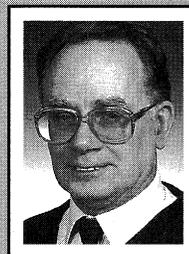


The impact of the ELSD on lipid research

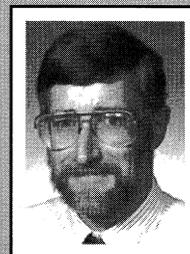
The evaporative light-scattering or "mass" detector (ELSD) is often portrayed as a new instrument, the potential of which might not be recognized by many lipid analysts. In fact, a commercial model has been available since the early 1980s. Indeed, the first paper describing the construction and principles of an evaporative light-scattering detector appeared more than 20 years ago (1), while the first paper describing an application to lipids was published in 1982 (2). One of the authors (WWC) purchased a detector of this type in 1984, after being impressed by a demonstration of its use to monitor the separation of milk triacylglycerols by reversed-phase high-performance liquid chromatography (HPLC) in the laboratory of the late Robert Macrae at Reading University in the United Kingdom. Having been accustomed to the problems of baseline drift with ultraviolet (UV) or refractive index detection, it appeared amazing to find a baseline that was absolutely steady even when steep gradients were applied. The technology can now be considered as mature, therefore, and at least three manufacturers offer commercial instruments. A number of brief reviews of the topic of evaporative light-scattering detection in lipid analysis have appeared together with one more comprehensive review (3).

The principle of detection is quite simple. The solvent emerging from the end of the HPLC column is evaporated in a stream of air or nitrogen in a heating chamber. The solute does not evaporate, but is nebulized and passes in the form of minute droplets through a light beam, which is reflected and refracted. The amount of scattered light is measured and bears a relationship to the amount of material in the eluent. There are no special wavelength requirements for the light source, and in some commercial instruments, it is simply a projector lamp. Although the first commercial instrument, manufactured by ACS Ltd. (Macclesfield, United Kingdom), was rather crude in that control of temperature and nebulizer gas flow

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was poor, it worked reliably for one of the authors for nearly 10 years.

Such detectors can be considered as universal in their applicability, in that they respond to any solute that does not evaporate before passing through the light beam. Almost any solvent of sufficient volatility, including ketones, esters and chlorinated and aromatic compounds, can be used in complex gradients. Up to 20% of water is permissible. The detectors themselves give excellent results under gradient elution conditions and are simple and rugged in use, with little baseline drift during continuous operation even with abrupt changes in solvent composition. However, as with all detectors, there are disadvantages. A source of dry, filtered compressed air that is capable of delivering 1–5 L/min is required. Since a standard cylinder of air or nitrogen can be emptied in as little as four hours, we recommend either the use of an air compressor or a source of nitrogen gas generated from stored liquid nitrogen. The effluent containing the evaporated solvent must be conducted to the outside of the laboratory or into a fume hood. Although organic ions, such as triethylamine with acetic acid, are permissible at low levels, inorganic ions cannot be incorporated into the mobile phase.

Although the detector is destructive in that the sample is lost, a stream splitter can be inserted at the end of the column to divert much of the sample to a collection device.

Quantification

It is essential to recognize that the ELSD does not give a rectilinear response to analyte concentrations. Although this is inconvenient, it need not inhibit good quantification. In the most comprehensive practical and theoretical investigations, use was made of one commercial detector but the results appear to be applicable to others (4,5). These authors reported that the detector response increased sigmoidally with increasing analyte concentration in a manner that was dependent on changes in the size distribution of particles in the aerosol. Thus at low solute concentrations, the solute particles scattered light to a proportionately lesser extent. As the diameter of the droplets began to approach the wavelength of light, they no longer affected its passage and the response fell off rapidly. The detector response was close to linear over a concentration range of about two decades before tending to plateau. In a custom-built detector incorporating a laser light source, the response was found to be related to the solute mass raised to the power of 1.35 (6), a relationship which has proved useful in a number of analytical circumstances. At least one manufacturer is offering a "lineariser" in which this formula is used electronically to "improve" the results.

