Novel HPLC-Based Approach for the Global Measurement of Lipids

Marc Plante,[1] Art Fitchett,[2] and Mike Hvizd[2]
[1]Thermo Fisher Scientific, Chelmsford, MA, USA; [2]Thermo Fisher Scientific, Bannockburn, IL, USA

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Abstract

Lipids are a structurally diverse group of compounds that can be challenging to measure. Typically, the sample is first extracted using organic solvents prior to derivatization either to render the lipid more volatile for gas chromatography (GC) determination, or to introduce a chromophore for UV detection. Sometimes a combination of techniques, including GC with flame ionization detection (FID), high-performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD), and liquid chromatography-mass spectrometry (LC-MS) is used to more fully characterize the sample. Each form of detection has benefits and limitations. Sample preparation for GC lipid analysis often requires the addition of carefully chosen internal standards, extraction, and derivatization. Nonreactivity can lead to errors in accuracy and undetected analytes. MS requires expensive instrumentation and equipment maintenance can be costly. The Thermo Scientific Dionex Corona™ *ultra*™ charged aerosol detector is a mass-sensitive detector capable of directly measuring any nonvolatile and many semivolatile analytes. Unlike ELSD, it shows high sensitivity (low ng), wide dynamic range (>4 orders), high precision, and more consistent interanalyte response independent of chemical structure, making it an ideal detector for simultaneously measuring different lipid classes.

Several HPLC methods are presented here that illustrate the determination of different lipid classes, including a universal, reversed-phase (RP) method that can resolve steroids, free fatty acids, free fatty alcohols, phytosterols, monoglycerides, diglycerides, triglycerides, phospholipids, and paraffins in a single run. A method for single-peak phospholipid quantification is shown as an example of normal-phase (NP) LC. Practical examples are also presented, including total glycerides in biodiesel by NP-LC, phytosterols in natural oils, and fat soluble vitamins found in commercially-available supplements.

Introduction

Lipids are physiologically important and involved in intermediary metabolism (acting as both energy storage and energy molecules), membrane structures, signaling, and protection (antioxidants, thermal insulation, and shock absorption). Lipids consist of a variety of forms, which can be categorized into fatty acyls (e.g., fatty alcohols and acids), glycerolipids (e.g., mono-, di-, and triacylglycerides), glycerophospholipids (e.g., phosphatidyl choline, phosphatidyl serine), sphingolipids, sterol lipids (e.g., cholesterol, bile acids, vitamin D), prenol lipids (e.g., vitamins E and K), saccharolipids, and polyketides (e.g., aflatoxin B1).

GC is widely used for the analysis of lipids. But because many of them are nonvolatile, it is necessary to derivatize the lipids before GC analysis. This adds to the complexity of the analysis, requiring additional sample preparation and the use of internal standards.

Due to the structural diversity of many lipid classes, HPLC separations can be performed using a variety of chromatographic conditions, with RP and NP being the most widely used. The use of HPLC allows for a simpler chromatographic method because derivatization is not required, and mass detectors such as ELSD, MS, and charged aerosol are available. UV detection is not widely used, as lipids typically lack a chromophore for the required light absorption.

Methods outlined here allow for HPLC-charged aerosol detection analysis of different lipids in different matrices. Compounds must be nonvolatile for routine and reliable detection.

A universal lipids HPLC method is outlined that offers high selectivity across a wide array of lipid classes (steroids to paraffins) in one 72-min HPLC analysis. This method can be used to determine which lipids are present in a sample, and the gradient conditions can be optimized to focus the separation on a particular region. From this, it is possible to increase resolution while maintaining the ability to quantify the analytes.

Examples of determinations of algal oil components, phytosterols in red palm oil, and fat-soluble vitamins in commercial products are provided using this and other methods detailed below.

Quantification of phospholipids represents a challenge for RP-HPLC. As many analytes occur in physiological samples which contain different carbon chain lengths and amounts of unsaturation, RP-HPLC can yield many peaks for a single phospholipid compound. To assist in quantification, an NP-HPLC method was created to maintain these different substructures as a single analyte peak.

