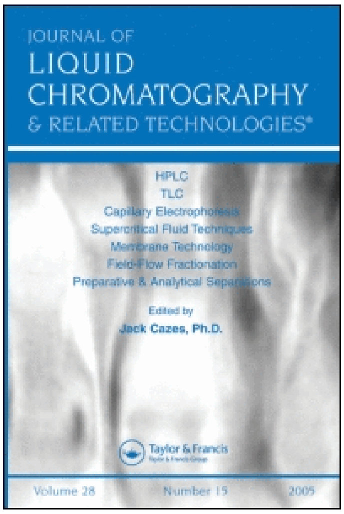

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COMPARISON OF THE EVAPORATIVE LIGHT SCATTERING DETECTOR (ELSD) AND REFRACTIVE INDEX DETECTOR (RID) IN LIPID ANALYSIS

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ABSTRACT

The properties of an evaporative light scattering detector (ELSD) and a refractive index detector (RID) in isocratic HPLC analysis of lipids are compared. The effect of molecular structure, especially the unsaturation level, on the detector response is studied using eight different lipid standards. Linearity and sensitivity are compared. Quantitation of an unknown sample is also performed.

The sensitivity of the ELSD was slightly better than that of the RID, the detector limits of triolein being 30 ng/injection and 50 ng/injection, respectively. The linearity of the ELSD was considered only approximate compared with that of the RID the response factors of the calibration curves being 0.9554 and 0.9999, respectively. The effect of the molecular structure on the detector response was higher with the RID than with the ELSD. The relative standard deviation (SD%) of responses was 23% for the RID and 12% for the ELSD. The unsaturation level had a greater effect on the detector response of the RID than on that of the ELSD.

This study indicates that a standard deviation (SD) of at least 13% is to be expected with the ELSD and an even higher SD with the RI detector when quantitating heterogeneous mixtures or unknown compounds.

INTRODUCTION

Mass detectors or "universal" detectors such as the refractive index detector (RID) and the evaporative light scattering detector (ELSD) are most commonly used in HPLC analysis of lipids. Lack of suitable chromophores in lipid molecules limits the use of other HPLC detectors such as UV and fluorescence detectors. ELSD has rapidly gained popularity among lipid analysts, one of its advantages being the possibility of using even complex gradients. Recently, Christie (1) reviewed the different detectors for HPLC analysis of lipids and concluded that, in spite of its limitations, the ELSD is superior to the other detector types available.

The theory of the ELSD has been discussed in several papers (1-6). It is a suitable detector for all types of compounds that are relatively non-volatile. In the light scattering detector the chromatographic solvent is first nebulized by a gas stream, and the vapor enters a heated tunnel, where the solvent evaporates. The leaving analyte particles pass through a narrow light beam, and the scattered light is collected by a photomultiplier. The response of the ELSD depends on the number and size of the analyte particles and is claimed to be largely independent of the chemical composition of the compounds studied. However, several parameters, such as molecular structure and the melting point of the molecule, have been reported to affect the response of the ELSD to some extent. The chromatographic conditions and the detector parameters also have an effect on the response (1-10).

Several ELSD applications to lipid class analyses have been presented (10-21). Individual standards for each compound have usually been used for quantitative analysis (12,14,17-19). However, several scientists (1,4,9,22) have suggested the possibility of accurate quantitative estimation of even unknown compounds, though careful calibration and detector parameter adjustment are necessary.

The present study compares the quantitative properties of the ELSD and the RID in lipid analysis. Special attention was focused on the effect of molecular structure on the detector response in order to estimate the accuracy of the detectors when used for quantitative analysis of unknown compounds. The variables studied were the molecular weight and chemical structure, especially unsaturation level, of the compound. The quantitative properties were estimated by analyzing a polyethyleneglycol polymer mixture as an unknown sample. Isocratic HPLC conditions were used, gradient elution system would give an additional parameter affecting the response of the ELS detector (9).

