Analysis of Lipids by HPLC-CAD

Marc Plante, Bruce Bailey, Ian Acworth, and David Clark
ESA—A Dionex Company, Chelmsford, MA, USA

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 to either render the lipid more volatile for gas chromatography (GC) determination or to introduce a chromophore for UV detection. Sometimes a combination of techniques is used to more fully characterize the sample, including GC with flame ionization detection (FID), high-performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD), and LC-mass spectrometry (MS). Each form of detection has benefits and limitations. Sample preparation for GC often requires the addition of carefully chosen internal standards, extraction, and derivatization for lipids. Errors in accuracy, as well as analytes not being detected, can result from nonreactivity. MS requires expensive instrumentation and maintenance costs can be high. The charged aerosol detector (CAD®) 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, thus 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 method that can resolve steroids, free fatty acids, free fatty alcohols, phytosterols, monoglycerides, diglycerides, triglycerides, phospholipids, and paraffins in a single run. For an example of normal-phase (NP) LC, a method for single-peak phospholipid quantification is shown. Practical examples are also presented, including total glycerides in biodiesel by normal-phase LC, phytosterols in natural oils, and fat soluble vitamins found in commercially available supplements.

INTRODUCTION

Lipids are a diverse group of molecules that are physiologically important and are 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 them 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 the many classes of lipids, HPLC separations can be performed using a variety of chromatographic conditions, with reversed-phase and normal-phase being the most widely used. The use of HPLC allows a simpler chromatographic method because derivatization is not required, and mass detectors such as ELSD, MS, and CAD 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-CAD 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 then 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.

Quantification of phospholipids represents a challenge for reversed-phase (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 phospholipid. To assist in quantification of these lipids, a normal-phase HPLC method was created to keep these different substructures as one analyte peak.

A method for the total quantification of glycerides in biodiesel is outlined that uses a normal-phase 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 that have been created can be found on www.coronaultra.com:

70-6995 Steroid Hormones
70-8305 Biodiesel Analysis by Normal-Phase HPLC and Corona CAD
70-8310 Simultaneous Analysis of Glycerides (mono-, di-, and triglycerides) and Free Fatty Acidsin 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
70-8334 Lipid Analysis by Reversed-Phase HPLC and Corona CAD: Paraffin Waxes
70-8335 Lipid Analysis by Reversed-Phase HPLC and Corona CAD: Algal Oil
70-9086 Phytosterols by HPLC with Corona ultra Charged Aerosol Detection
70-9094 Sensitive, Single-Peak Phospholipid Quantitation by NP-HPLC-CAD

UNIVERSAL LIPIDS METHOD BY RP-HPLC-CAD

Corona® *ultra*™ Parameters

Gas:	35 psi via nitrogen generator
Filter:	Corona
Range:	500 pA
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 3 parts chloroform, with 1 part methanol added later.

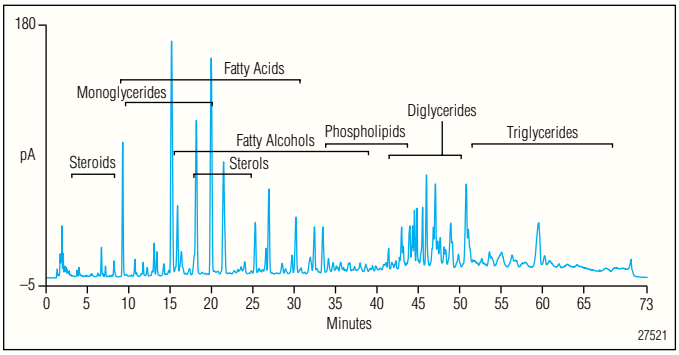

Figure 1. Algal oil sample by RP-HPLC-CAD showing lipid class regions identified in previous work.

