

Quantitative HPLC Analysis of Lipids

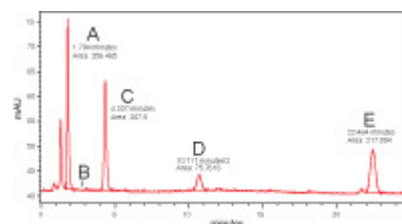
By Jerry Fireman

A recent study by Dr. Robert A. Moreau, of the Eastern Regional Research Center of the Agricultural Research Service, USDA, evaluated a charged aerosol detector (CAD), a new type of high-performance liquid chromatography detector,⁽¹⁾ with several normal phase and reverse phase HPLC systems commonly used for the quantitative analysis of lipid classes and lipid molecular species. The CAD detected common lipids such as triacylglycerols, cholesterol esters, and free sterols to a minimum limit of detection of about 1 nanogram per injection. The mass to peak area ratio was nearly linear from about 1 nanogram to 10 micrograms per injection.

Quantitative analysis of lipids becoming more important

Quantitative analysis of lipids is a currently a topic of strong interest, partly due to studies that have shown that plant sterol esters (PSE) can reduce the risk of cardiovascular disease by improving blood lipid profiles. In a study by Joseph Judd, David Baer, and Beverly Clevidence of the USDA, Shirley Chen and Gert Meijer of Unilever Best Foods NA, Richard Muesing of George Washington University, and Matthew Kramer of Biomedical Consulting,⁽²⁾ 53 men and women were fed controlled diets with two different types of salad dressings each containing 3.6 g PSE. Diets having dressing without PSE and with PSE were provided for three weeks and crossed over randomly. Switching from a self-selected baseline diet to a controlled diet with no PSE resulted in a significant reduction of low-density lipid cholesterol (LDLC) of 7.9, high-density lipid cholesterol (HDLC) of 3.1%, and triglyceride (TC) of 9.2%. Daily consumption of 3.6 g PSE resulted in further decreases in LDLC of 9.7% and TG of 7.3% with no effect on HDLC.

As the benefits of PSEs and other lipids are demonstrated, the ability to quantitatively analyze them becomes more important. Gas chromatography (GC) provides a powerful quantitative analysis method. However, for lipids to be detected and quantified by GC, they must have a boiling point within the GC temperature range. Many common lipids, such as phospholipids, have boiling points that are too high to separate and analyze accurately with GC. The commonly-used ultraviolet (UV) detector is not quantitative for lipids because analytes must possess at least one carbon-carbon double bond to be detected: Many lipids are saturated and invisible to UV or have varying levels of saturation. Refractive index (RI) detectors work well in a number of HPLC quantitative analysis applications, however, they are limited to isocratic analysis with a single solvent while gradient analysis with multiple solvents works better with most lipids because it provides superior separation. For these reasons, evaporative light scattering detectors (ELSDs) have become the standard method of quantitative analysis of lipids on HPLCs. Several successive generations of ELSD detectors have provided increasing levels of sensitivity and durability.



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Figure 1. Separation of nonpolar lipid class standards using a normal-phase HPLC method and the CAD detector.⁽⁶⁾

Matreya Non-polar Lipid mix B

A = Cholesteryl oleate (20 ng)

B = Methyl oleate (20 ng)

C = Triolein (20 ng)

D = Oleic Acid (20 ng)

E = Cholesterol (20 ng)

Note that B was totally evaporated and D was partially evaporated in during detection and the other three lipids had similar detector response.



Figure 2. The Corona charged aerosol detector.

Search for improved analytical tools and methods

In the CAD, HPLC column eluent is first nebulized with nitrogen and the droplets are dried to remove the mobile phase, generating an aerosol stream composed of particles of all non-volatile constituents. A secondary stream of nitrogen becomes positively charged as it passes a high-voltage, platinum corona wire. The secondary stream merges with the primary particle stream to turbulently mix the gas ions with the aerosol particles. The secondary stream's charge transfers to the opposing stream of analyte particles. The charge is transferred to a collector where it is measured by a highly-sensitive electrometer, generating a signal in direct proportion to the quantity of analyte present.