A method for total quantification of glycerides in biodiesel is outlined that uses an NP-HPLC system to obtain results that are comparable to the current ASTM-GC method, is simpler to perform, and is less costly to operate.

Applications of Interest

These and other lipids applications can be found at www.coronaultra.com:

70-6995	*Steroid Hormones*
70-8096	*Phytosterols by HPLC with Corona* ultra *Charged Aerosol Detection*
70-8305P	*Total Glycerides of Biodiesel by Normal-Phase HPLC and Corona* ultra
70-8310P	*Simultaneous Analysis of Glycerides (mono-, di-, and triglycerides) and Free Fatty Acids in Palm Oil*
70-8322P	*Lipid Analysis by Reversed-Phase HPLC and Corona CAD: Natural Oils*
70-8323	*Lipid Analysis by Reversed-Phase HPLC and Corona CAD: Triglycerides*
70-8332	*Lipid Analysis by Reversed-Phase HPLC and Corona CAD: Free Fatty Acids*
70-8333	*Lipid Analysis by Reversed-Phase HPLC and Corona CAD: Free Fatty Alcohols*
70-8334P	*Lipid Analysis by Reversed-Phase HPLC and Corona CAD: Paraffin Waxes*
70-8335	*Lipid Analysis by Reversed-Phase HPLC and Corona CAD: Algal Oil*
70-9094P	*Sensitive, Single-Peak Phospholipid Quantitation by NP-HPLC-CAD*

Universal Lipids Method by RP-HPLC-Charged Aerosol Detection

Thermo Scientific Dionex Corona *ultra* Parameters

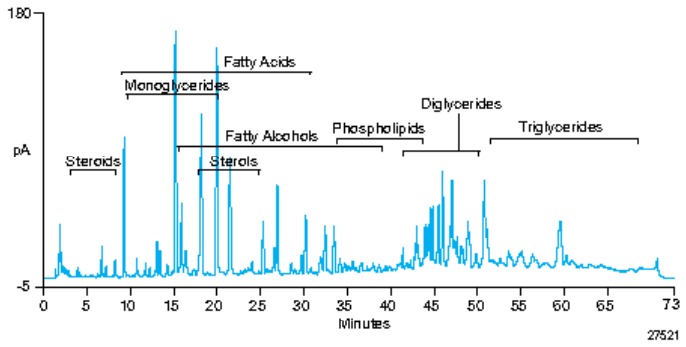
Filter:	Corona
Nebulizer Heater:	30 °C

HPLC Parameters

Mobile Phase A:	Methanol/water/acetic acid (750:250:4)
Mobile Phase B:	Acetonitrile/methanol/tetrahydrofuran/acetic acid (500:375:125:4)
Gradient:	0–70% B to 46 min; 70–90% B to 60 min; 90% B to 65 min; 0% B from 65.1 to 72 min
Flow Rate:	0.8 mL/min
Run Time:	72 min
HPLC Column:	Halo® C8, 150 × 4.6 mm, 2.7 µm
Column Temperature:	40 °C
Sample Temperature:	10 °C
Injection Volume:	10 µL

Standards were prepared at 1 mg/mL in methanol/chloroform (1:1), and extremely hydrophobic samples were first dissolved in three parts chloroform, with one part methanol added thereafter.

Figure 1. Algal oil sample by RP-HPLC-charged aerosol detection showing lipid class regions identified in previous work.



