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Journal of Chromatography A, 1035 (2004) 221–225
JOURNAL OF CHROMATOGRAPHY A
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Post-column addition as a method of controlling triacylglycerol response coefficient of an evaporative light scattering detector in liquid chromatography–evaporative light-scattering detection

Sylvie Héron [a,*], Michel Dreux [b], Alain Tchapla [a]

[a] *Groupe de Chimie Analytique, Universite de Paris-Sud (XI), EA 3343, LETIAM, IUT d'Orsay, Plateau du Moulon, F 91400 Orsay, France*
[b] *Sedere, 9 Rue Parmentier, F 91140 Alfortville, France*

Received 22 December 2003; received in revised form 17 February 2004; accepted 17 February 2004

Abstract

The non-linear response is generally the main limitation to the general quantitative use of evaporative light-scattering detection (ELSD). In the particular case of triacylglycerol (TG) analysis, we present a preliminary paper dealing with the use of post-column additives as a means of monitoring the response of such a detector. As TG can form molecular association complexes (ligand–ligate associations) with either cholesterol, urea or silver nitrate, we report the influence of the concentration of each of these chemical compounds in the liquid phase directed towards the ELSD system. The results show that the response coefficient *b* of the calibration curve either decreases from 1.25–1.30 to 0.51 or increases from 1.25–1.30 to 1.78 according to the nature and concentration of post-column additive. The use of cholesterol as additive, at a discrete concentration, may lead to a linear response curve ($b = 1$), i.e. to the direct proportionality of ELSD response versus the TG concentration, making quantitative analysis of such solutes easier. On the other hand, to improve sensitivity, the addition of silver nitrate may be chosen for an increase in *b* value.

Keywords: Evaporative light-scattering detection; Detection, LC; Triacylglycerols

1. Introduction

Evaporative light-scattering detection (ELSD) is increasingly being used in liquid chromatography (LC) as universal detection method, as long as the solutes are less volatile than the LC eluent [1,2].

The response varies with the scattering domain and for a large range of sample sizes, the peak area (*A*) is usually related to the sample mass (*m*) by the following relationship [1]:

$$A = am^b \quad (1)$$

where *a* and *b* are coefficients which depend on droplet size, concentration, nature of solute, gas and liquid flow-rates, molar volatility, etc.

Eq. (1) reflects the non-linear relationship ($b \neq 1$) between *A* and *m*. Consequently, it is necessary to use double logarithmic coordinates (Eq. (2)) in order to have a linear calibration curve with a slope value of *b*.

$$\log A = \log a + b \log m \quad (2)$$

This equation depends on the chromatographic conditions and it is well known [2] that high *b* value is consistent with low particle size (Rayleigh diffusion) while low *b* value corresponds to high particle size (Mie and refraction–reflexion domain). Moreover, this explains why in the reversed-phase (RP) mode lower *b* values (<1.5) than in the normal-phase (NP) mode (>1.5) were reported [1].

Different attempts to overcome this non-direct linearity have been previously reported. Three experimental approaches for solving this challenge can be considered.

The first one is related to the nature of gaseous nebulization atmosphere. Thus, by using helium instead of dinitrogen or air as nebulization gas, a modification can be obtained in the response coefficient (*b* value close to 1) and

* Corresponding author. Tel.: +33-1-6933-6130; fax: +33-1-6933-6048.
E-mail address: heron@iut-orsay.fr (S. Héron).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.02.052

the limit of detection improved [3,4]. Similarly, the heating or the cooling of the nebulizing gas (He, N_2 or air) and/or the nebulization chamber leads to a change in the response coefficient b in aqueous as well as in non aqueous liquid chromatography [4].

The second approach consists in regulating the ratio between the flow-rates of mobile phase and nebulization gas. Thus, the use of a nebulizer optimized for micro and capillary LC gives a linear dependence between the peak area and the sample mass [5–8].

The third could be related to the aerosol droplet size regulation.

More sophisticated detection using a nucleation process in the final step of the detection process has demonstrated the importance of solute coagulation possibility for a scattering system. As a consequence, condensation nucleation light-scattering detection (CNLSD) leads to a direct linearity response, particularly when using an additional diffusion screen which selects particles according to their size [9,10]. Recent studies have also shown a response enhancement for ELSD on adding triethylamine and formic acid to the mobile phase [11–13] or a response decrease using trifluoroacetic acid (TFA) instead of TFA–NH_4OH [14].

Lastly, one could consider that lower values of b which have been reported in the reversed-phase mode than in the normal-phase mode are related to the same origin.

