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LIGHT-SCATTERING DETECTION IN LIQUID CHROMATOGRAPHY

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SUMMARY

A novel detection system for liquid chromatography is described which consists of post-column solute precipitation and light-scattering measurement of the formed precipitate. Its efficiency is demonstrated by the sensitive detection of non-polar lipids of biological origin separated by reversed-phase chromatography. A linear relationship between the square root of the detector response and sample concentration was found for several lipid solutes. Detection sensitivity below 10^{-6} g has been achieved with the present system. Some response-affecting factors were also investigated.

INTRODUCTION

During recent years, certain advances in selective detection have contributed significantly to expanding the scope and applications of high-performance liquid chromatography (HPLC). In many instances, detector selectivity reduces or eliminates the problems of interfering compounds in complex sample matrices[1,2], thus making less demands on modern liquid chromatography (LC) for sample purification. This feature has been particularly exemplified by successful utilizations of spectrophotometric, fluorimetric, and electrochemical detectors.

Among the spectroscopic principles employed in LC detection, light-scattering measurements have been almost non-existent. Long before HPLC matured as an important analytical method, Tappan[3] described the use of nephelometry to measure the protein content in eluates from Sephadex columns. More recently, advances in laser technology and associated optics have permitted observations of low-angle light-scattering from large molecules separated by gel chromatography[4,5]; subsequent to such measurements, accurate molecular weights and other important parameters of the macromolecules can be determined.

The present communication describes the design and selected applications of a small-volume light-scattering detector for HPLC. Whereas detector performance has been demonstrated for non-polar lipids in this publication, selective detection of other types of compounds is feasible through a suitable combination of chromatographic conditions and post-column chemistry. Possibilities for precipitation of

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solutes include decreasing solubility, reaction to produce associated precipitates, and reaction to form insoluble complexes.

Analytical aspects of light-scattering detection which were carried out with standard lipid solutes include response *versus* concentration measurements, determination of sensitivity and investigations of parameters affecting response (ionic strength of the precipitation agent and the ratio of column effluent and precipitation agent flow-rates).

In the chromatographic analysis of lipids, detection conditions were found to be fully compatible with the choice of optimum column parameters. This is demonstrated by the efficient reversed-phase chromatography of lipid samples derived from a vegetable oil and the Folch extract of human serum.

EXPERIMENTAL AND RESULTS

Detector design

The instrumental arrangement used in this study is illustrated in Fig. 1. Precipitation agents are added to the column effluent through a tee to initiate precipitation of solutes. A mixing coil following the tee is necessary for adequate mixing of the two solvents. The mixture then enters the detection cell where the scattered light is measured.

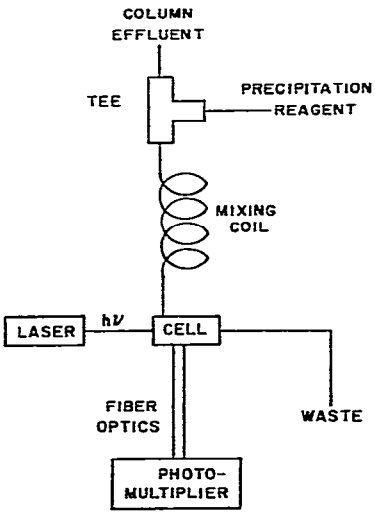


Fig. 1. Schematic diagram of detection system.