Phytosterols

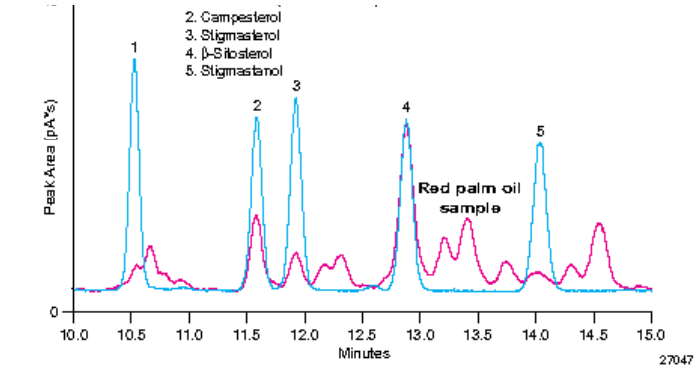
Dionex Corona *ultra* Parameters

Filter:	Medium
Nebulizer Heater:	30 °C

HPLC Parameters

Mobile Phase A:	Methanol/water/acetic acid (750:250:4)
Mobile Phase B:	Acetone/methanol/tetrahydrofuran/acetic acid (500:375:125:4)
Gradient:	0–30% B to 3 min; 30–38% B to 20 min; 0% B to 20.1 min; 0% B from 20.1 to 25 min
Flow Rate:	0.8 mL/min
Run Time:	25 min
HPLC Column:	Halo C8, 150 × 4.6 mm, 2.7 µm
Column Temperature:	40 °C
Sample Temperature:	10 °C
Injection Volume:	5 µL

Figure 2. Red palm oil sample (462 µg, red), and phytosterols standards (156 ng, blue) chromatogram, by RP-HPLC-charged aerosol detection. The phytosterol contents found in the sample were consistent with those reported in the literature.[1]



Fat-Soluble Vitamins by RP-HPLC-Charged Aerosol Detection

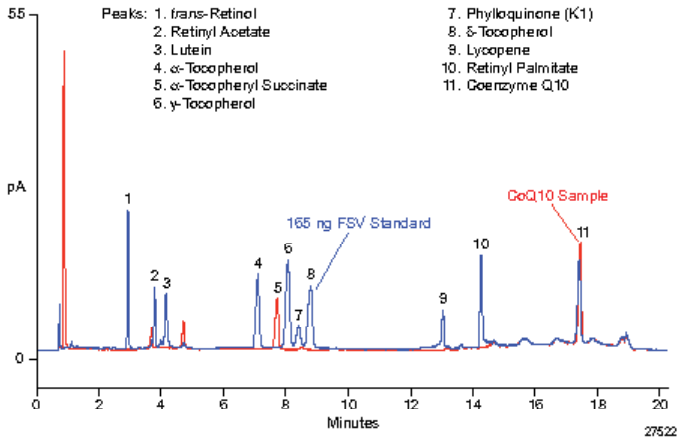
Dionex Corona *ultra* Parameters

Filter:	Corona
Nebulizer Heater:	30 °C

HPLC Parameters

Mobile Phase A:	Methanol/water/acetic acid (750:250:4)
Mobile Phase B:	Acetonitrile/methanol/tetrahydrofuran/acetic acid (500:375:125:4)
Gradient:	30–50% B from 0 to 1 min; 60% B to 5 min; 65% B to 10 min; 90% B to 12 min; 100% B to 17 min; 30% to 17.1 min; hold until 20 min
Flow Rate:	1.5 mL/min
Run Time:	20 min
HPLC Column:	Halo C8, 150 × 4.6 mm, 2.7 µm
Column Temperature:	40 °C
Sample Temperature:	10 °C
Injection Volume:	10 µL

Figure 3. Commercial Coenzyme Q10-Vitamin E succinate sample (red), overlaid with fat-soluble vitamin standard, 165 ng on column (o.c.), with 66 ng of Vitamin K1, (blue) HPLC-charged aerosol detection chromatograms.