MATERIALS

Chemicals and reagents

The responses of the two detectors were studied using the following lipid standards manufactured by Nu Chek Prep Inc., USA: triacylglycerols (18:3, 18:2, 18:1, 18:0 and 16:0), diacylglycerol (18:1), monoacylglycerol (18:1), cholesterol and cholesterol stearate. Tri-, di- and monoacylglycerols are glycerolipids with one to three fatty acid chains, whereas cholesterol and cholesterol stearate have sterol ring-molecular structure. A polyethyleneglycol polymer mixture was a PEG 1000 standard (BDH Limited, UK).

The mobile phase was HPLC grade tetrahydrofuran (Rathburn Chemicals Limited, UK). Butylated hydroxytoluene, BHT (0.025%), was used as stabilizer.

Instrumentation

The HPLC system was a Waters 6000A HPLC pump equipped with a Waters 700 Satellite WISP autosampler (Waters, USA). The refractive index detector was a Waters 410 and the light scattering detector a Cunow DDL21 (Cunow Department DMS, France). The gas used in the ELSD was compressed air passed through a 0.45 μm filter before the detector. The detector temperature was 54°C and the air pressure 1.0 bar. The photomultiplier sensitivity was adjusted to the mean value (600 mVolts) of the photomultiplier gain area (400 - 800 mVolts). The Maxima 820 program (Waters, USA) on a 386SX PC was used for data compilation and processing.

Columns

The lipid classes were separated using size exclusion chromatography. The optimization of the detector parameters for this chromatographic system has been reported previously (23). The size exclusion column series used in the analysis of lipid standards included one 100 Å and two 50 Å columns (PLGEL, 30 x 0.8 cm I.D., Polymer Laboratories Inc., USA). The unknown sample was quantitated with only a precolumn (PLGEL, 10 x 0.8 cm I.D.). The flow rate of the eluent was 0.6 ml/min at ambient temperature.

METHODS

The detector sensitivity and linearity were studied using four different standards: triolein, diolein, monoolein and cholesterol. The linearity of the ELSD was evaluated using concentrations of 0.05-4.5 μg/injection (nine concentration levels), those for the RID being 0.3-130 μg/injection (ten concentration levels).

The effect of molecular structure on the detector response was studied by evaluating the relative response factors for eight compounds for the ELSD using sample amounts of 0.1, 0.8 and 3 μg/injection. For the RID, the sample amounts were 1, 10 and 100 μg/injection. The selected concentrations were in the optimal working area of the two detector types. The responses for each compound were analyzed as duplicates, and relative response factors were calculated as area counts/µg injected.

The unknown sample was also quantitated with three different concentrations for each of the detector types. A single peak was obtained in the chromatogram by using only precolumn for separation. Triolein was used as an external standard in the quantitative analysis.

RESULTS and DISCUSSION

General

All the results are mean values of two injections, the variation of response between the injections being less than 3% (lowest injection amounts less than 9%) for the RID. For the ELSD, the variation of responses was less than 6% (lowest injection amounts less than 17%).

The ELSD and RID chromatograms of the standard mixture (triolein, diolein, monoolein and cholesterol) are shown in FIGURE I. In the RID chromatogram the significantly higher response of the ring-structured cholesterol (peak 4) than of the glycerol-structured lipids is obvious.

The sensitivity of the detectors was established by measuring the detection limits (signal to noise ratio 2:1). The sensitivity of the ELSD was slightly better than that of the RID model. The detection limit for triolein was 30 ng (0.034 nmol) with the ELSD and 50 ng (0.056 nmol) with the RID. Note, however, that these values are valid only for these particular detector models. For the ELSD, the detector parameters and chromatographic conditions also have a significant effect on the detector sensitivity (1-10).

