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Comparison of the responses of triacylglycerols with an evaporative visible light scattering detector used in conventional, micro and capillary liquid chromatography☆

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Abstract

During recent years, there has been a growing interest in the development of packed nano- and microchromatographies. It needs coupling the systems used to detectors adapted for microchromatographies. In the reported results, the properties of two different nebulizers of an evaporative visible light scattering detector have been studied under different flow-rate conditions (conventional HPLC, micro-LC and capillary LC). In micro-LC and capillary LC, it is shown that the calibration curves gained for triacylglycerols analyzed in non-aqueous reversed-phase liquid chromatography could be statistically assimilated to a linear relationship even if they rigorously follow a well-known power relationship such as area$=a\cdot\text{mass}^{b}$. This makes the establishment of the calibration curves easier in miniaturized chromatographies than in conventional ones. Depending on the chromatography used, the linear range falls between either 2–50 ng or 14–80 ng. The limit of quantification was 5 ng ($S/N=10$). On the other hand, a study of the response of modified nebulizer versus flow-rate has shown that the modified evaporative visible light scattering detector is more complex than detectors whose response is really proportional to the concentration or to the mass.

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

1. Introduction

Nowadays, the detector most frequently used in liquid chromatography (LC) is the spectrophotometric UV absorption detector, easy to use, allowing the choice of the best wavelength for the solutes and gradient solvent analysis conditions. For quantitative studies, it gives linear responses when using diluted solutes. However, this detector cannot commonly be used for compounds which do not absorb in the near UV (200–400 nm) or which have weak chromophores in this UV range. Moreover it does not allow one to use any solvent for the mobile phase because many of them possess a cut-off above 200 nm. Thus, in the 190–220 nm range, quantitative analysis is difficult.

To overcome these difficulties, some laboratories made advances in the early development of the evaporative light scattering detection (ELSD) [1,2], using either a visible light-source (EVLSD) [3,4] or laser light-source (ELLSD) [5–7]. The evaporative light scattering detector has a significant advantage

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as compared to the UV one. Indeed, it is especially attractive for analyzing compounds which do not absorb above 200 nm, such as, for instance, saturated hydrocarbons, carbohydrates, steroids, surfactants or mono-, di- and triacylglycerols [8–12]. However, using conventional high-performance liquid chromatography (HPLC) conditions, the disadvantage of using ELSD, for a quantitative analysis, comes from the observed non linear relationship between the peak area, *A*, and the injected sample mass, *m* [7–12]: $A=am^b$. This seems related to the droplet size inhomogeneity which depends on surface tension, viscosity, density and flow-rate of the mobile phase [13]; it also depends on the make-up gas flow-rate and on the geometry of the nebulizer [14]. Finally, problems arises because light is much less scattered by small droplets as their diameters near that of the wavelength of light [15]. Consequently, it is necessary to draw a logarithm plot of the response area versus the sample mass for quantitative analysis. This led to a failure in using it for a lot of analysts. Thus, the control of homogeneity of the droplets size due to nebulization conditions appears to be of a fundamental importance. The use of micro- or capillary columns which accommodate low flow-rates could lead to solve this problem partially. In this case, the supplementary advantages of miniaturization of the HPLC are to allow the analysis of minute samples, to improve the signal-to-noise ratio, to use low amounts of mobile phase. In another way, as the amount of stationary phase packing the micro-column is very small, its other advantage is to use scanty or specific stationary phases, which could give new selectivities.

Unfortunately, using micro- and capillary chromatography conditions, it is necessary to modify the nebulizer in the available detectors. Thus Hoffmann et al. [16] showed that the response of such laboratory-made detector was not linear in the nanogram region, for ELLSD. On the contrary, recent studies about the properties of modified EVLSD have shown that in conditions of miniaturized HPLC (i.e., using micro-flow-rate) the peak area *A* has a direct linear dependence on the sample mass *m*, when such a detector was used in direct injection mode in the range of flow-rate of 0–100 μl/min [17]. The same results (a linear response over two different ranges of sample amount) were also recently reported for similar experimental conditions using ELLSD [18]. However, these last two experiments were not done using usual chromatographic conditions. Results were gained without accounting the peak broadening effects due to the intra- and extra-column phenomena. Finally, it has been shown that the response of ELSD is highly dependent on the mobile phase flow-rate in conventional and microbore HPLC. An increase of the signal has been observed at low flow-rates and interpreted by losses of solutes on the walls of the drift tubes, whereas a decrease at high flow-rates has been explained by the formation of smaller drops [12,19]. Consequently, ELSD does not give the same responses at low and high flow-rates.