To maximize the response and the linear range, it is necessary to adjust the flowrate of the nebulizer gas and the temperature of the evaporator chamber to the optimum to generate

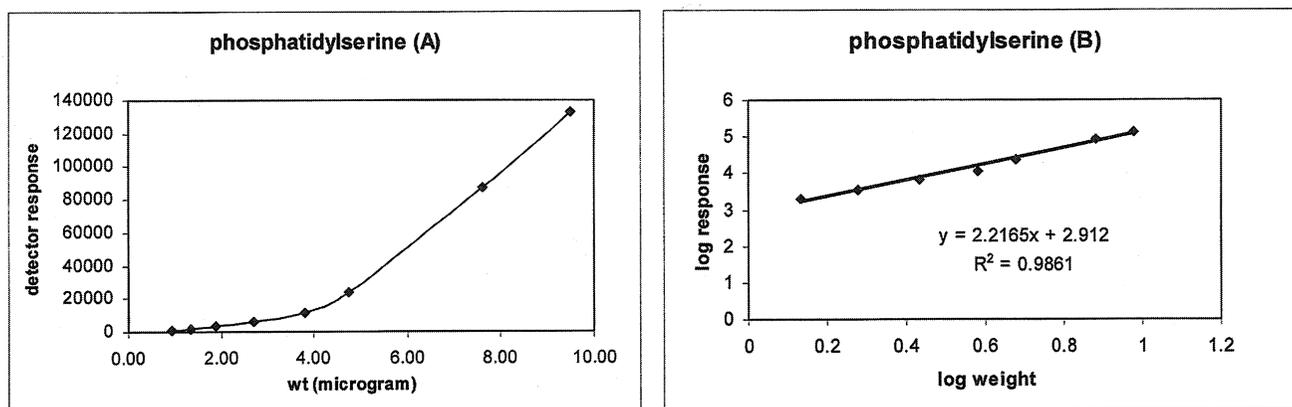


Figure 1. Calibration curve for phosphatidylserine with an ELSD and normal-phase chromatography (7). A = plot of response vs. sample size; B = log response vs. log sample weight.

aerosol particles that are relatively uniform in size. Most solvents give similar responses, though some differences may be noted with chlorinated solvents. Finally, the response is dependent on the refractive index of

the analytes and thus might be expected to vary with changes in structural features of lipids. Thus each lipid class has a different response, but molecular species within a lipid class do not vary significantly. However,

sensitivity is closely related to peak width, so optimal quantitative results are obtained under gradient elution conditions when peak widths are relatively constant. Apparent response factors for molecular species tend to increase under isocratic elution conditions.

In practice, the first step in quantitative work with an ELSD is to work out the optimal conditions for the desired separations. Subsequently, a calibration is conducted using lipid standards which are as close as possible in composition to the material to be analyzed. The operating parameters for the instrument, such as gas pressure, evaporator temperature and attenuation, must also be rigorously standardized. If the elution conditions or detector settings have to be changed later for any reason, a tedious recalibration is necessary.

Figure 1 shows calibration curves for phosphatidylserine obtained under conditions of normal-phase chromatography (7). The sigmoidal nature of the response is evident from the graph of response vs. sample size, and it is also apparent that the response approaches linearity at the higher masses. As might be anticipated, a linear relationship is obtained for the graph of log response vs. log sample weight. The same type of curvilinear relationship has been found in many studies. It should be noted that some lipids of relatively low molecular weight, such as fatty acid methyl