Fig. 2 shows a more detailed schematic diagram of the detection cell assembly. Solutes enter a glass light-scattering cell (approximately 17-μl volume) through a stainless-steel aperture. A 0.5-mW helium–neon laser (Model 155, Spectra Physics, Mountain View, Calif., U.S.A.) operating at 633 nm provides the incident light beam. A laser was chosen as the light source to provide easy alignment with the cell and thus minimize background scatter from the inner walls of the cell. Scattered light is transmitted from the cell to the photomultiplier tube (Type R 372, Hamamatsu, Japan)

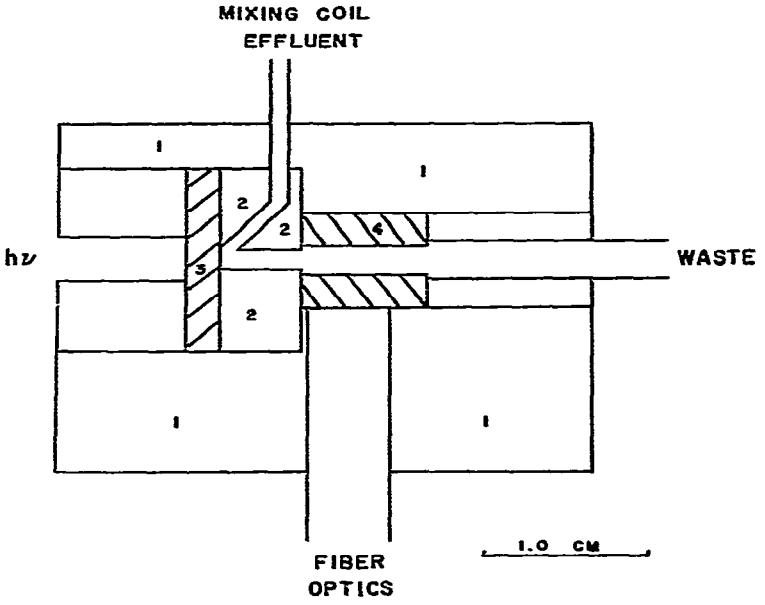


Fig. 2. Simplified schematic of light-scattering detection cell. 1 = Stainless-steel detector block; 2 = stainless-steel entrance aperture; 3 = Pyrex window; 4 = Pyrex scattering cell.

via fiber optics (Edmund Scientific, Barrington, N.J., U.S.A.). Although the fiber optics are arranged at 90° to the incident laser beam, the cell geometry and fiber optics allow collection of scattered light from a larger solid angle. Associated electronic equipment is a high-voltage power supply (Model 244, Keithley, Cleveland, Ohio, U.S.A.) for the photomultiplier tube, and an electrometer (Model 616, Keithley) for signal amplification.

Analytical performance

Detection parameters were investigated with non-polar lipids using a 30 cm × 3.9 mm I.D., μBondapak C_{18} reversed-phase column (Waters Assoc., Milford, Mass., U.S.A.). The mobile phase consisted of a 2:1 (v/v) mixture of acetone and methanol. Post-column precipitation of solutes was accomplished by adding an ammonium sulfate solution to the column effluent. The resulting change in solvent composition was sufficient to precipitate non-polar lipids. Cholesterol, cholesterol esters and triglycerides (Applied Science Labs., State College, Pa., U.S.A.) were used as standards.

High-pressure reciprocating piston pumps (Model M 6000, Waters Assoc.) were employed to deliver both the mobile phase and the precipitation agent. Complete degassing of both solvents was found necessary to prevent the formation of bubbles upon their mixing. All investigations were carried out using a mobile-phase flow-rate of 1.0 ml/min.

It can be expected that the detector response is affected by both solvent composition and dynamic conditions of detection. The effect of flow-rate of the precipitation agent on detector response for three different solutes is shown in Fig. 3. Flow-rates below 0.5 ml/min are of little value because of the volumes of the mixing

coil and the light-scattering cell. While cholesterol shows maximum response at 1.5 ml/min, the signals generated by cholesterol esters and triglycerides gradually decrease with increasing flow-rate of the precipitating agent. Decreased response at low flow-rates is probably due to a partial solubility of the lipid in the solvent medium (insufficient water content), while the decreased responses at higher flow-rates appear to result from excessive dilution of the precipitate. Cholesterol requires more aqueous medium for a more complete precipitation than cholesterol esters or triglycerides. Although detection of individual lipids can be somewhat optimized through adjustment of flow-rate, low values must be avoided to prevent anomalous shapes of chromatographic peaks.

