Analysis of Lipids by RP-HPLC Using the Dionex Corona *ultra*

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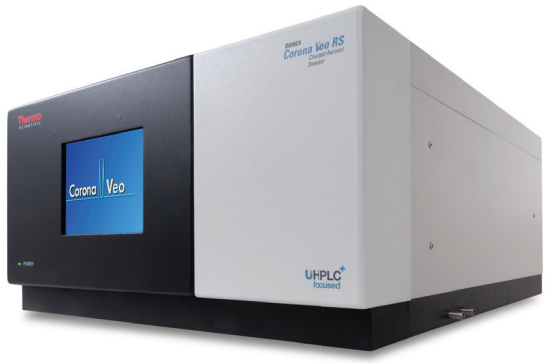
Key Words
Dionex Corona, Halo HPLC Column, CAD, lipids, reversed-phase HPLC

Abstract

With the increasing interest in lipidomics, analytical methods are required to quantify samples with high sensitivity and selectivity. We have developed a 72-minute, reversed-phase high pressure liquid chromatography (HPLC) method, using the Thermo Scientific™ Dionex™ Corona™ Charged Aerosol Detector (CAD) in combination with a fused-core (Halo) C8 150 × 4.6 mm (2.7 µm) HPLC column. This method has broad selectivity that can separate and quantify lipids, containing a wide range of hydrophobicity. Free fatty acids (lauric to stearic acid), fatty acid-esters and alcohols (tetradecanol to docosol), phospholipids (LPC, DPPC, DPPE, PE, PS, PC, sphingomyelin), acylated glycerols (mono-, di-, and tri-acylglycerols and milk fats), and paraffins (octadecane to octacosane) have been characterized using this single method. Typical dynamic ranges cover approximately three orders of magnitude contained within (10–10,000 ng on column (o.c)) and limits of detection (LOD) values are < 30 ng o.c.

Introduction

Lipids are a structurally diverse group of naturally-occurring, water-insoluble compounds that can, for convenience, be divided into the following eight categories: fatty acyls (e.g., fatty acids), glycerolipids (e.g., monoacylglycerides, diacylglycerides, 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).[1] Even within a particular category, there can be great structural complexity. For example, although triglycerides are composed of one glycerol molecule and three fatty acid molecules, differences in fatty acid chain length, degree of unsaturation, position of unsaturation and position on the glycerol backbone, result in numerous triglyceride variants.



Typically, much of the chromatography for lipids has been performed using normal phase methods, where the solvent is less polar than the stationary phase. Normal phase HPLC separates largely on differences in polarity between different analytes and their interactions with the stationary phase and solvents.[2] The solubility of lipids in normal phase solvents makes sample preparation simpler than for reversed phase systems. However, certain solvent combinations can allow for non-polar sample solvents to be used in reversed phase chromatography, which offers different selectivity than is achieved using normal phase.

In this presentation we show chromatography based on reversed-phase HPLC combined with the Dionex Corona CAD. CAD is a mass-dependent detector and responds to all non-volatile analytes, independent of chemical structure. Reversed-phase HPLC separates analytes based on their different interactions with the solvents through hydrogen bonding and dipole-dipole attractions. This yields different selectivity than normal-phase HPLC.[2] This method is capable of separating the relatively hydrophilic steroids to the completely hydrophobic paraffins using a single run even resolving analytes that are closely related in structure. The Dionex Corona CAD, with greater sensitivity over that of evaporative light scattering detection, can also reveal analytes that may not be detected by other means (e.g. mass spectrometry, ultraviolet or flow injection).

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In this presentation a number of examples are presented including: analysis of free fatty acids, fatty alcohols, a natural oil fingerprint, a tissue sample, as well as the separation and quantitation of ten fat-soluble vitamins. Acceptable calibration curves can be generated from data obtained using this method. This allows for the quantitation of known components contained in complex matrices. Sample preparation is simple, with dissolution in methanol / chloroform, varying from a ratio of 1:1 to 1:3, depending on the solubility of the sample matrix.

