An Improved Global Method for the Quantitation and Characterization of Lipids by High Performance Liquid Chromatography and Corona Charged Aerosol Detection

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Overview

Purpose: To develop an improved global HPLC method for the high-resolution characterization and quantitation of a variety of lipids from different lipid classes.

Method: A ternary gradient HPLC method, using the Thermo Scientific™ Accucore™ C18 HPLC column, and the Thermo Scientific™ Corona™ ultra RS™ charged aerosol detector is outlined.

Results: The global method was evaluated for selectivity, calibration, sensitivity, and precision. Five different lipid classes were analyzed demonstrating the selectivity of the method, and calibration curves over four orders of magnitude were created. For examples, two oil samples were analyzed.

Introduction

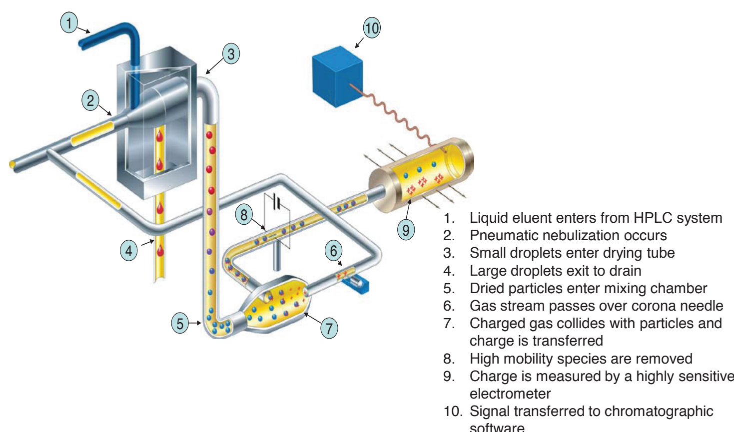
Lipids constitute a wide variety of compounds and for convenience can be classified based on their chemical structure (e.g., fatty acids, triglycerides, waxes, steroids, phospholipids etc). Lipids play numerous important roles including biological (e.g., for fuel storage, insulation, and membrane structure), industrial (e.g., lubricants and fuels), and cooking. One of the most common methods used to measure lipids is gas chromatography, with many of the lipids requiring derivatization prior to separation and detection. Another approach uses high performance liquid chromatography (HPLC), but their lack of a chromophore leaves lipids unresponsive to detection by the most common HPLC detector, ultraviolet absorbance. HPLC also can be used with universal detectors, such as evaporative light scattering (ELS), but lack of sensitivity, poor linearity, and a narrow dynamic range are often too limiting. The previous version of this method lacked resolution of triglycerides, which is now improved using the method presented here.

To overcome the poor resolution of triglycerides lipids were separated on an Accucore C18, a solid core column, using a ternary gradient and then measured using a Corona ultra RS charged aerosol detector. This approach was capable of resolving and detecting numerous classes of lipids (esters, all acylglycerols, fatty alcohols and acids, and paraffins) in a single analysis with only simple dilution as a sample pretreatment.

The Corona ultra RS detector provides the most sensitive and most uniform response factors of all universal HPLC detectors. As shown in the system schematic in Figure 1, it uses nebulization to form droplets that are then dried to particles. Particles are charged and measured using an electrometer. This mechanism enables detection at single-digit nanogram on-column. Since the mobile phase requirements for the charged aerosol detector are similar to those for mass spectrometry (MS), the same separation methods can be used with MS for peak identification.

A complete lipid composition of samples can now be generated using a single HPLC method. The method was used to characterize complex oil samples, such as algal oil and emu oil.

FIGURE 1. Schematic and functioning of charged aerosol detection



Methods

Standard and Sample Preparations
Stock standards were generally prepared at a concentration of 10 mg/mL in methanol/chloroform (1:1). More chloroform was used (up to 1:3) for more hydrophobic compounds (large paraffins).

Samples were dissolved at 10 mg/mL concentration in either methanol/chloroform (1:1) or methanol/tetrahydrofuran (1:1), depending on solubility. Samples were centrifuged at 10,000 g for 3 minutes to clarify.