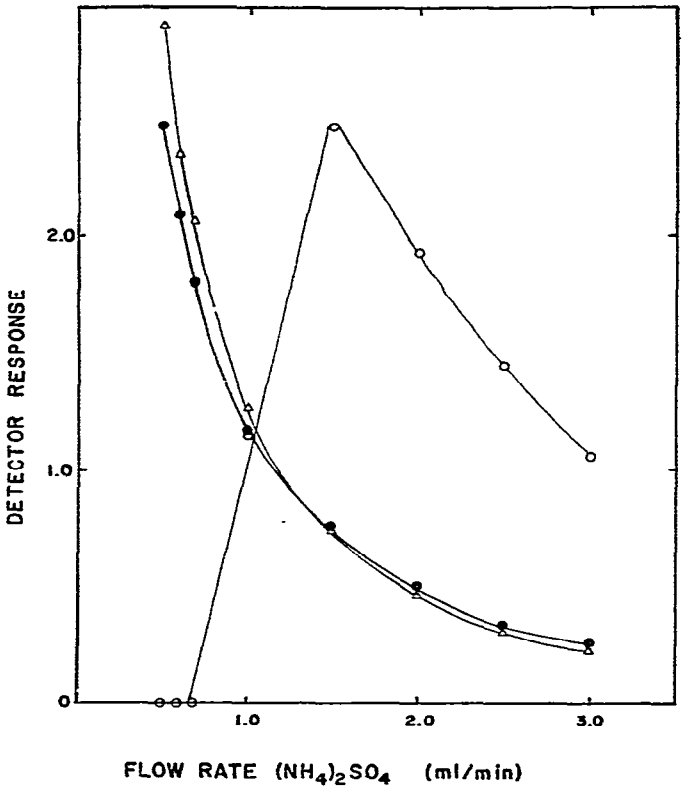


Fig. 3. Dependence of detection response on flow-rate of precipitation agent. Conditions: column flow-rate, 1.0 ml/min; ammonium sulfate concentration, 0.025 *F*. Detector responses were measured as relative peak heights of the solutes chromatographed (approximately 10 μg each). ○ = Cholesterol; △ = cholesteryl palmitate; ● = triolein.

The effect of the ionic strength of the precipitation agent on the detector response is demonstrated in Fig. 4. Whereas for cholesterol only a modest enhancement of response (50% increase) is seen at high salt concentration, a seven-fold increase is observed for triolein (other triglycerides and cholesterol esters behave similarly). A "salting-out" effect is most likely responsible for the enhanced response. At ammonium sulfate concentrations of 0.2 *F* and above, detector operation becomes