Thus, the main goal of this article is to study the properties and to compare the response of a modified EVLSD system, when using it with micro-columns (internal diameter, I.D.=1 mm) and capillary columns (I.D.=300 μm). A study of responses versus linear velocity has also been made in order to know if, in these cases, it is proportional to the concentration of solute, or proportional to mass.

Accounting that triacylglycerols (TGs) are a very important class of compounds (with applications in cosmetic, food industries or in pharmaceutical investigations [20]) and also our knowledge in the field [21–25], we have developed micro- and capillary column studies for this type of solutes. Previous studies have shown in conventional HPLC that triacylglycerols give logarithmic responses with similar slopes *b* for ELLSD [26]. So we report here their respective quantitative response using micro- and capillary LC conditions with modified EVLSD.

2. Experimental

2.1. Chemicals

Benzo[*a*]pyrene (BaP) and triacylglycerols (TGs) are from Sigma (Saint-Quentin Fallavier, France) (see Table 1).

2.2. Solvents

Acetonitrile and acetone were HPLC grade (SDS, Peypin, France). They were filtered through a 0.5-mm Millipore filter (Whatman, Hillsboro, OR, USA)

Table 1
Carbon number and double bonds of compounds used

Name	Carbon number: double bonds	Abbreviation
Tricaproin	6: 0	CoCoCo
Trilaurin	12: 0	LaLaLa
Trimyristin	14: 0	MMM
Triolein	18: 1	OOO
Trilinolein	18: 2	LLL
Trilinolenin	18: 3	LnLnLn

before use and degased with Helium (Air Liquide, Jouy en Josas, France) during the experiments.

2.3. Equipment

Chervet et al. [27] have defined names for the different HPLC techniques based on inner diameter of the column and flow-rate range. In this work, three techniques have been studied and compared.

2.3.1. Conventional HPLC

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), a Model Sedex 45 light-scattering detector with a ''classical'' nebulizer (Sedere, Alfortville, France) and a Chromjet integrator (TSP, Les Ulis, Courtaboeuf, France).

A Brownlee Spheri 5 ODS (5 μm) 250×4.6 mm column was used (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA).

The mobile phase flow-rate was 0.85 ml/min.

2.3.2. Micro-liquid chromatography

The chromatographic system consisted of the same pump, a micro-injection valve equipped with a 200/60 nl internal loop, Model CI4W 0.06/0.2 μl (Valco Europe, Schenkon, Switzerland), a conventional Shimadzu UV SPD-10A detector equipped with a micro-cell of 35 nl (Tokyo, Japan), a Model Sedex 45 light-scattering detector with a micro (μ)-nebulizer (Sedere), a Chromjet (TSP) or a Shimadzu CR-6A integrator (Touzart et Matignon, Les Ulis, Courtaboeuf, France).

A Brownlee Spheri 5 RP18 (5 μm) 250×1 mm column was used (Perkin Elmer). Like in previous reported results (with ELLSD) [16,18,23,24] the position of the mobile phase transfer capillary versus the outer tubing of the nebulizer has been optimized for the micro-nebulizer from the EVLSD system of Sedere. Following the thesis of Guerrero [17], for available micro-nebulizer the inner capillary is 0.25 mm outside the metallic needle. For capillary LC conditions, a second nebulizer was used for which the inner capillary must be 1 to 2 mm inside the nozzle of the nebulizer to obtain an optimal response.

The mobile phase flow-rate (100 to 10 μl/min) was obtained directly by the pump.