All these experimental methodologies involve changing the nature of the aerosol as well as the size distribution of the scattering particles.

The goal of this work is to demonstrate the variations (Δb) of the slope b in order to obtain either an increase in sensitivity (high b value) or a linear response ($b = 1$). This has been achieved using the post-column addition of different ligates which may change the nature of the scattering particles in terms of size as well as distribution [15].

Such a modification has never been studied with ELSD and it corresponds to a new and easy way of "solute aggregate modification" at the column output.

Due to our interest in the field of triacylglycerol (TG) analysis [16–21], these solutes have been chosen as solute model for these preliminary studies. The choice of the additives was governed by complexation studies carried out in the field of lipids analysis. Three different substances were chosen, due to their potential activity to form molecular complexes (ligand–ligate associations) with TGs.

It is well known that silver ion gives complexes with unsaturated compounds. One of the possible applications is argentation-chromatography development. This liquid chromatography is commonly used to separate TGs or fatty acid esters, according to the number of double bonds borne by solutes, based on the polar charge-transfer complexes formed between the Π-electrons of the double bonds and silver ions [16,22]. More recently, studies in LC–MS have shown that excellent ionization of ester constituents of jojoba oil (saturated and unsaturated ones) can be achieved following the post-column addition of silver ions before MS detection [23]. Surprisingly, the authors demonstrated that saturated esters give the same molecular adduct $[M + Ag]^+$ than unsaturated ones in LC–MS. For the intact wax ester, complexation occurs even when no unsaturation is present in the hydrocarbon chains. Thus, after nebulization, the presence of molecular complex between Ag^+ and saturated chain was clearly established [23].

Urea is also known to form complexes with fatty acids [24,25].

Finally, calorimetric and spectroscopic studies have led to the conclusion that TGs form complexes with cholesterol [26].

Therefore, silver nitrate, urea and cholesterol were tested as mobile phase additives. In order to avoid modification of the solutes retention times, post-column addition of the compounds, via a peek tee was employed. The flow-rate of the post-column addition was maintained at 50 μl/min, corresponding to a 5% increase in the mobile phase flow-rate, thus avoiding too large a loss of resolution due to an additional extracolumn effect.

2. Experimental

2.1. Chemicals

Tricaprin CCC (TG 10) and trilinolein LLL (TG 18:2) chosen as typical saturated and polyunsaturated TGs were obtained from Sigma (Saint-Quentin Fallavier, France) as were cholesterol and urea. Silver nitrate was obtained from Carlo Erba (Milan, Italy).

Acetonitrile (Acros, Noisy le Grand, France) and methylene chloride (Carlo Erba) were of HPLC grade.

2.2. Equipment

The chromatographic system consisted of a model 1050 pump (Hewlett-Packard, Palo Alto, CA, USA), a model 7125 injection valve with a 20 μl loop (Rheodyne, Cotati, CA, USA), and a model Sedex 75 light-scattering detector (Sedere, Alfortville, France). The nebulizing gas was air at 3.6 bar (corresponding to a flow-rate of 1.92 l/min), the nebulization temperature was 37 °C or 55 °C, according to the post-column additive, and the gain (PM) was maintained at 11. Chromatograms were recorded with Azur (v3.0) acquisition software (Datalys, Saint Martin d'Heres, France).

The column temperature was controlled using an Igloo-Cil oven (Cluzeau, Sainte-Foy-la-Grande, France) and set at 20 °C.

A Kromasil C_{18} (5 μm) 150 mm × 4.6 mm column was used (Touzart and Matignon, Les Ulis, France). The mobile phase flow-rate was 1 ml/min.

For the post-column addition, a model 114 M pump in micro mode (Beckman, Gagny, France) and a pulsation damper (Touzart et Matignon) were used and connected between the column output and the nebulizer via a polyether ether

ketone (PEEK) tee. The post-column addition flow-rate was 50 μl/min. Therefore, the total flow-rate directed towards the ELSD system was 1.05 ml/min.

2.3. Methods

The mobile phase composition (CH_3CN–CH_2Cl_2, 65:35) was chosen to give moderate retention times for the triacylglycerols [16–21]. For TG 10 and TG 18:2 the retention factors were 1.9 and 6.4, respectively.

The TGs were dissolved in CH_3CN–CH_2Cl_2 (50:50).

The calibration curves were established using between five (range 40–5 ppm) and eight (range 100–5 ppm) different TG concentration levels. The difference in the calibration ranges was due to the saturation of the signal with some of the post-column additives.