Liquid Chromatography

HPLC System: Thermo Scientific™ Dionex™ UltiMate™ 3000 DGP-3600RS pump, WPS-3000RS autosampler, and TCC-3000RS column oven
HPLC Column: Accucore C18, 2.6 µm, 3.0 × 150 mm
Column Temperature: 40 °C
Mobile Phase A: Methanol/water/acetic acid (600:400:4)
Mobile Phase B: Tetrahydrofuran/acetonitrile (50:950)
Mobile Phase C: Acetone/acetonitrile (900:100)
Flow Rate: 1.0–1.5 mL/min
Injection Volume: 2–10 µL
Detector: Corona ultra RS
Nebulizer Temperature: 15 °C
Filter: 3
Data Rate: 10 Hz
Power Function: 1.00
Flow Gradient:

Time (min)	Flow Rate (mL/min)	%A	%B	%C
-10	1.0	90	10	0
0	1.0	90	10	0
20	1.5	15	85	0
35	1.5	2	78	20
60	1.5	2	3	95
65	1.0	90	10	0

Data Analysis
All HPLC chromatograms were obtained and compiled using Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, 7.1 SR 1.

Results

Calibrations
Calibration curves were generated using standard solutions (prepared as above), and analyzed in triplicate. The data were fit to second-order polynomials using data plotted on inverted axes (amount on the y-axis, peak area on the x-axis), as it provides for better correlations for second-order polynomial fits on Corona detector data. Calibrations using quadratic fits were performed for amounts ranging from 10 to 10,000 ng on column (ng o.c.) for paraffins, fatty alcohols, fatty acids, esters, and acylglycerides. An example of linear fits was provided using the fatty acids with different power function values, which are used to internally correct for the curvature of the charged aerosol response curves.

Chromatograms of these four classes of lipids are shown in Figure 2. All of the analytes were resolved within their group as well as between analytes in different groups, demonstrating the specificity of the method.

Fitted, quadratic calibration curves for seven paraffins are shown in Figure 3, and linear calibration curves for the fatty acids, each with its indicated power function value (PFV), is shown in Figure 4. The spread of analyte response seen in these figures is the result of differences in analyte volatility. Since the Corona detector responds to mass of analyte particles passing through the detector, the more volatile an analyte, the less particle mass is produced, and therefore less response.

The results of the quadratic calibrations are provided in Table 1, including analyte retention time, limit of quantitation (LOQ) based on a signal-to-noise ratio of 10.0, precision of calibration over the entire dynamic range, and the correlation coefficient for each analyte calibration.

FIGURE 2. Overlays of HPLC-Corona detector chromatograms of paraffins, fatty alcohols, fatty acids, and esters, at 2500 ng o.c.

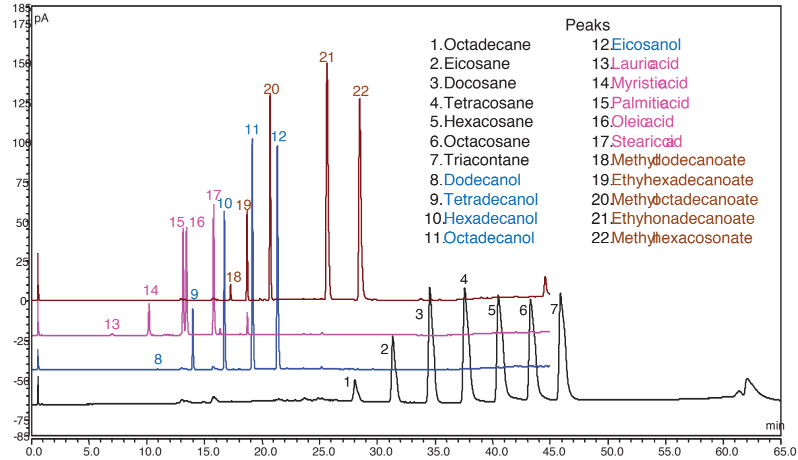


FIGURE 3. Second-order polynomial fits for paraffins, from 10 to 10,000 ng o.c., injected in triplicate

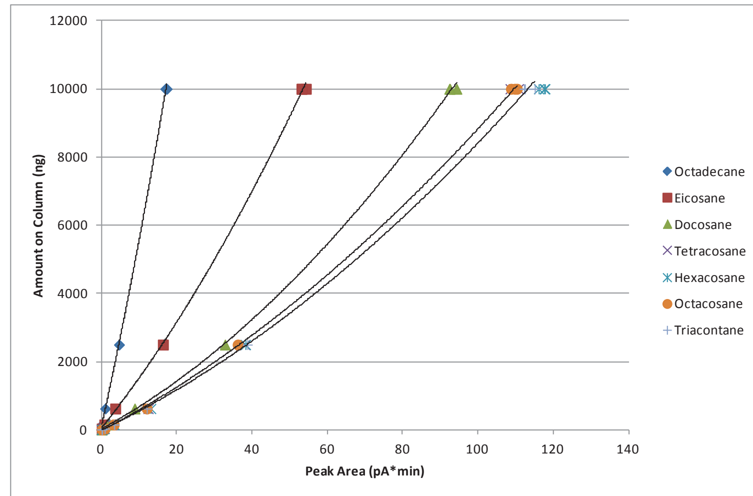


FIGURE 4. Linear calibration plots for free fatty acids, from 10 – 5000 ng o.c., using power function values (PFV).