2.3.3. Capillary liquid chromatography

The system was the same that for micro-LC. A μ-flow processor (Acurate, Model AC-70-CAP, LC Packings, Amsterdam, The Netherlands) placed between the pump and the injector was used to split the flow down to 10–0.5 μl/min [28].

LC-Packing Hypersil C_{18} BDS (3 μm) 150 mm× 300 μm (LC Packings) was used.

To avoid extra-column dispersion particularly critical in micro-LC and capillary LC [27,29,30], all the connecting tubes were in polyether ether ketone (PEEK) with inner diameters of 60 μm, the connections were zero dead volumes PTFE connectors.

2.3.4. Evaporative visible light scattering detection

All studies have been made with a constant temperature (33°C) and nebulization gas (air) at 2.3 bar. As the different gains are not proportional, each series of data has been made with a constant gain, this later could be different from one series to another.

2.4. Procedure

The quantitative analysis of TGs has been studied in non-aqueous reversed-phase (NARP) with acetonitrile–acetone (30:70) as mobile phase, allowing one to inject simultaneously five standards in order to reduce errors.

The flow-rates (F) have been calculated taking into account the ratio of the squared column diameters, in order to be able to compare the results [30]. The linear velocity (u) has been systematically calculated.

2.5. Calculations

2.5.1. Methodology and statistical treatment

(i) The detector response is given by measurements of the area and height of each peak. (ii) Each solution is injected not less than six times in order to have six final values after having applied Dixon's criterion which removes doubtful values [31,32]. (iii) Calculation of the mean of the six values for plotting response curves. (iv) Regression analyses of results were made as following: in order to know if a linear model fits the experimental points, a linear regression analysis of the two linearly expected variables (i.e., peak area and mass or logarithm of area and logarithm of mass) has been made. A high correlation coefficient (r^2) not being sufficient enough to characterize a linear response, we studied the *F*-test (Snedecor) and the *y* residuals (Test of Shapiro and Wilk) [31,32]. When these three criteria are positive a linear response between investigated variables correctly describes the properties of the EVLSD system. Moreover, from the statistics of the linear regression, the two hyperbolic curves corresponding to the confidence intervals for the expected value of the response function have been systematically evaluated. This permits to define the range of *b* values for which the response can be considered as linear.

2.5.2. Detector response study

The study of the mathematical model $A=am^b$ has been analyzed by linear regression of the plot log $A=b$ log $m+$log a which gives an estimation of *b* and log *a* values. The significance of the estimation values ''*b*'' in comparison with 1 (corresponding to a linear response of the detector: $A=am$) has been established by means of the statistical variables ''$b-1$'', for a significance level of 95% (Student's *t*-test). If there is no significant difference between *b* and 1, we can consider the model is really linear (in the following part of the article, we will say that *b* is statistically equal to 1). If the hypothesis of a significant difference between *b* and 1 is accepted, the response is $A=am^b$.

In this latter case, we have studied how the plots are placed comparatively to the two confidence interval curves gained from a linear regression treatment of the experimental values. In the mass range where the plots fall between the two confidence interval curves corresponding to their linear regression treatment, it is possible to consider linear the response of the detector versus the injected amount. In such a mass range, the 95% confidence level of the slope of this linear regression (using Student's *t*-test, standard deviation and degrees of freedom) have been calculated.

3. Results and discussion

3.1. Influence of the effluent flow-rate on the response

In order to be sure of the evolutions of the curves obtained with EVLSD, a UV detector was used in series for reference. So, a compound which could be detected by the two detectors was used: BaP. The influence of effluent flow-rate on the response was determined for flow-rate ranging from either 10 to 100 μl/min ($u=0.03–0.33$ cm/s) (micro-LC) or 2 to 8 μl/min ($u=0.07–0.29$ cm/s) (capillary LC).

Fig. 1 shows the response area and response height of the peak obtained for BaP with the UV detector versus the linear velocity. Fig. 1a is for micro-LC conditions, Fig. 1b for capillary LC conditions. These figures are references. They confirm UV detector gives a response which is proportional to concentration, i.e., area decreases when linear velocity increases and height is nearly constant [33].