We have checked that irrespective of the number of concentration levels [i.e. the range studied (100–5 ppm or 40–5 ppm)], the *b* value was the same.

Each area value was the average of three reproducible injections.

3. Results and discussion

3.1. Preliminary experiments

3.1.1. Choice of reference solutes

As previously reported [8], *b* values obtained for different TGs may be different, due either to the liquid or solid nature of pure TG, or their different solvatation by the mobile phase. Thus, in order to characterize the response variation (Δb or $\Delta b/b$) given by post-column ligate addition, we have chosen in this present preliminary study to investigate the effect of some ligates with only two representative TGs: one totally saturated, i.e. CCC and one polyunsaturated, i.e. LLL.

3.1.2. Choice of solvent for the post-column additives

Depending on the additive, the dissolution solvent was either the mobile phase CH_3CN–CH_2Cl_2 (65:35) (for cholesterol) or pure CH_3CN (for urea and silver nitrate). For the latter case, we have checked that the addition of a flow-rate equal to 50 μl/min of pure CH_3CN (i.e. concentration of the additive equal to 0 in the figures) to the mobile phase flow-rate (equal to 1 ml/min in CH_3CN–CH_2Cl_2, 65:35) does not modify the response coefficient *b*.

3.1.3. Studies without post-column addition

In the first instance, the study of peak area versus the amount of injected TGs was made without post-column addition. The *b* values were 1.27–1.36 for CCC and 1.21–1.26 for LLL. These values are in agreement with the fact that there is little difference in *b*, within one chemical family but such values differ strongly from the common *b* value, which is generally higher than 1.50 in normal-phase chromatography and lower than 1.50 in reversed-phase chromatography [27]. Such a difference may be explained according to Schultz and Engelhardt [28] by solvatation of TGs. Particles that scatter the light are composed of TGs associated to solvent as aggregates. Consequently, with an increase in the particle size a decrease in the slope *b* is observed [1,2].

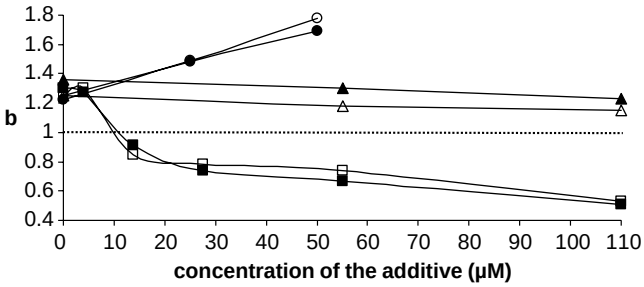

Fig. 1. *b* vs. concentration of different additives for the two triacylglycerols. TG 10: full symbols; TG 18:2: open symbols; (■) and (□) cholesterol in CH_3CN–CH_2Cl_2 (65/35); (●) and (○) silver nitrate in CH_3CN; (▲) and (△) urea in CH_3CN.

The variations we observe for *b* using a post column addition correspond to the difference between the *b* value obtained without a post column addition and with a post column addition. Two opposite variations are observed, depending on the additive nature and concentration.

3.2. Effects of the additives

3.2.1. Additive decreasing the b slope (cholesterol and urea)

The study of peak area versus the amount of injected TGs was carried out with post-column addition of cholesterol or urea at various concentrations. The *b* values versus the concentration of the additive are given Fig. 1.

The maximum value (110 μM) corresponds to the maximum ligate concentration for which there is no problem of solubility for the total flow-rate (1.05 ml/min).

The same observations can be made about the two triacylglycerols and the two ligates:

(i) A decrease in the value of the slope *b* is noticed when the concentration of cholesterol or urea is increased (Fig. 1). The lowest *b* values could be 59% of the initial *b* value (without ligate addition) and such a variation is very significant.

(ii) When the concentration of ligate decreases, the response coefficient tends to the *b* value obtained without post-column addition.

(iii) Without the additive [i.e. with pure CH_3CN or CH_3CN–CH_2Cl_2 (65:35) post-column addition at 50 μl/min] no significant *b* slope variation was noticed between the mobile phase flow-rate (1 ml/min) and the total flow-rate (1 ml/min of mobile phase + 0.05 ml/min of pure CH_3CN or CH_3CN–CH_2Cl_2 (65:35)].