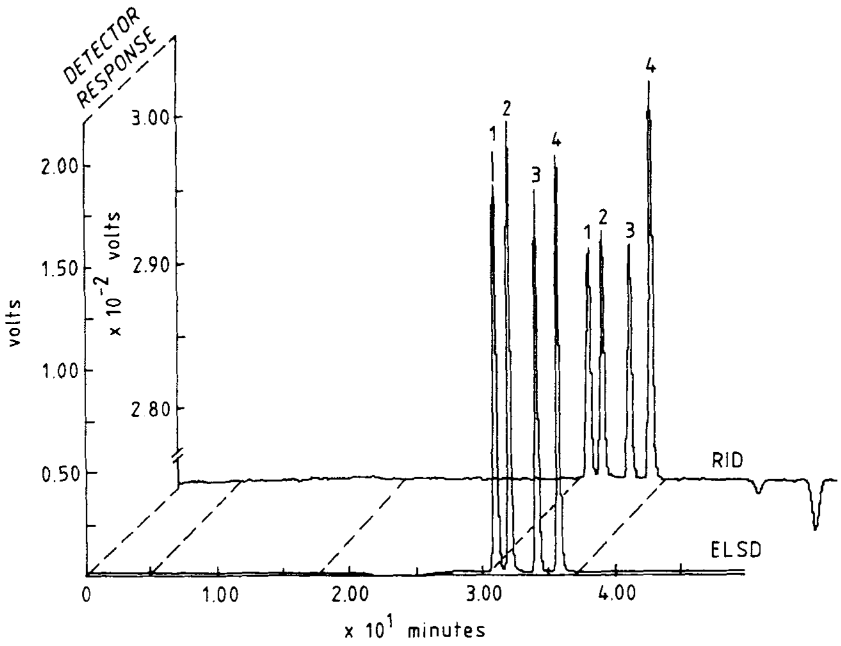

FIGURE I. ELSD and RID chromatograms of lipid standard mixture. Chromatographic conditions described in the text (Methods). 1. Triolein, 2. Diolein, 3. Monoolein and 4. Cholesterol. Sample amount ELSD: 3µg/compound/injection, RID: 25µg/compound/injection.

Calibration curves of the lipid standards

The calibration curves of the four standards are shown in FIGUREs II (ELSD) and III (RID). The RID calibration curve of cholesterol differs markedly from the other three curves, whereas this difference is not seen with the ELSD.

Linear regression equations of the calibration curves regression are shown in Tables I (ELSD) and II (RID). The geometric regression parameters of the ELSD are given in Table III. The better linearity of the RID is marked, the r^2 values being 0.9999 (linear curve fitting). The ELSD, on the other hand, fol-

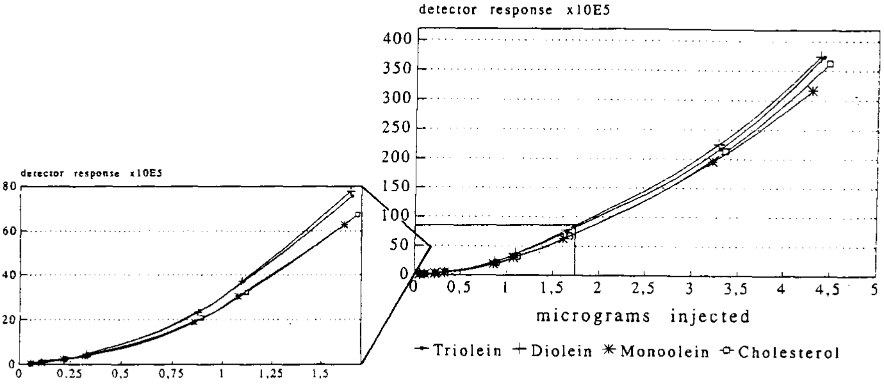

FIGURE II. ELSD calibration curves of lipid standards.

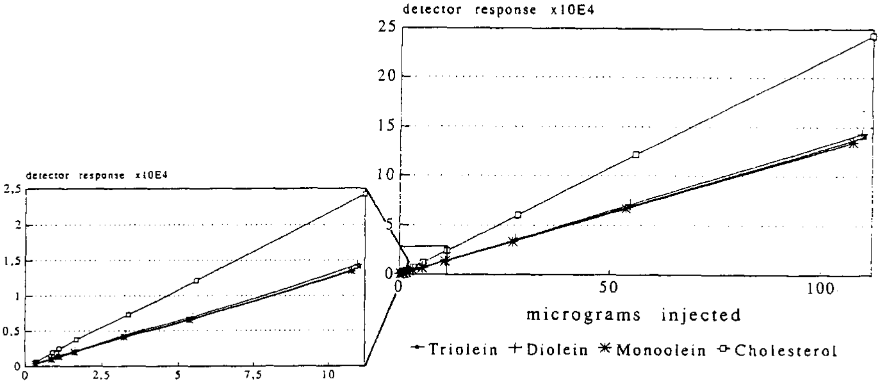

FIGURE III. RID calibration curves of lipid standards.