Fig. 2 is the same plot for EVLSD with a μ-nebulizer used under micro-LC conditions (Fig. 2a) and under capillary LC conditions (Fig. 2b).

For micro-LC conditions (Fig. 2a), the plot area versus linear velocity is characteristic of response proportional to the concentration, whereas the plot height versus linear velocity is rather characteristic of response proportional to mass. This does not allow one to make any conclusions as to the classification of this detector. This can be compared to some gas chromatography detectors described by Guiochon and Guillemin as being more complex detectors because in principle they are not linear [34].

For capillary LC conditions (Fig. 2b), responses are random and the μ-nebulizer is not suitable for

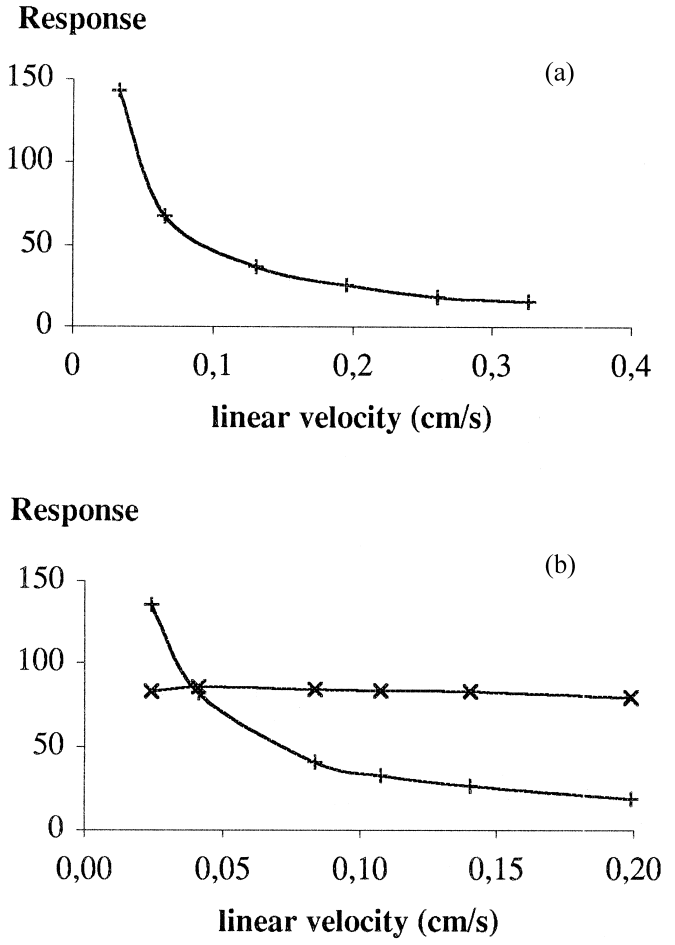

Fig. 1. (a) Peak area of BaP (arbitrary units) with UV detector vs. linear velocity under micro-LC conditions. (b) Peak area (+) and peak height (×) of BaP (arbitrary units) with UV detector vs. linear velocity under capillary LC conditions.

flow-rate under capillary LC conditions (i.e., 2 to 8 μl/min).

One of the differences between a nebulizer for conventional HPLC and a μ-nebulizer for micro-LC is the presence in this latter case of a silica tube insert in the μ-nebulizer which plays a role on the response of the detector. Indeed, previous studies have shown that modification of position of this silica tube modifies responses from no signal to maximum intensity [17]. So, the used μ-nebulizer was optimized (by the position of the silica tube) for working in micro-LC (see Experimental).

So we have been obliged to use a second μ-nebulizer for which we have shifted the silica tube of 1–2 mm towards the inside of the nozzle in order to gain responses for capillary LC conditions. In this latter case, for capillary LC conditions (2 to 6 μl/min) (Fig. 3b) it offers responses proportional to mass. The same μ-nebulizer used in micro-LC conditions (Fig. 3a), gives two different types of response, depending on the range of the flow-rate. In the 40–100 μl/min (u=0.13–0.33 cm/s) range, responses are proportional to concentration, whereas for flow-rate lower than 40 μl/min, plots are in contradiction.