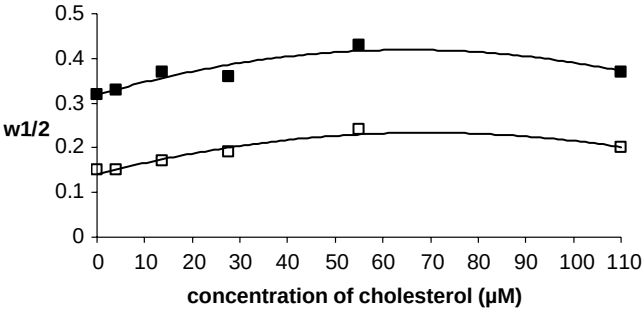

Fig. 2. Peak width of triacylglycerols vs. concentration of cholesterol in CH_3CN–CH_2Cl_2 (65/35) (□) TG 10; (■) TG 18:2.

(iv) A linear response of TGs was obtained with a post addition of a 10 μM solution of cholesterol. In this case, Eq. (1) becomes:

$$A = am \tag{3}$$

and a direct linearly as with UV detection is observed.

(v) When the b value decreases, an increase in the peak width $w_{1/2}$ is observed (Fig. 2).

The change in the response may result from a modification of the aerosol's and/or analyte's characteristics.

A decrease in the value of b means that the particle size distribution is increasing [2].

The cholesterol post-column addition leads to a decrease in b value which could correspond to a reflection–refraction optical domain, occurring with a large particle size. This can be explained by the formation of a triacylglycerol–cholesterol association, leading to an increase in particle size. A low b value is consistent with an increase in solute complexation as previously reported using other analytical methods [16,22–26].

The same studies have been made with taurocholic acid (skeleton close to that of cholesterol) as additive and the same conclusions can be done ($b = 1.03$ for CCC and 1.1 for LLL for post-column addition of 55 μM taurocholic acid) (results not shown).

In the same way, the decrease in the b value with the post-column addition of urea may suggest such an intermolecular complex formation with triacylglycerols.

A second effect due to the post-column ligate addition to the mobile phase is observed; the variation of the peak width $w_{1/2}$ is in agreement with the standard deviation σ corresponding to a Gaussian curve in ELSD [29]. In ELSD, the apparent standard deviation σ_a is related to the true value by the following relationship:

$$\sigma_a^2 = \frac{\sigma^2}{b} \tag{4}$$

leading to a peak width smaller than the chromatographic peak width. So, when b decreases, the apparent standard deviation increases. Therefore, the peak width increases. With cholesterol as additive, a significant increase may be observed (60%; $w_{1/2}$ varies from 0.15 to 0.24 s with an intermediate cholesterol concentration of 55 μM) (Fig. 2).

3.2.2. Additive increasing the b slope (silver nitrate)

The study of peak area versus the amount of injected TGs has been also done with post-column addition of silver nitrate at various concentrations. The b values versus the concentration of silver nitrate are given Fig. 1.

The same observations can be made for the two triacylglycerols CCC and LLL:

(i) An increase in the slope b value is noticed as the concentration of the silver ions is increased (Fig. 1).
(ii) log a decreases as b increases.
(iii) When the concentration of additive decreases, the response coefficient tends towards the b value obtained without post-column addition.

The post-column addition of silver nitrate leads to changes which are the opposite of those observed with cholesterol, taurocholic acid and urea.

In the presence of silver ions, a molecular complex smaller in size than the TGs in the mobile phase without additive occurs, the response coefficient (b) value is larger in the presence of silver ions. This means that the scattering particles diameter is lower when post-column silver ion. The electrically charged triacylglycerols–silver complex leads to particles of smaller diameter than those involving solvated or aggregated triacylglycerols.

The b value variations are not consistent with a variation of surface tension associated (or non-associated) to nucleation phenomena which may be used successfully [30].

Such a ligand–ligate association may also be used for the transformation of a volatile analyte into one which is non volatile as previously mentioned in the first paper devoted to this new detection principle based on light scattering by particles in the gas phase [31].

The results reported here may explain also the lack of reproducibility sometimes noted with ELSD [28]; it could be due to small quantities of impurities present in the mobile phase or gas phase and it justifies the special grade of solvent required in ELSD as well as the purity of the nebulizing gas.

4. Conclusion

This preliminary work demonstrated that a very small quantity of an additive, as a post-column addition, may change the ELSD response coefficient.

Involving a single class of solute (triacyglycerols), experimental results clearly demonstrate that the ELSD response coefficient can be modulated. Using a post-column addition, an appropriate compound may complex the solutes being studied.

Two different strategies can be employed to perform a quantitative analysis. Depending on the nature and the amount of additives, either the ELSD response can be

linearized ($b = 1$ using cholesterol) or maximized (highest b values using silver nitrate).

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