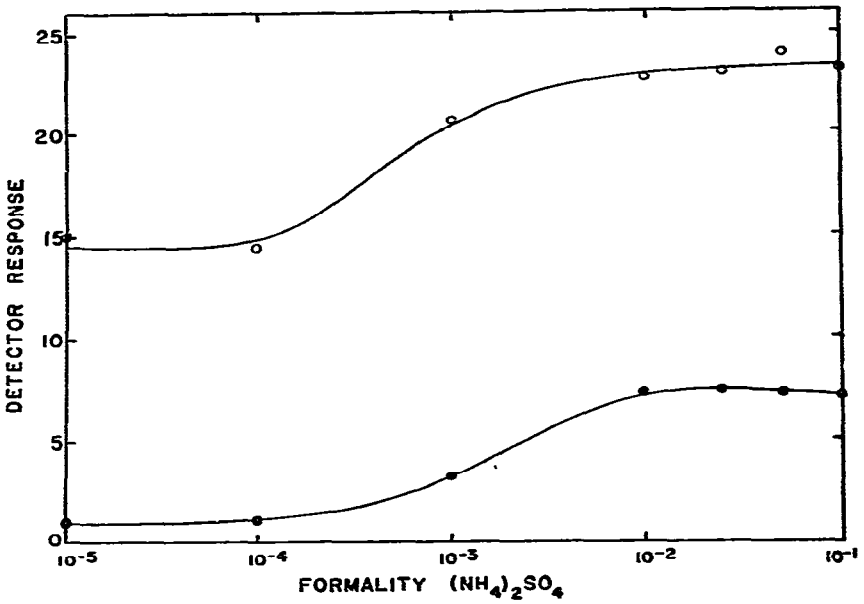


Fig. 4. Dependence of detector response on ammonium sulfate concentration in precipitation agent. Conditions: column flow-rate, 1.0 ml/min; precipitation agent flow-rate, 2.0 ml/min. Detector responses were measured as relative peak heights of the solutes chromatographed (approximately 10 μg each). ○ = Cholesterol; ● = triolein.

difficult (increased noise level), probably owing to increased viscosity and incomplete mixing.

Investigations of the detector response as a function of sample size indicate that the response is proportional to the square of the sample weight. Fig. 5 shows the square root of detector response plotted against the sample weight for several compounds. These curves intercept the origin and are essentially linear with small samples, but negative deviations from linearity are seen at greater values. It is very likely that these deviations are caused by an "end-absorption" effect, where the cell is not uniformly illuminated throughout its entire length owing to the larger amounts of precipitate.

Optimization of detector parameters is not particularly difficult, and satisfactory operation is rapidly achieved. A high-sensitivity recording of several standard lipids is shown in Fig. 6. Detection limits for the system as described are typically 0.5 μg.

Applications

Reversed-phase chromatography combines well with the precipitation detection system, putting no particular constraints on optimal chromatographic or detection conditions. Fig. 7 shows the separation of triglyceride components of linseed oil with retention order essentially determined by carbon number and number of unsaturations[6]. Fig. 8 is the chromatogram of a Folch extract of human blood serum[7]. Although no identification of the components of this extract was carried out, it is