So, the EVLSD response with a μ-nebulizer is

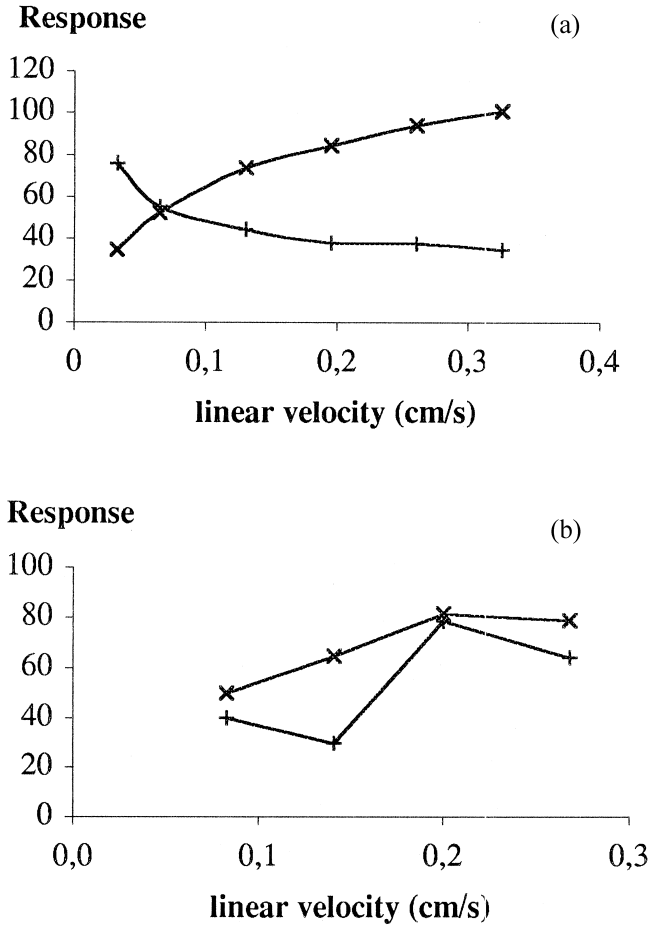

Fig. 2. (a) Peak area (+) and peak height (×) of BaP (arbitrary units) with EVLSD vs. linear velocity under micro-LC conditions. (b) Peak area (+) and peak height (×) of BaP (arbitrary units) with EVLSD vs. linear velocity under capillary LC conditions.

different if working under micro-LC or capillary LC conditions. It is also more complex than detectors whose response is really proportional to concentration (UV detectors) or to mass (amperometric detectors).

Studies are in progress [35] to try to show how this complexity depends on the droplet size obtained during the nebulization.

In other respects, these studies have confirmed that the position of the silica tube in the μ-nebulizer is the predominant parameter to control to have no random responses under micro-LC or capillary LC conditions. For ELLSD, the same conclusions have just been recently published [16,18,36].

3.2. Influence of the injected amount on the response of the evaporative visible light scattering detector

A study of peak area and peak height versus the amount of injected TGs has been made in conventional HPLC, micro-LC and capillary LC in order to compare the results.

The equations of the curves $A=am^b$ have been systematically calculated. Results are shown in Table 2. The b value ranges obtained in conventional HPLC show that TG curves cannot be considered as linear ($b\neq1$; 1 does not fall into b range).