PHYTOSTEROLS

Corona *ultra* Parameters

Gas:	35 psi via nitrogen generator
Filter:	Medium
Range:	100 pA
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

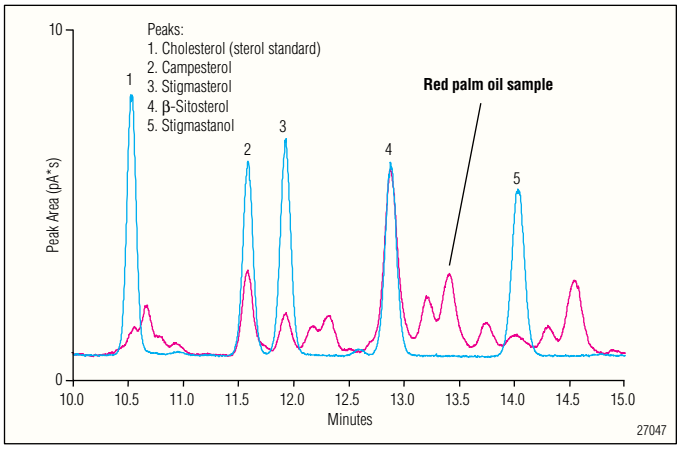

Figure 2. Red palm oil sample (462 µg, red), and phytosterols standards (156 ng, blue) chromatogram, by RP-HPLC-CAD. The phytosterol contents found in the sample were consistent with those reported in the literature.[1]

FAT-SOLUBLE VITAMINS BY RP-HPLC-CAD

Corona *ultra* Parameters

Gas:	35 psi via nitrogen generator
Filter:	Corona
Range:	100 pA
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

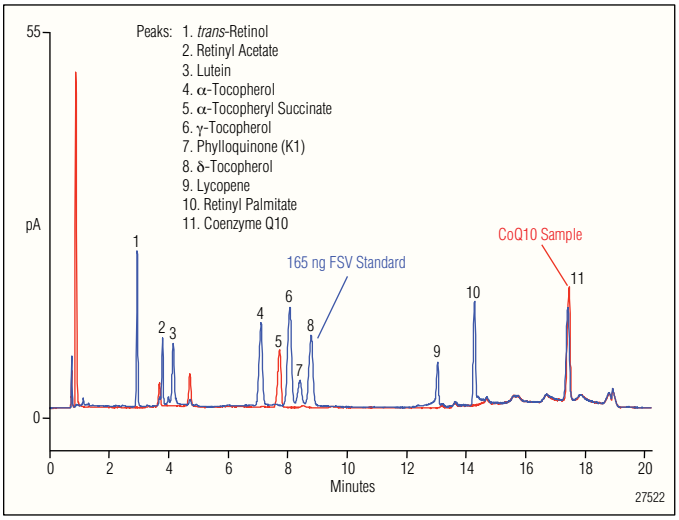

Figure 3. Commercial CoQ10-Vitamin E succinate sample (red), overlaid with fat soluble vitamin standard, 165 ng o.c., with 66 ng of Vitamin K1, (blue) HPLC-CAD chromatograms.

SINGLE-PEAK PHOSPHOLIPIDS BY NP-HPLC-CAD

Corona *ultra* Parameters

Gas:	35 psi via nitrogen generator
Filter:	High
Range:	100 pA
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

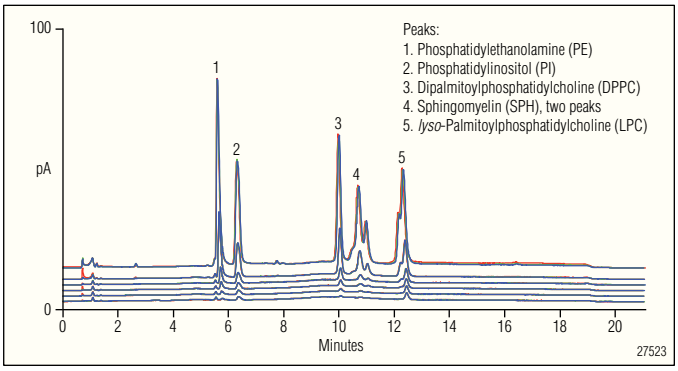

Figure 4. NP- HPLC-CAD chromatograms of five phospholipid standards as near-single peaks, 16–2000 ng o.c., n=3.

BIODIESEL ANALYSIS: MATERIALS AND METHODS

Corona *ultra* Parameters

Gas:	35 psi via nitrogen generator
Filter:	Corona
Range:	500 pA
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.