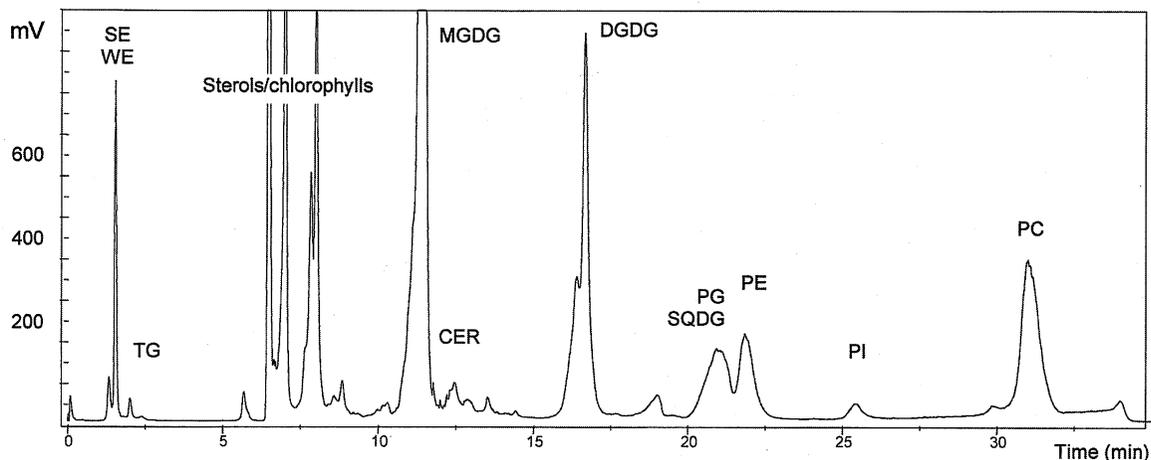


Figure 2. A sample HPLC-ELSD chromatogram showing the separation of nonpolar and polar lipid classes from the leaves of *Arabidopsis thaliana*, obtained by the procedure described in Reference 7. Abbreviations: SE, sterol fatty acyl esters; WE, wax esters; TG, triacylglycerols; MGDG, monogalactosyldiacylglycerols; CER, glucocerebrosides; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine

esters, can evaporate partially in an ELSD so that accurate quantification is impossible; this behavior is highly dependent on the model of commercial instrument. A further practical hint is that it is advisable to have the sample size such that it is neither over- nor underloaded, and ideally with the main peaks at 80% or more of full-scale deflection.

It is possible to use an internal standard to improve direct quantification with an ELSD. For example, a synthetic phospholipid, phosphatidylmethylethanolamine (dipalmitoyl), has been used in this way in order that the absolute amount of phospholipid in an extract could be determined in addition to the relative compositions of each phospholipid class. This lipid is only present naturally at very low levels in tissues so endogenous material does not interfere with the standard.

Areas of lipid research most affected by HPLC-ELSD

Innumerable references to applications of the ELSD in lipid separations have appeared, so we have had to be highly selective in choosing those to discuss here because of space limitations. The authors' own experiences and biases may also be apparent.

Simultaneous analysis of nonpolar and polar lipid classes

Because of the diverse range of polarities encountered among the various lipid classes, development of HPLC-ELSD methods for the analysis of such analytes was difficult but was a task of such importance that it was tackled quickly. The first published method (8) employed a silica gel column, and a ternary gradient system (isooctane/tetrahydrofuran/isopropanol/chloroform/water), to separate nonpolar and polar lipid classes from animal tissues. Others have adapted this system in many ways (often replacing isooctane with hexane) to optimize separation of lipids from many types of animals, plants (Figure 2) and microbes, retaining the essential hexane/isopropanol/water blend (isopropanol appears to be the only solvent capable of mediating the change from hexane- to water-based mixtures), but including various volatile acids, bases, or buffers (9). A reason for omitting chloroform from normal-phase hexane/isopropanol/water systems is that UV detection in the 205–210 nm range can then be used in tandem with the ELSD, providing useful information about the degree of unsaturation of individual lipid components (10). Diol or PVA-SilTM (bonded-polyvinyl alcohol) columns are now being widely used instead of silica gel.

The choice of solvent in which to dissolve and inject the sample is very important when the procedure involves simultaneously analyzing both nonpolar and polar lipid classes via HPLC-ELSD. Traditionally, HPLC textbooks have recommended dissolving the sample in the same solvent blend used for the initial portion of the gradient. For the hexane/isopropanol/water gradients, this would require dissolving the sample in hexane, in which many polar lipids are not very soluble. In order to remedy this problem, most samples can be dissolved in chloroform or chloroform/hexane mixtures. However, when samples are dissolved in these polar solvents, resolution of the nonpolar lipids is best when the injection volume is kept to the minimum (i.e., 5 to 10 mL). When accurate resolution of nonpolar lipids is a high priority, it may also be beneficial to dissolve the sample in hexane and use methods such as those described in the next section.

Analysis of nonpolar lipid classes

Nonpolar lipid classes (including sterol esters, wax esters, triacylglycerols, diacylglycerols, free fatty acids, sterols, tocopherols, and several others) have been successfully analyzed by several HPLC-ELSD gradient and isocratic systems. We have recently

published two different approaches. One involves the use of a cyanopropyl column, and a binary gradient of hexane/methyl *t*-butyl ether (11). The other employs a diol column, and a binary gradient of hexane/isopropanol/acetic acid (10). Unfortunately, in each of these nonpolar methods, lipids less polar than sterolfatty acyl esters elute very early, often in the solvent peak, and are not adequately resolved (i.e., β -carotene, squalene, and other hydrocarbons).