Single-Peak Phospholipids by NP-HPLC-Charged Aerosol Detection

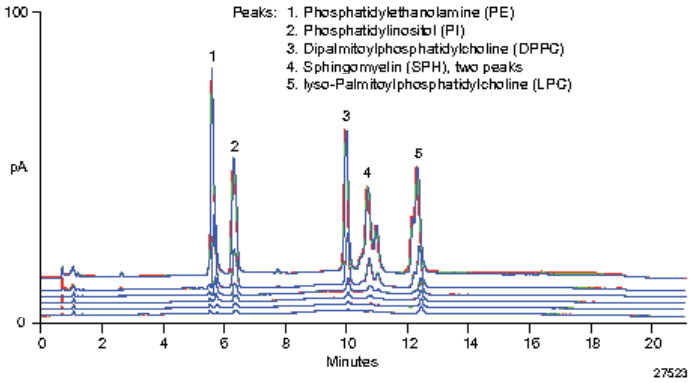
Dionex Corona *ultra* Parameters

Filter:	High
Nebulizer Heater:	30 °C

HPLC Parameters:

Mobile Phase A:	*n*-Butyl acetate/methanol/buffer (800:200:5)
Mobile Phase B:	*n*-Butyl acetate/methanol/buffer (200:600:200)
Buffer:	Water (18.2 MΩ-cm), 0.07% triethylamine, 0.07% formic acid
Flow Rate:	1.0 mL/min
Gradient:	0–100% B in 15 min; 100% B to 17 min; 0% B from 17.1 to 21 min
Run Time:	21 min
HPLC Column:	Alltech® Allsphere™ silica 100 × 4.6 mm, 3 µm
Column Temp:	35 °C
Sample Temp:	10 °C
Injection Volume:	10 µL

Figure 4. NP- HPLC-charged aerosol detection chromatograms of five phospholipid standards as near-single peaks, 16–2000 ng o.c., n = 3.



Biodiesel Analysis by Charged Aerosol Detection: Materials and Methods

Dionex Corona *ultra* Parameters

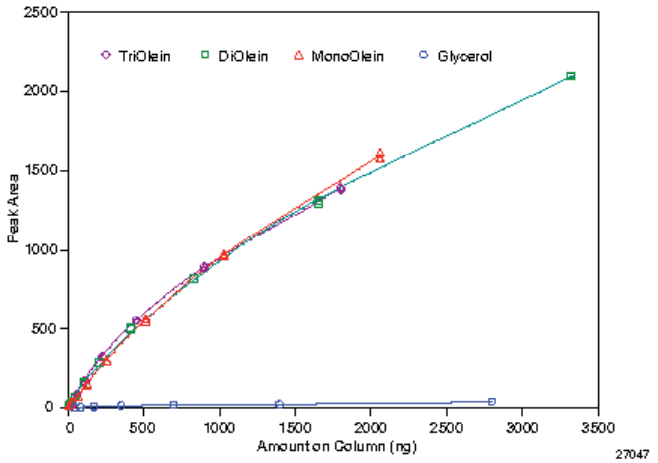
Filter:	Corona
Nebulizer Heater:	30 °C

HPLC Parameters

Mobile Phase A:	iso-Octane/acetic acid (1000:4)
Mobile Phase B:	iso-Octane/2-propanol/acetic acid (1000:1:4)
Mobile Phase C:	Methyl-t-butyl ether/acetic acid (1000:4)
Mobile Phase D:	iso-Octane/n-butyl acetate/methanol/acetic acid (500:666:133:4)
Gradient:	Available at http://www.coronaultra.com, Application Note #70-8035
Flow Rate:	1.0–1.2 mL/min
Run Time:	40 min
HPLC Column:	SGE Exsil™ CN, 250 × 4.0 mm; 5 µm
Column Temperature:	30 °C
Sample Temperature:	10 °C
Injection Volume:	10 µL

- All RSDs <2% for all analytes at all concentrations above 100 ng o.c.
- All acylglycerides had similar correlation curves (Figure 5), demonstrating uniform response factors attributable to the normal-phase solvents. Using a mobile phase that is completely organic in composition across the gradient (unlike aqueous reversed-phase) provides little change in evaporation rates, yielding a more uniform mass response.
- All recoveries were between 89–107% over spiked amounts of 0.01–0.05% of all acylglycerides and glycerol.

