinity LC, a recently introduced UHPLC system with up to 1200 bar (~18,000 psi) pressure and flow rates up to 5 ml/min, with the Agilent G4218A ELS detector. The ELSD is considered a destructive detector, with respect to recovery of effluent from the separation as might be appropriate for purification. When mass spectrometers, also similarly destructive, are present it is necessary to split the effluent into two or more streams depending on the specifics of the method and whether fraction collection is also desirable. When MS detectors are also used, it can be desirable to use a post-UV split for ELSD/MSD, and add a diluting or detection enhancing post-split flow to the MSD. The make-up solvent mixture can be optimized to enhance detectability of target components, and might include different organic solvents (from that used for the optimal separation) and/or organic or inorganic pH modifiers that might also enhance detectability, as shown in .

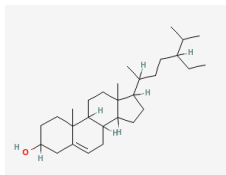


Figure 2. beta-sitosterol $C_{29}H_{50}O$
Limited unsaturation or chromophoric functional groups limits UV performance in this compound class.

Results and Discussion

Name	Emp.form.	# C=C bonds	Calc. Mc. Wt.
Cholesterol	$C_{27}H_{46}O$	1	386.366
ergosterol	$C_{28}H_{44}O$	(3) 2 conj.1 isol.	396.350
brassicasterol	$C_{28}H_{46}O$	2, non-conjugated	398.366
Campesterol	$C_{28}H_{48}O$	1	400.382
campestAnol	$C_{28}H_{50}O$		402.398
Stigmasterol	$C_{29}H_{48}O$	2, non-conj.	412.382
delta-5-avenasterol	$C_{29}H_{48}O$		412.382
b-Sitosterol	$C_{29}H_{50}O$	1	414.398
sitostAnol	$C_{29}H_{52}O$		416.414
Lanosterol	$C_{30}H_{50}O$	2, non-conj.	426.398

Table 1. Pertinent details of the typical phytosterol materials found in vegetable and/or animal fats and oils. Cholesterol occurs significantly in only one vegetable source – palm oil. Lanosterol does not typically occur in plants or animal lipid fractions and was used as a retention marker. beta-Sitosterol, Campesterol and Stigmasterol are typically the most abundant phyto's extracted from oilseeds. Brassicasterol is unique to rapeseed/canola.

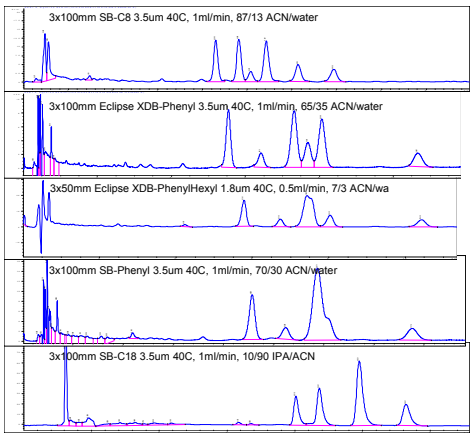
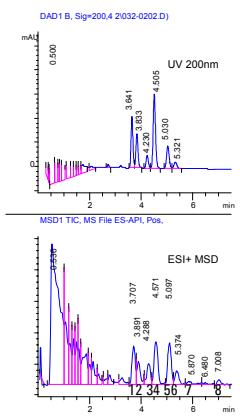


Figure 3. Optimized phytosterol separation on ZORBAX SB-C8 compared to some other ZORBAX ligands in the SB (StableBond) and XDB-Eclipse families.

Figure 4, below, peak identities based on the ZORBAX SB-C8 conditions, via positive ESI-MS. No post-UV addition was required to optimize the phytosterol response.

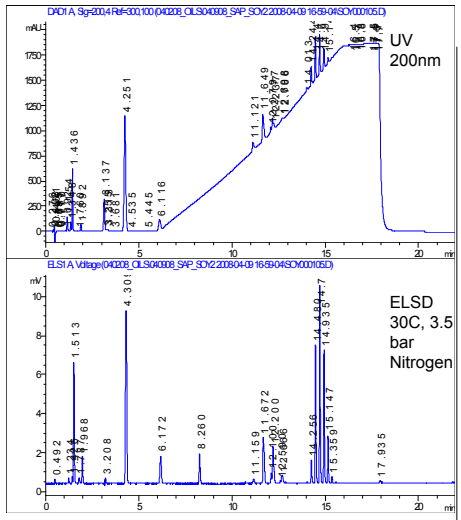


3x100mm SB-C8 3.5um 40C
1ml/min 87/13 ACN/water
Sample: mixture of working stds of various purities. Some id's (6,7,8) speculative

Proposed ID	
Sterol Mix	**m/z +ESI**
1 cholesterol	(369.3)
2 lanosterol	(409.3)
3 campesterol	(383.4)
4 stigmasterol	(395.4)
5 beta-sitosterol	(397.4)
6 dihydrolanosterol	(411.4)
7 sitostanol	(399.3)
8 tetrahydrolanosterol	(413.4)

m/z values are consistent with $[M-H_2O]^+$

Results and Discussion



FAME's----monogly diglyc. triglyc.

Figure 5. Soy triglycerides and products of a slow in-vial FAME conversion reaction, separated via a gradient optimized method for gross composition. Conditions: 3x100mm SB-C8 40C 3.5um 1ml/min solvent A 75/25 ACN/wa, Solvent B 1/1 IPA/ACN. solvent A to 5 minutes, then to 100% B at 15 minutes to 17. Because of the low UV wavelength required for sensitive detection of these compounds, the IPA in solvent B (required to elute the di- and tri-glycerides at these temperatures) caused significant baseline drift that was not observed in the ELSD. The UV signal is still useful, especially for the low levels of some of the less abundant fatty acid methyl esters (FAME's) produced during the saponification reaction. Sample taken at 90 minutes into ambient rxn 100mg/L KOH/MeOH. Less than 10% TG's remaining after 180 minutes.

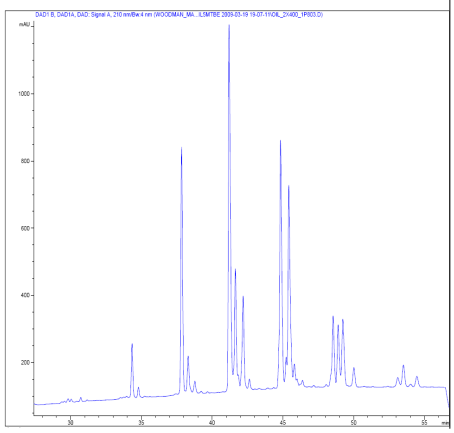


Figure 6. High resolution separation of triglycerides using the Agilent 1290 Infinity LC and ZORBAX RRHD columns. Conditions: 30ug., 0.29ml/min, 10-40% MTBE/ACN over 43 minutes. Up to 730 bar on ZORBAX SB-C18, 2.1x400mm, 1.8 µm, 20°C. Utilizing lower column temperature and C18 support with 1.8um particles, and with MTBE solvent to lower viscosity, a column set offering approximately 100,000 plates in actual use. For MS monitoring (not shown) APCIpos MSD was particularly effective with post-column addtion of 200ul/min MeOH/0.03M Ammonium Formate.

Conclusion

The Agilent 1290 Infinity LC system equipped with ancillary Agilent ELS and MS detection provided a rapid separation with good resolution, good sensitivity and high confidence in the proposed identity of compounds where standards were not present.
Future work includes expansion of the number of lipid classes, additional peak identity support, and recommended sample preparation and cleanup prior to HPLC analysis.