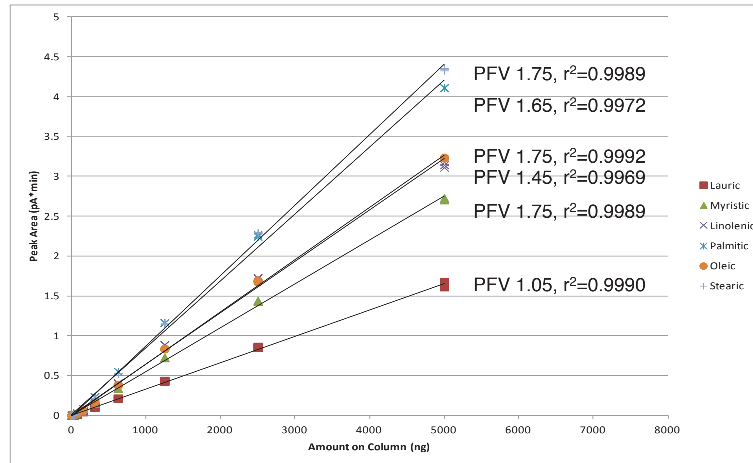


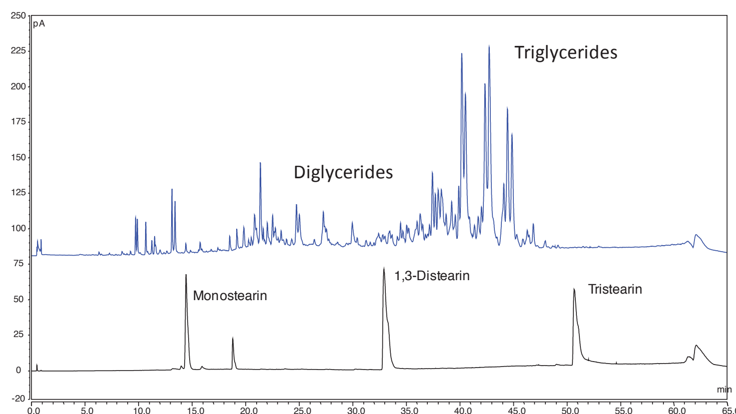
TABLE 1. Calibration data for five lipid classes by quadratic fits using inverted axes

Compound	Retention Time (min)	LOQ (ng o.c.)	Calibration RSD (%)*	Quadratic Correlation Coeff. (r^2)
1. Octadecane	28.05	225	1.75	0.9998
2. Eicosane	31.34	80	1.99	0.9998
3. Docosane	34.56	33	1.90	0.9999
4. Tetracosane	37.56	25	2.66	0.9997
5. Hexacosane	40.49	20	2.91	0.9997
6. Octacosane	43.27	20	2.55	0.9998
7. Triacontane	45.88	20	2.78	0.9997
8. Dodecanol	11.07	1515	3.37	0.9996
9. Tetradecanol	14.10	35	5.90	0.9990
10. Hexadecanol	16.80	10	5.49	0.9990
11. Octadecanol	19.17	2	7.21	0.9979
12. Eicosanol	21.32	2	8.73	0.9967
13. Lauric acid	6.99	190	4.94	0.9984
14. Myristic acid	10.15	10	2.52	0.9997
15. Palmitic acid	13.09	5	2.08	0.9998
16. Oleic acid	13.38	3	1.65	0.9999
17. Stearic acid	15.73	1	2.79	0.9997
18. Methyl dodecanoate	17.22	300	1.98	0.9998
19. Ethyl hexadecanoate	18.66	40	6.03	0.9985
20. Methyl octadecanoate	20.66	10	4.11	0.9994
21. Ethyl nonadecanoate	25.62	4	3.29	0.9996
22. Methyl hexacosonate	28.48	4	2.87	0.9997
Monostearin	14.42	20	2.03	0.9998
1,3-Distearin	32.85	20	5.33	0.9998
Tristearin	50.60	18	2.35	0.9995

Sample Results

Samples of algal oil and emu oil were prepared and 5 μL aliquots were injected and analyzed. Algal oil, an important source of a variety of lipids and other compounds, proved to be a complex sample, as the shown in the chromatogram in Figure 5. An overlay of stearylglycerols shows the position of highly retained acylglycerols. The method provides high resolution throughout the chromatogram. As can be seen in this chromatogram, the triglyceride region is also highly resolved.