TABLE I

Linear Regression Equations of the ELSD, cons*inj.vol=a+b*R.
(0.05-4.5μg/injection at nine concentration levels)

Lipid Standard	Slope, b (10^{-7})	Y-intercept, a	r^2
triolein	1.52	0.268	0.9599
diolein	1.45	0.277	0.9585
monolein	1.65	0.284	0.9478
cholesterol	1.56	0.324	0.9483

TABLE II

Linear Regression Equations of the RID, cons*inj.vol=a+b*R
(0.3-130μg/injection at ten concentration levels)

Lipid Standard	Slope, b (10^{-4})	Y-intercept, a	r^2
triolein	7.87	-0.00130	0.9999
diolein	7.66	0.00268	0.9999
monoolein	8.00	0.00572	0.9999
cholesterol	4.61	0.00288	0.9999

TABLE III

Geometric Regression Equations of the ELSD, cons*inj.vol=a*R^b
(0.05-4.5μg/injection at nine concentration levels)

Lipid Standard	b(10^{-1})	a(10^{-4})	r^2
triolein	6.877	0.330	0.9805
diolein	6.282	0.827	0.9938
monoolein	6.118	1.166	0.9976
cholesterol	6.159	1.079	0.9971

lows better the pattern of the geometric regression equation, the r^2 values being better (mean value 0.9923) for geometric than for linear (mean value 0.9554) regression equations. The response of ELSD is usually reported to increase sigmoidally with increasing sample size (2-5, 10).

The response factors (RF) of the RID were very similar with the varying sample amounts, as seen in Table IV, and no trend is detectable. The response factor of the ELSD in contrast changes very markedly when the amount of injection increases. This strong increase in response factors (RF) indicates the change in the intensity of scattered light with increasing concentration. The particle size changes with the changing concentration of the column eluent, and the mechanism of scattering changes from the non-efficient (Rayleigh and Mie areas) scattering toward the more efficient (reflection and refraction) scattering area (2-5). This change in mechanism is apparent in the strong increase in RF and also explains the sigmoidal shape of the calibration curves.

The slopes of the linear regression equation varied more with the RID ($4.6 - 8.0 \times 10^{-4}$) than with the ELSD ($1.5\text{-}1.7 \times 10^{-7}$), thus indicating the weaker effect of molecule-structure on the response. The values in Tables I-III were calculated on the Maxima program, where the x-axis represents the detector response and the y-axis is the sample amount. This is the set-up to that in FIGUREs II-III.

Effect of chemical structure on the detector response

The relative RFs of the lipid standards using three different injection levels are combiled in Table IV. The mean values, standard deviations (SD) and relative standard deviations (SD%) are calculated for all eight compounds studied (mean n=8, SD n=8 and SD% n=8) and separately for the triacylglycerol standards (mean TAG, SD TAG and SD% TAG).

TABLE IV

Response Factors (RF) of Lipid Standards

Standard/ number of double bonds in fatty acids	ELSD/RF x10^5			RID/RF x10^4		
	Amount of Injection/µg			Amount of Injection/µg		
	0.1	0.8	3	1	10	100
Tristearate/0	11	23	62	0.13	0.11	0.11
Trioleate/3	14	27	62	0.14	0.13	0.13
Trilinoleate/6	7.4	27	70	0.13	0.15	0.15
Trilinolenate/9	8.2	31	81	0.16	0.17	0.17
mean TAG	10	27	69	0.14	0.14	0.14
SD TAG	3.2	3.3	9.0	0.014	0.03	0.03
SD% TAG	32%	12%	13%	10%	18%	18%
Diolein	9.1	27	69	0.13	0.13	0.13
Monoolein	6.1	22	61	0.12	0.13	0.13
Cholesterol	7.5	23	63	0.22	0.22	0.22
Cholesterol stearate	8.5	25	51	0.17	0.17	0.17
mean n=8	8.9	26	65	0.15	0.15	0.15
SD n=8	2.5	3.0	8.7	0.03	0.03	0.035
SD% n=8	28%	12%	13%	22%	23%	23%