Analysis of polar lipid classes

The term "polar lipid" usually includes phospholipids and glycolipids. Numerous HPLC-ELSD methods have been reported for the analysis of phospholipids, and most of these include isocratic or binary gradients based on either hexane/isopropanol/water or acetonitrile/methanol/water (3,9). Because all phospholipids contain an acidic phosphate group, and some contain other charged moieties, many successful HPLC-ELSD systems contain volatile acids, bases, or buffers to maintain constant charge. In addition, the high levels of lysophospholipids in some biological tissues make chromatograms more complex. Some types of phospholipids, such as phosphoinositides (derivatives of PI that include additional phosphates on the inositol), are very polar, and methods for their successful analysis have only recently been reported (12).

Another often-overlooked phospholipid class is sphingomyelin (an abundant polar lipid in most animal tissues such as muscle and blood) and other phosphosphingolipids. Many of the above methods reported for the analysis of common glycerolphospholipids also resolve sphingomyelin in the same chromatograms. We recently reported an HPLC-ELSD method for the quantitative analysis of a new type of phosphosphingolipid, ceramide-phosphorylethanolamine, in several fungal species (13).

Only a few methods for the HPLC-ELSD analysis of glycolipid classes have been reported. Most plant tissues, especially green photosynthetic tissue, contain high levels of glycolipids, and several gradient methods

have been reported for the combined analysis of both glycolipids and phospholipids (7,14-16). One of the problems in this field, which has not yet been adequately solved and needs more attention, is that most plant tissues contain three different types of monoglycolipids (monogalactosyldiacylglycerols, glucocerebrosides, and sterol glycosides) that are very difficult to resolve adequately in gradient HPLC-ELSD systems.

Analysis of unusual lipid classes

In addition to the common types of glycerolipid classes mentioned above, HPLC-ELSD methods have been reported for less common lipids such as oxylipins, synthetic fat substitutes, and hopanoids. The last example is a case where the presence of hopanoids in several bacterial species (and in the symbiotic nitrogen-fixing root nodules of several tree species) eluded detection for many years, until the lipids were examined *via* HPLC-ELSD (17).

Analysis of molecular species of acylglycerols

Many methods have been published for the separation of molecular species of triacylglycerols and phospholipids *via* reversed-phase HPLC-ELSD. Most of these have employed a C-18 column and predominantly methanol- or acetonitrile-based mobile phases with considerable success (18). For certain samples, such as fish oils, the extremely large number of peaks of individual triacylglycerol molecular species has resulted in very complex chromatograms with unacceptable resolution of many of the peaks. An alternative perspective on the problem was the development of silver-ion HPLC columns, which separate groups of molecular species into fractions based on numbers of C-C double bonds only and not by chainlength (19). The combination of silver-ion chromatography and reversed-phase chromatography has been used successfully to resolve samples containing large numbers of triacylglycerol molecular species, but incorporation of silver nitrate into mobile phases in some cases causes other problems (18).

Because of the similar charges of most types of phospholipids, the

HPLC-ELSD methods for the separation of their molecular species have been applicable to several phospholipid classes (20). Currently, the only successful method for the analysis of molecular species of phosphatidic acid requires the use of inorganic buffer salts (precluding detection *via* ELSD), and it employs UV detection (21). In addition, methods for the HPLC-ELSD analysis of molecular species of lysophospholipids have been reported (22). The ELSD has also been used for the analyses of molecular species of digalactosyldiacylglycerols (23).

Conclusion

The major advantage of all HPLC-ELSD methods is that they allow the quantitative analysis of all types of nonvolatile lipids. Although the analysis of certain types of lipids (e.g., fatty acid methyl esters and

free sterols) is technically feasible via HPLC-ELSD, we would be remiss if we did not point out that GLC-FID methods are more sensitive and are still the method of choice. A few types of lipids possess strong chromophores (e.g., cinnamic acid esters) or fluorophores (e.g., tocopherols), and detection of these types of lipids by ELSD may not be the most sensitive method, but only with detection via ELSD is it possible to compare the levels of these compounds with all other lipids in a sample. Nonetheless, we are confident that when the book *The History of Lipid Analytical Technology in the Twentieth Century* is written, it will not only include chapters on GC, TLC, and HPLC but will also document the impact of ELSD.

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Disclaimer

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