On the other hand, for the same TGs studied under

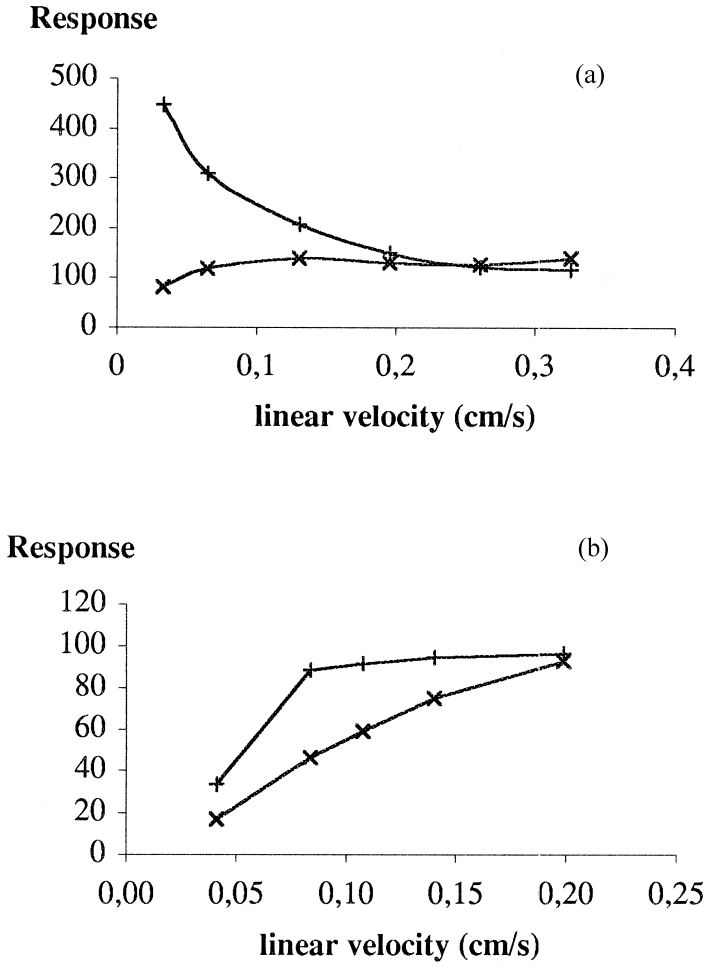

Fig. 3. Same legends as in Fig. 2 with a second μ-nebulizer.

Table 2
b value range for a mobile phase of MeCN–acetone (30:70)

Triacylglycerols	*b* value range, coefficient of equation: $A=am^b$ (significance level of 95%)		
	Conventional HPLC (F=0.85 ml/min)	Micro-LC (F=40 μl/min)	Capillary LC (F=3 μl/min)
LaLaLa	1.7–1.99	–	1.10–1.23
MMM	1.76–2.5	0.85–1.13	1.06–1.25
OOO	1.07–2.5	–	1.09–1.31
LLL	1.36–1.72	0.97–1.31	1.06–1.21
LnLnLn	1.49–2.14	0.96–1.19	1.08–1.19

micro-LC and capillary LC conditions, *b* coefficients are systematically lower than those obtained in conventional HPLC. With regard to statistics, the *b* coefficient is either statistically equal to 1 (micro-LC) or different from 1 in capillary LC. However in this latter case, the curve $A=am^b$ is between the two confidence interval curves of the linear regression for injected amounts inferior to about 70 ng (function of the TG). An example of the plots is given Fig. 4.

So, we have chromatographic conditions for which the TG response in EVLSD versus the injected amount can be statistically considered as linear. The range of linearity is between 2–50 ng injected, corresponding to a relative concentration of 10 to 250 ppm.

The same treatment was made for the result gained under micro-LC conditions. In this case, the range of linearity is between 14 and 80 ng, i.e., 70 to 400 ppm in relative concentration.

All data of the tables and the figures are responses in peak area. The same conclusions have been established with peak height. The relative standard deviation in peak areas or heights was between 2 and 8%.

As the responses depend on the mobile phase, the same study has been made with pure acetonitrile as mobile phase and for solutes BaP and a TG with short chain (CoCoCo), soluble in acetonitrile (Table 3). In micro-LC or capillary LC, the b coefficients are statistically different from 1, but as previously shown the curve $A=am^b$ falls between the two confidence interval curves of linear regression, we can statistically assimilate the response to a straight line.