FIGURE 5. HPLC-Corona detector chromatogram of algal oil (blue), 10 mg/mL in methanol/chloroform (1:1) and the stearylglycerols standards (black)



Emu oil, with a number of purported health benefits, consists largely of triglycerides (TAG). A sample of AEA-certified (American Emu Association) emu oil was prepared and analyzed, and the chromatogram is shown in Figure 6. Relative peak area amounts were integrated and compared against emu oil product specifications, as presented in Table 2. The triglycerides are grouped together and labeled by equivalent carbon number, ECN. ECN is the value calculated by 2C-2n, where C is the number of carbon atoms, and n is the number of double bonds in a TAG. The results are consistent with expectations. The lower amounts of TAG (ECN 50) are overestimates, which is attributable to the non-linear detector response of this experiment. At lower amounts of analyte, the response factor is larger than at higher amounts (providing more peak area per amount of analyte).

With the determination of appropriate power function values, changing the gradient to increase analyte separation, and with comparable standards for this analysis, the method is capable of highly sensitive and fast characterizations and quantitation of specific analytes.

FIGURE 6. Chromatogram of 5 µL injection of emu oil, 10 mg/mL in methanol/tetrahydrofuran (1:1), with equivalent carbon numbers (ECN) for triglycerides

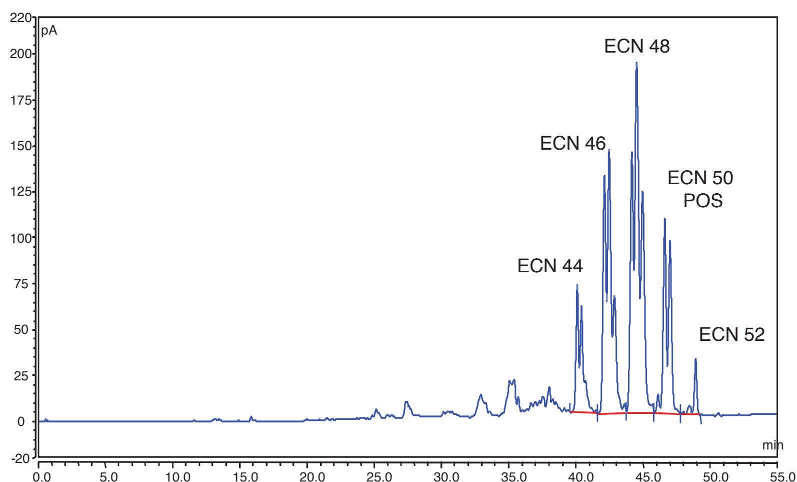


TABLE 2. Nonlinear, relative experimental results for emu oil against product specifications for triacylglycerols

Equivalent Carbon Number (ECN)	TAGs	Specification Amount[1] (Mass-%) Range, Mean	Experimental Amount Found (Area-%)	Recovery at Mean (%)
ECN-42	LLL, OLLn, PLLn	0.1-2.5, 1.0	--	--
ECN-44	OLL, PLL	4.0-16.0, 10.0	10.3	103
ECN-46	OOL, POL, SLL	15.6-40.8, 28.2	29.1	103
ECN-48	OOO, POO, PPO, SOL	33.2-63.2, 48.2	41.9	86.9
ECN-50	POS	3.4-8.2, 5.8	8.1	283

L = linoleic, Ln = linolenic, P = palmitic, S = stearic, O = oleic

Conclusions

- A general, universal method for the simultaneous analysis of different classes of lipids, including paraffins, fatty acids, alcohol, esters, and acylglycerols is presented.
- Sensitivity is dependent on the normal boiling points of the analytes, with 300 ºC being a general limit for detectability by charged aerosol detection.
- Sensitivity for the majority of analytes was found to be between 2 and 20 ng o.c.
- A complex lipid sample, algal oil, demonstrated the resolution of the method over the entire range of compounds present in this sample. Emu oil demonstrated the quantitative possibilities of the method, even without standards.
- The power function can be used to create linear calibration curves when required.

References

1. Emushop.com. http://www.emushop.com/specs.html, specifications of triacylglycerol content of emu oil by AOCS HPLC method Ce 5b-89 (last accessed 12 Feb 2013)

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