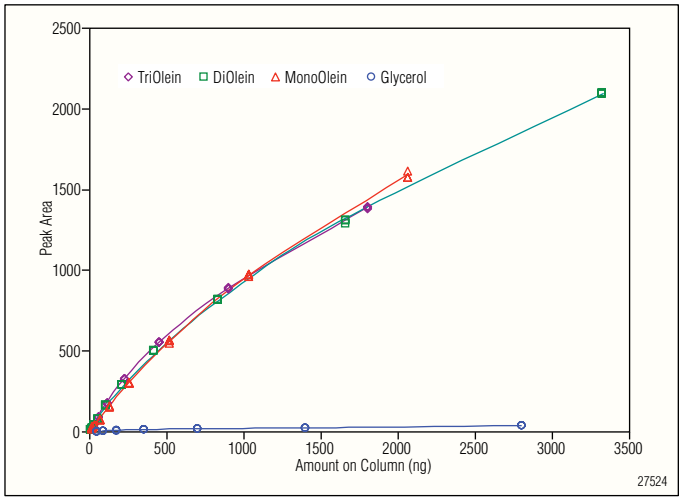

Figure 5. Standard correlation curves for three acylglycerides and free glycerol, 7–3300 ng o.c.

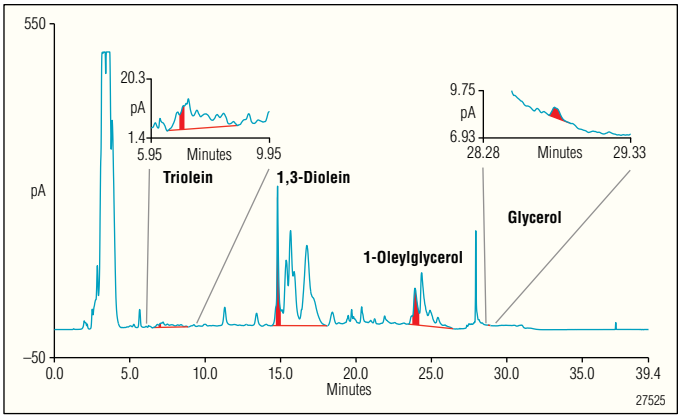

Figure 6. Biodiesel sample, 880 µg on column, by NP-HPLC-CAD. Biodiesel B100 (100 µL) diluted in 900 µL of iso-octane/2-propanol (98:2) and mixed. Sample was not derivatized.

RESULTS AND DISCUSSION

A universal lipids method is presented that 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 Corona *ultra* detector, provides a complete characterization of the lipid content within a sample. 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.

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

REFERENCES

1. Bonnie, T.Y.P.; Choo, Y.M. Valuable Minor Constituents of Commercial Red Palm Olein: Carotenoids, Vitamin E, Ubiquinones, and Sterols. Proceedings of the 1999 PORIM International Palm Oil Congress (Chemistry and Technology), 97–108.
2. Agilent (U.S.A.) HPLC for Food Analysis—A Primer, 2001. http://www.chem.agilent.com/Library/primers/Public/59883294.pdf
3. Rombaut, R. et al. *J. Dairy Sci.* **2005,** *88,* 482.

Dionex Corporation
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

North America
U.S. / Canada (847) 295-7500

South America
Brazil (55) 11 3731 5140

Europe
Austria (43) 1 616 51 25 Benelux (31) 20 683 9768; (32) 3 353 4294
Denmark (45) 36 36 90 90 France (33) 1 39 30 01 10 Germany (49) 6126 991 0
Ireland (353) 1 644 0064 Italy (39) 02 51 62 1267 Sweden (46) 8 473 3380
Switzerland (41) 62 205 9966 United Kingdom (44) 1276 691722

Asia Pacific
Australia (61) 2 9420 5233 China (852) 2428 3282 India (91) 22 2764 2735
Japan (81) 6 6885 1213 Korea (82) 2 2653 2580 Singapore (65) 6289 1190
Taiwan (886) 2 8751 6655

www.dionex.com

ISO 9001 REGISTERED
LPN 2738-01 3/11