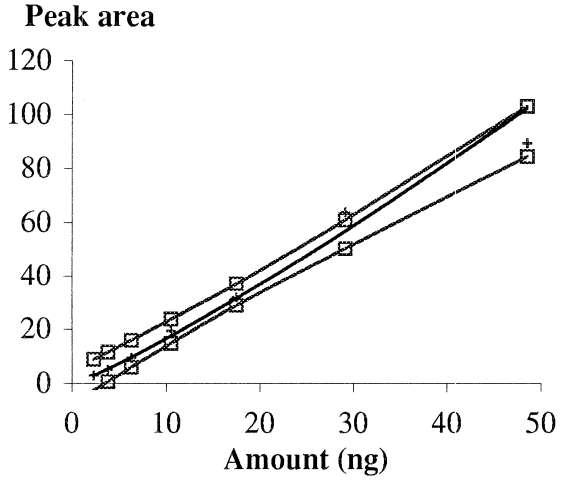

Fig. 4. True response area (+) of TG MMM and confidence interval of linear regression (□) vs. injected amount. Capillary LC conditions: flow-rate: 3 μl/min (u=0.11 cm/s); mobile phase MeCN–acetone (30:70).

Table 3
b value range for a mobile phase of 100% MeCN

Solutes	*b* value range, coefficient of equation: $A=am^b$ (significance level of 95%)	
	Micro-LC (F=40 μl/min)	Capillary LC (F=3 μl/min)
BaP	1.12–1.32	0.96–1.24
CoCoCo	1.15–1.31	1.04–1.23

So in micro-LC or capillary LC, the responses versus the amount can be considered statistically linear for a range of injected amount different according the nature of the solute.

Slope values obtained for the different TGs in the linear range have been determined (Fig. 5). LaLaLa, OOO and LLL have statistically the same slope, whereas MMM and LnLnLn have very different slopes. As the TGs do not have the same carbon number neither the same number of double bonds, this difference in responses must be due to different solvation of the compounds as reported by Andersson and Blomberg [36] and Alexander IV [18], or the liquid or solid nature of pure TGs.

The limit of quantification (i.e., 10-times the noise) for MMM, TGs which have the lowest response was 5 ng in capillary LC. In these conditions the detection limits (S/N=3) was lower than 1.7 ng, which is the smallest value published to date for this kind of solute.

So, if we consider oils containing 10 to 20 main TGs, taking into account the polydispersion of the sample, we have injected 200 nL, we can make a quantitative analysis by using a linear law for solutions of oils at 1.25–2.5 mg/l.

We also have to report that during our own experiment [37–39] Andersson and Blomberg [36] and Greibrokk and co-workers [40,41] have developed the same study using a modified ELLSD system. Their results published very recently are similar to above reported results with EVLSD.

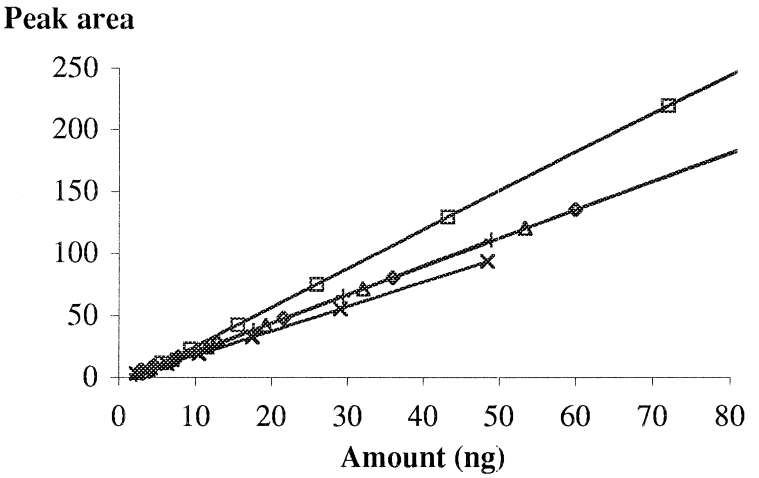

Fig. 5. Response area of TG (arbitrary units) vs. injected amount in the linear range. Same conditions as in Fig. 4. +: LaLaLa; ×: MMM; △: OOO; ◊: LLL; □: LnLnLn.

EVLSD allows one to work in conventional HPLC, micro-LC or capillary LC in so far as the μ-nebulizer has been optimized for these latter conditions. Moreover, in miniaturized chromatography, we have shown for TGs that responses can be statistically assimilated to a linear making quantitative analysis easier.

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