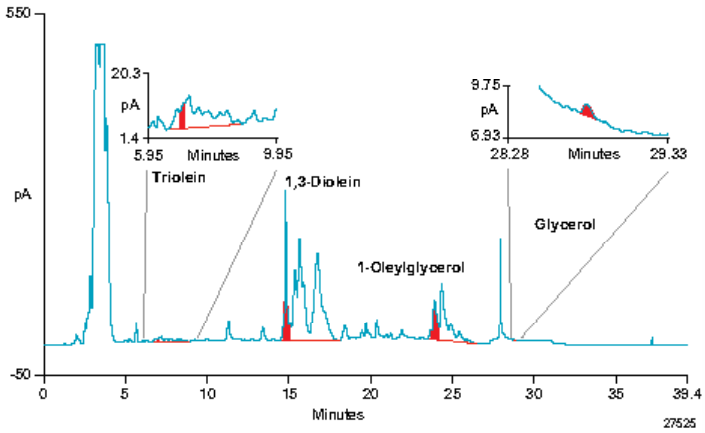
Figure 5. Standard correlation curves for three acylglycerides and free glycerol, 7–3300 ng o.c.



Results and Discussion

The method presented here can be used to separate eight classes of lipids in a single run. An example of this is provided in Figure 1, showing a chromatogram of algal oil. This method, combined with the sensitivity of the Dionex Corona *ultra* detector, provides a complete characterization of the lipid content within a sample. Paraffins were found to elute after the triglycerides. Incoming oils, which can vary from different sources and batches, can be quickly characterized to determine potential cleanup steps that may be necessary to allow a more predictable esterification process. This method can also be used for in-process analyses along each step of the biodiesel manufacturing process. Two other reversed-phase methods are also outlined: one providing for the quantification of phytosterols in a natural matrix (red palm oil); and the second for the determination of fat-soluble vitamins. Chromatograms are shown in Figures 2 and 3, respectively.

Figure 6. Biodiesel sample, 880 µg o.c., by NP-HPLC-charged aerosol detection. Biodiesel B100 (100 µL) diluted in 900 µL of iso-octane/2-propanol (98:2) and mixed. Sample was not derivatized.



An NP-HPLC method is shown for the analysis of phospholipids with a chromatogram containing five different phospholipids shown in Figure 4. This method was adapted from an ELSD method,[3] with solvents substituted to optimize conditions for the Dionex Corona *ultra* detector, yielding greater sensitivity and precision. This method showed good correlations, with r^2 values > 0.999 for all compounds. Precision was acceptable at <4 % RSD for amounts greater than 10 ng o.c. LOQ values—based on a signal-to-noise ratio of 10—were found to be 10 ng o.c. for PE, PI, and DPPC, 20 ng o.c. for LPC, and 30 ng o.c. for SPH. These values provide approximately 3–4 times greater sensitivity than the original ELSD method.

With a simple dilution of a B100 biodiesel sample, a total glyceride content was determined using the HPLC method, described in the last method. A calibration curve is provided in Figure 5, and a sample chromatogram is shown in Figure 6. The same sample was also characterized by the ASTM GC method. The results for the HPLC and GC methods compared favorably, with total glycerides being 0.088% for the HPLC method, and 0.081% for the GC method, using the same, glycerol-equivalent conversion factors.

Conclusions

- The Dionex Corona *ultra* detector can be used to quantify lipids of many classes down to low-level amounts, typically <10 ng o.c., using both reversed-phase and normal-phase HPLC conditions.
- Calibration curves from charged aerosol detection provide greater accuracy down to lower amounts o.c. than ELSD, which typically loses accuracy below 50–100 ng o.c. The calibration curves also provide a uniform equation across the entire dynamic range of the analysis.These methods offer a flexible analytical platform to characterize and quantify lipids in a variety of samples.

References

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3. Rombaut, R.; Camp, J. V.; Dewettinck, K. Analysis of Phospho- and Sphingolipids in Dairy Products by a New HPLC Method. *J. Dairy Sci.* **2005,** *88,* 482–488.

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