Experimental

Corona *ultra* Parameters	
Gas:	35 psi via nitrogen generator
Filter:	None
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:

General Lipids

Time	%A	%B
0.00	100.0	0.0
46.00	30.0	70.0
60.00	10.0	90.0
65.00	10.0	90.0
65.10	100.0	0.0
72.0	100.0	0.0

Fat-soluble Vitamins

Time	%A	%B
0.00	70.0	30.0
1.00	50.0	50.0
5.00	40.0	60.0
10.00	35.0	65.0
12.00	90.0	10.0
17.00	100.0	0.0
17.10	70.0	30.0
20.00	70.0	30.0

Flow Rate:	0.8 mL/min	1.5 mL/min
Run Time:	72 min	20 min
HPLC Column:	Halo C8, 150 × 4.6 mm, 2.7 µm	
Column Temperature:	40 °C	45 °C
Sample Temperature:	0 °	10 °C
Injection Volume:	10 µL	10 µL

Sample Preparation

Samples were prepared by diluting 1 mg of analyte in 1 mL of methanol / chloroform (1:1 – 1:3). Extremely hydrophobic samples were dissolved in 3 parts chloroform, with 1 part methanol added. Fat-soluble vitamins were dissolved in ethanol/ butylated hydroxyanisole (10 mg/L) at stock concentrations of 100 or 1000 µg/mL.

Fat-Soluble Vitamins Calibration

Calibration plots of ten fat-soluble vitamins are shown in Figure 1. Three vitamin supplements were extracted and quantified, with the percent of label claim shown in Table 1.

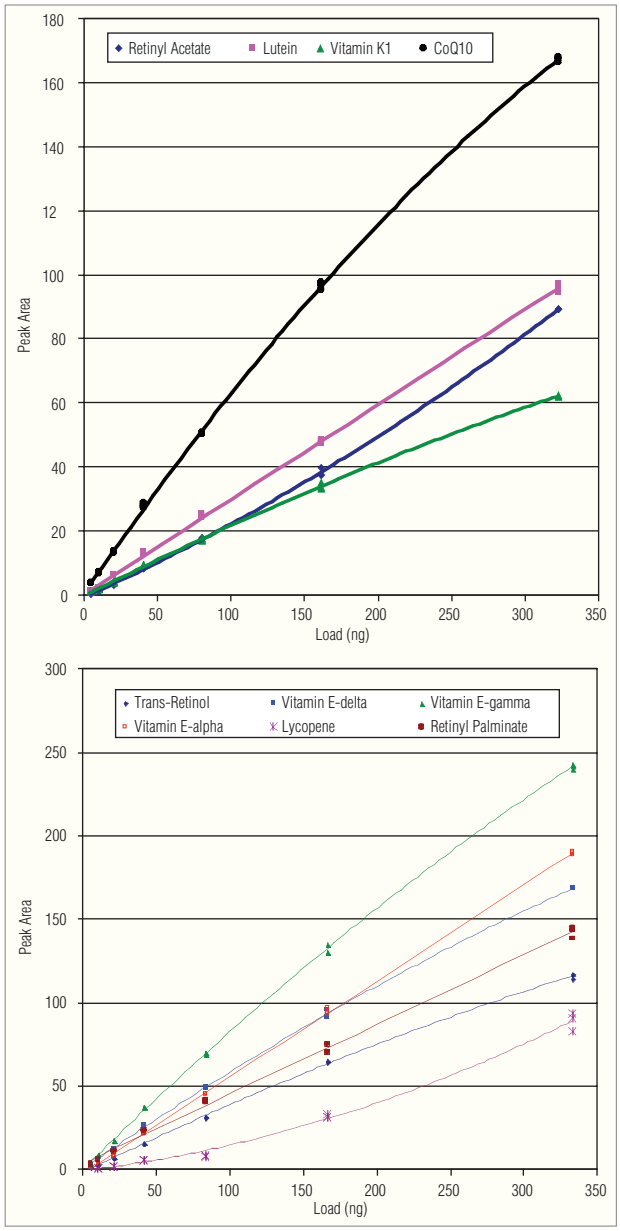

Figure 1. Calibration plots of ten fat-soluble vitamins. All relative standard deviations (RSDs) were < 5% for all amounts above 20 ng o.c. This indicates good precision for the method results and LOD values were determined at less than 10 ng o.c.