The effect of the molecular structure on the detector response was assessed using different types of glycerol-structured lipids (tri-, di- and monoacylglycerols) and ring-structured lipids (cholesterol and cholesterol stearate). The molecular weights (MW) of the compounds varied from 356 for monoolein to 892 for tristearate. Within this range, MW had no detectable effect on the responses. The effects of MW in the 285-895 range have been reported in an earlier paper (23) in which the ELSD response of light molecules, such as free fatty acids, were found to be significantly lower owing to evaporation of the compound in the evaporation tube. This effect has also been discussed by Coulombe (7).

There was a markedly higher variation in the relative responses with the RID, the SD% of the eight compounds studied being approximately 23% while the corresponding figure with the ELSD was approximately 13% (Table IV, SD% n=8). The high SD% of the smallest amount injected (0.1 µg, 28 %) is obviously due to the high variation between injections in low concentration areas of the ELSD. This result indicates the weaker effect of the molecular structure on the ELSD detector response, as reported previously (1,4).

The effect of the unsaturation level on the detector response was studied using triacylglycerol standards of approximately equal molecular weight (equal number of carbon atoms) but with a varying amount of double bonds in the molecule (0-9 double bonds in the fatty acid chains).

With the RID, the differences between the response factors was expected since the unsaturation level affects the refractive index of the compound. The response factor of the triacylglycerols increased with an increasing number of double bonds in the molecule the SD% of the compounds studied being 18% (Table IV, SD% TAG). Differences between the response factors are seen also with the ELSD, the SD% of the triacylglycerol standards being approximately 13%. The response factors increase with an increasing number of double bonds in the molecule. Weather this is due to a change in the refractive index or to change in some other parameter affecting the mechanism or intensity of the scattered light was not established in this study. Previous reports have indicated that the response of the ELSD is slightly dependent on the refractive index of the analyte (1,3,4,10,12), but this effect has been reported to be of no significance (1,3,12).

Quantitation of an unknown sample

The quantitative properties of the two detector types were also evaluated using a polyethyleneglycol mixture (PEG 1000) as a sample of unknown composition. Quantitation was performed using triolein as an external standard.

TABLE V

Quantitation of polyethyleneglycol mixture PEG 1000.

Detector	Sample amount µg	calculated solution conc. µg/µl	original sol. conc. µg/µl	variation +/- (%)
ELSD:				
PEG	0.1	0.0065	0.0209	-0.0144 (69%)
	0.8	0.0231	0.0209	+0.0022 (11%)
	3	0.0813	0.1044	-0.0231 (22%)
RID:				
PEG	1	0.0660	0.1044	-0.0384 (37 %)
	10	0.8264	1.0440	-0.2176 (21 %)
	100	0.8990	1.0440	-0.1450 (14 %)

The results of the standard compound studies suggested that the quantitation of the RID should be based on a linear equation and that of the ELSD on a geometric equation.

The quantitative results are given in Table V. The variation from the original sample concentration using the ELSD was 11%, whereas the lowest variation of the RID was 14%. Quantitation was performed with three different sample amounts. The results for the highest and lowest sample amounts were calculated using the marginal areas of the standard curve. With the ELSD the variation from the original sample concentration was very high (70%) when very low amounts of sample were used.

CONCLUSIONS

The molecular structure of the sample has a detectable effect on the response of both the RID and the ELSD, but the effect is smaller with the ELSD than with the RID. The ELSD can therefore be considered more reliable than the RID for the quantitative analysis of unknown compounds.

It has been previously reported that the ELSD response per unit mass is approximately constant, at least for compounds that condense as liquids and that belong to a given chemical group (1-5). The present study compared the responses of the different detectors in conjunction with three different types of molecules: glycerolipids with varying amounts of fatty acids and varying amounts of double bonds in the molecule, molecules with sterol rings and a polyethyleneglycol polymer. The results indicate that accurate quantitative analysis of lipids requires individual response factors for each of the compounds studied. However, when quantitating heterogeneous mixtures or unknown compounds, a standard deviation of at least 13% is to be expected with the ELSD and even higher values with the RI detector.

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