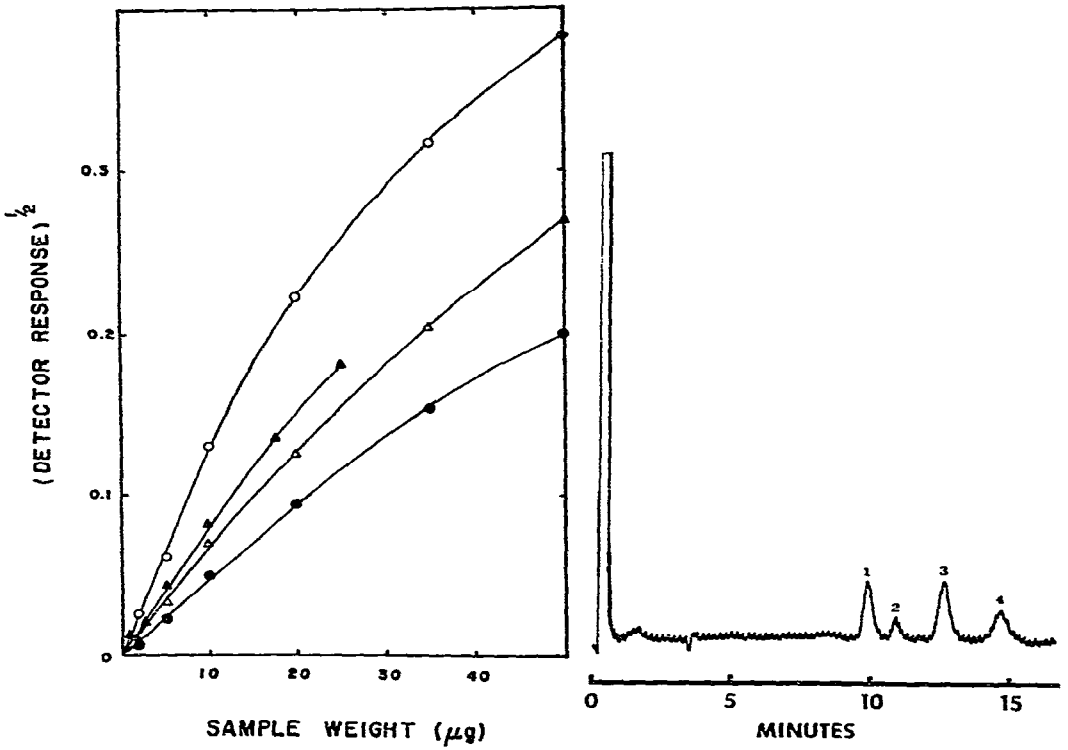


Fig. 5. Dependence of square root of detector response on sample size. Conditions: column flow-rate, 1.0 ml/min; precipitation agent concentration, 0.1 *F* ammonium sulfate; precipitation agent flow-rate, 2.0 ml/min. Detector responses were measured as relative peak heights of the solutes chromatographed. ○ = Cholesterol; ▲ = cholesteryl linoleate; △ = triolein; ● = tristearin.

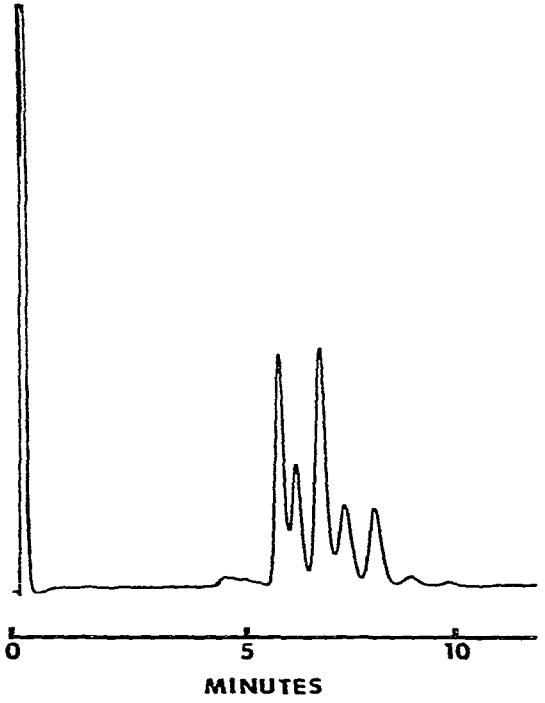
Fig. 6. Chromatogram of standard lipids at high sensitivity. Conditions: column flow-rate, 1.0 ml/min; precipitation agent concentration, 0.025 *F* ammonium sulfate; precipitation agent flow-rate, 0.5 ml/min. 1 = Triolein (2 μg); 2 = cholesteryl linoleate (1 μg); 3 = cholesteryl palmitate (2 μg); 4 = tristearin (2 μg).

probable that the initial peak is cholesterol and the later group of peaks is due to triglycerides.

DISCUSSION

The LC detector described in this publication is based on the inherently sensitive measurement technique of light scattering. It is a selective detector with many desirable characteristics. Most importantly, the detector is simple in both design and operation. Varying degrees of selectivity can be achieved through a proper choice of precipitation agent. The detection principle should be compatible with gradient elution techniques.

Lack of sensitive detection has long been experienced in LC of lipids where the less suitable general-purpose detectors are often the only alternative[8,9]. The light-scattering detector described here provides the necessary sensitivity for detection of non-polar lipids while being non-destructive of sample. The lipids investigated in this