All of the correlations, fit to second-order polynomials, resulted in coefficients between 0.994–0.999.

Table 1. Vitamin supplements were extracted and quantified.

Product	Vitamin E-alpha succinate*	Vitamin E-alpha	CoQ10
Whole Foods® CoQ10 200 mg	109.8%	N/A	72.7
Solgar VM-75®	97.2%	N/A	N/A
CVS® Vitamin E 400 IU Capgels	N/A	97.6	N/A

*Vitamin E-alpha succinate calculated based on Vitamin E-alpha correlation.

Examples

Free Fatty Acids

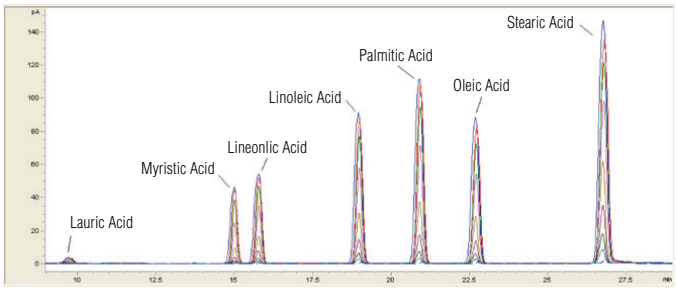

Figure 2. Lauric to stearic acids, including unsaturated acids in methanol/chloroform (1:1), 100–11,000 ng on column. Greater response was found with larger-molecular weight analytes, due to decreases in vapor pressure.

Fatty Alcohols

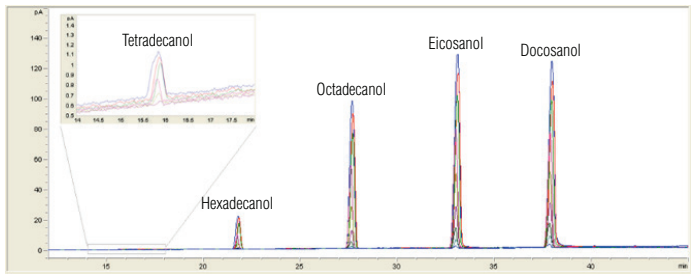

Figure 3. Tetradecanol to docosanol in methanol/chloroform (1:1), 100–12,000 ng on column. Greater response was found with larger-molecular weight analytes, due to decreases in vapor pressure.

Natural Oils

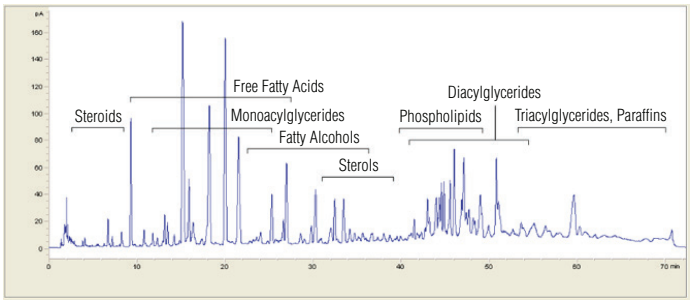

Figure 4. Algal oil from hexane wash, dissolved in methanol/chloroform (1:1), 10 µg on column. Lipid classifications were determined from other, independent standard analyses.

Tissue Lipids

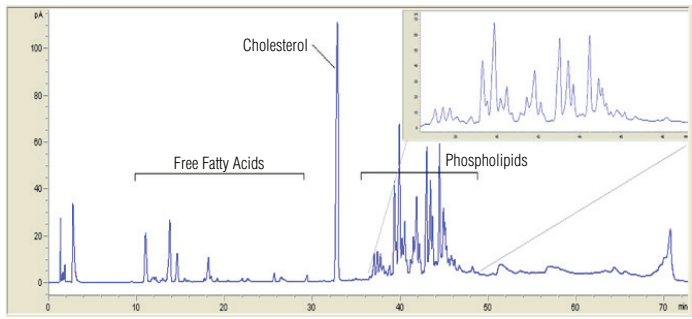

Figure 5. Extract from rat brain in methanol / chloroform (1:1), showing the expected components of fatty acids, phospholipids, and cholesterol. The phospholipid region is expanded in the inset to show additional detail.