Dr. Moreau has recently been involved in developing methods to analyze intact lipids, such as triacylglycerols (sometimes called triglycerides) and phytosterols (plant sterols) in vegetable oils and phospholipids in biological membranes. He is particularly interested in determining differences in lipids in different types of vegetable oils, such as corn, olive, and sunflower oils. With normal-phase HPLC analysis, lipids are separated into "lipid classes" such as triacylglycerols, diacylglycerols, free fatty acids, and free sterols. Each of these lipid classes contains a mixture of molecules (molecular species), usually with different combinations of esterified fatty acids. After separating lipid classes via normal-phase HPLC, Moreau uses reverse-phase HPLC to separate each class of lipid, such as triacylglycerols into their individual molecular species. Examples of triacylglycerol molecular species include glycerol-trioleate, the most abundant triacylglycerol molecular species in olive oil, and glycerol-linoleate-linoleate-oleate, the most abundant triacylglycerol molecular species in corn oil. In some edible oils such as fish oils, there may be as many as a hundred different molecular species of triacylglycerols, whereas in oils rich in mono-unsaturates, such as olive oil and canola oil, the number of triacylglycerol molecular species may be less than ten.

Using the CAD

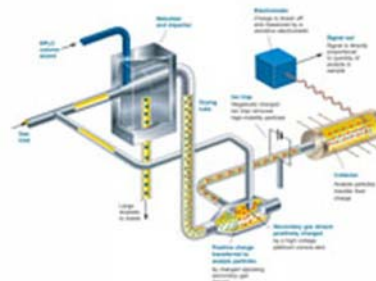
Dr. Moreau had previously developed numerous separation methods based on the use of ELSD^(3,4,5) and followed these establishing protocols in evaluating the new CAD technology. He used the Corona CAD for HPLC (ESA Biosciences, Chelmsford, Mass.). The CAD detector is simple to set up and operate. One of the few parameters that need to be adjusted on the detector is maximum signal strength: Moreau used the highest possible value (range – 500 pA). He purchased standard mixtures of polar and nonpolar lipids from Matreya LLC, Pleasant Gap, Penn. The polar lipid mix includes cholesterol, phosphatidyl ethanolamine, lecithin, and lysolecithin while the nonpolar lipid mix consists of cholesteryl palmitate, tripalmitin, palmitic acid, and cholesterol. The two characteristics that he was most interested in comparing to ELSD were the minimum detection limits and mass to peak area ratio.

Data was sent to a software program that allows Dr. Moreau to manipulate the chromatograms and zoom in on the peaks to identify small amounts of analyte, which are often injected in microgram to nanograms levels. He also constructed calibration curves that plot mass against peak area and are used to perform quantitative analysis. Quantitative analysis is easiest when the calibration curves are linear; however, this is not essential. He examined the effects of different HPLC solvents on minimum limits of detection and the mass/signal ratio. The data obtained from the CAD is not linear but it is usually close to linear over a typical range of

results and it can be linearized by fitting it to a quadratic equation. The CAD detected common lipids such as triacylglycerols, diacylglycerols, glycolipids, phospholipids, and sterols. Lower molecular weight lipids and the volatile fatty acid methyl esters as expected were not detected by the CAD.

CAD shows high sensitivity and linearity

The minimum limits of detection of the CAD with lipids varied with different mobile phase solvents. Using solvent systems that were predominantly hexane, the minimum limits of detection of triacylglycerols, cholesterol esters, and free sterols were about 1 nanogram per injection. This compares to 50 to 100 nanogram minimum limits of detection with an ELSD. Using hexane predominantly, the mass to peak area ratio was nearly linear from the range of about 1 nanogram to about 10 micrograms per injection. The mass to peak ratio was substantially more linear with CAD when compared with ELSD, especially around the lower limits of detection. Three other solvents commonly used for HPLC lipid analysis (methanol, isopropanol, and acetonitrile) caused higher levels of background noise and higher minimum limits of detection. These preliminary results indicate that the CAD has the potential to become a valuable tool for the quantitative HPLC analysis of lipids.



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Figure 3. Diagram of the Corona CAD.

About the Author

Jerry Fireman, president of Structured Information, has written extensively in the area of scientific instrumentation and drug discovery. ESA Biosciences, the manufacturer of the Corona CAD used in this study, specifies that, as with mass spectroscopy, the detector be used only with volatile mobile phase additives. While CAD presents a strong alternative for quantitative analysis of lipids, long-term studies are needed to confirm that the results are reproducible and that the instrument is durable and reliable.

References:

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