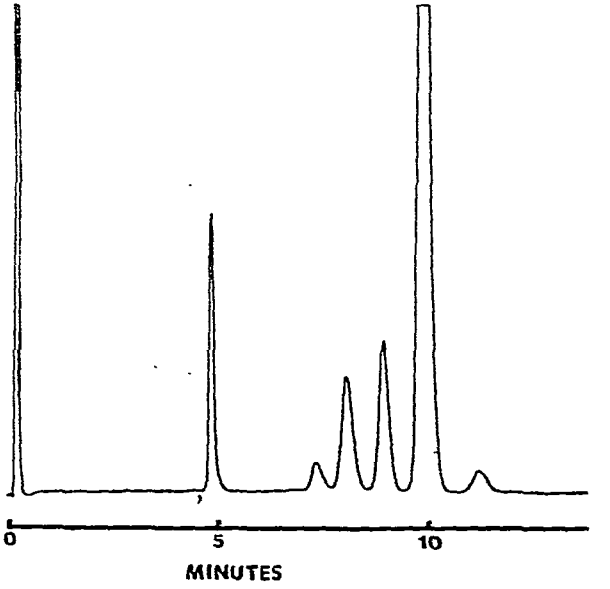


Fig. 7. Chromatogram of linseed oil (100 μg). Conditions: column flow-rate, 1.0 ml/min; precipitation agent concentration, 0.025 *F* ammonium sulfate; precipitation agent flow-rate, 2.0 ml/min. Detected peaks are various triglycerides.

Fig. 8. Chromatogram of non-polar lipids of human serum (sample size equivalent to 50 μl serum). Conditions: same as in Fig. 7 except that precipitation agent flow-rate was 0.5 ml/min. The first peak (retention time = 5 min) is cholesterol, the other peaks are triglycerides and cholesterol esters.

work show comparable response factors —a fact of definite practical value in their analysis.

The selected applications shown in this work suggest additional utility of this detector to various analytical problems concerning lipids and related classes of compounds. Thus, more specific biomedical applications may be extended toward the study of lipid storage diseases and other conditions where alterations in lipid metabolism are likely. Speed, sensitivity, and simplicity of this method may be clinically significant for the simultaneous determination of cholesterol, its esters, and triglycerides. In addition, detection sensitivity should allow investigations of biological specimens with lower lipid content (*e.g.*, cerebrospinal fluid) and plasma lipids from infants where the sample size is limited.

The separation of vegetable oil components (Fig. 7) suggests applications to profiling plant and animal fats, waxes, triglycerides, sterols, non-polar terpenes, etc. A combination of light-scattering detection with efficient reversed-phase LC on small-particle columns can be an attractive substitute for gas chromatography of such mixtures which often need to be carried out at temperatures above 300°.

The practical limit of detection for lipids is approximately 0.5 μg with the current system. Major sources of noise were the instability of pumps (pulsing) and

incomplete mixing of the mobile phase and precipitation agent. Noise from both sources could undoubtedly be minimized if syringe-type pumps were used. Thus, even lower detection limits appear feasible. Frequent degassing of the solvents (approximately every 2 h) was found necessary. This problem should also be minimized through the use of syringe pumps.

Certain changes in detector design should further improve its analytical characteristics. Investigations of mixing coil dimensions and volume may contribute to sensitivity enhancement while retaining chromatographic efficiency. Redesign of the detector cell geometry should reduce the "end-absorption" effect and extend linearity to higher concentrations.

The present study gives an example of precipitation based on a decreased solute solubility due to an altered solvent composition. However, this represents just one mode of forming precipitates, and further applications of the light-scattering detection principle in conjunction with solute chemical alterations should be feasible. For example, many classical analytical precipitations could be used to detect various ions. Another method of precipitation may involve the reaction of solute leading to an insoluble product or by-product. An example is the reaction of permanganate with an oxidizable solute to produce insoluble manganese dioxide.

In conclusion, the light-scattering detector appears to be a valuable addition to the family of LC detectors, and it may serve well in cases where other existing detection techniques fail. Through the adaption of many of the qualitative precipitation reactions generally known from the literature, various levels of selectivity can be attained. These may range from very specific and functional group detections to "selectivity" in a broader sense. It should be emphasized that a maximum utilization of the light-scattering detector will always be strongly dependent on proper design of post-column chemistry.

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