Fat-soluble Vitamins

Peak #	Compound	LOQ (ng)
1	Trans-Retinol	4
2	Retinyl Acetate	7
3	Lutein	10
4	Vitamin E- delta	5
5	Vitamin E- gamma	6
6	Vitamin K1	15
7	Vitamin E- alpha	9
8	Lycopene	42
9	Retinyl Palmitate	15
10	CoQ10	7

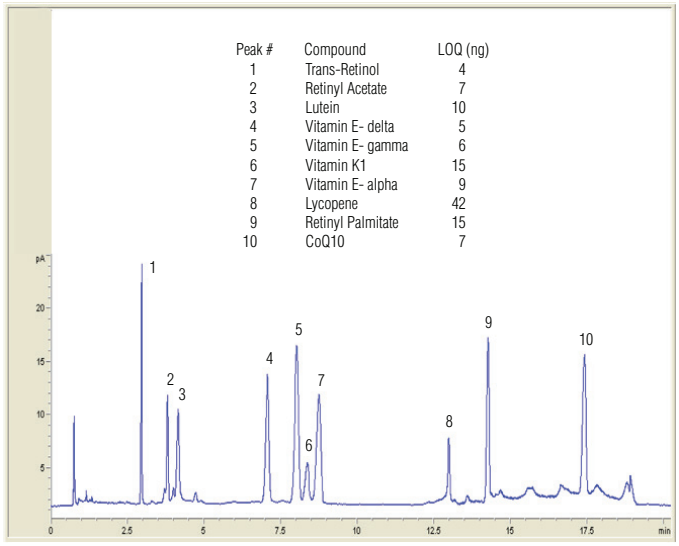

Figure 6. HPLC-CAD of ten fat-soluble vitamins, in ethanol/BHA (10 mg/L), each at 165 ng on column. Lycopene (#8) exhibited fronting, which increased the limit of quantitation (LOQ).

Discussion and Conclusions

This method shows great selectivity for many different lipid compounds, with sufficient dynamic range to measure both major and minor constituents simultaneously (e.g. the natural oils chromatogram) and without the need for additional standards when response factors are shown to be consistent.[3] The high resolution of the chromatography enables the separation of many compounds. The mobile phase is also compatible with mass spectrometry, allowing for the direct identification of these compounds by *m/z* ratios.[3] This is especially useful for drug discovery efforts, where minor components that are identified and isolated are often found to possess biological activity,[4] or may be very potent pharmaceutical candidates.[5]

Vapor pressure can affect the sensitivity of some analytes, as shown in the chromatograms for the free fatty acids and fatty alcohols. As analyte vapor pressure increases, fewer particles form which decreases the amount of response. For the free fatty acids, the lowest molecular weight compound that showed response is Lauric acid. To improve response of acidic or basic semi-volatile analytes, the addition of volatile buffer salts can transform relatively volatile analytes into non-volatile salts, which can then be determined with the Dionex Corona CAD.[6]

For a sample of unknown lipids, this method can be run as an initial screen; gradient optimization can then be used for analysis of a particular suite of lipids. The method is also flexible: the gradient can be adjusted to optimize for separation and run time, as was shown with the vitamin sample analysis.

The method can be used for quantitative analysis. Calibration curves were created for 10 fat-soluble vitamins, each with a correlation coefficient > 0.994, fit to second-order polynomials. For all analytes evaluated here, the LOQ, based on S/N = 10, was < 10-45 ng on column, which is lower than can be achieved with evaporative light-scattering detectors (ELSD). Precision was acceptable, with RSDs < 7% across all amounts above 20 ng o.c. and for all fat-soluble vitamins evaluated here. Percent recovery values on vitamin products showed quantitative results, with ~98% label claim for Vitamin Es and 74% label claim for a CoQ10 sample was found, possibly attributable to incomplete extraction of the latter from the product matrix.

This method is routinely used to separate a wide variety of lipids in many matrices, including milk, plant oils, tissues, and vitamin supplements. Preliminary results obtained through this initial screening process allow for further chromatographic optimization to characterize the specific lipids relevant to the sample.

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