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Fluorescence probe assisted post-column detection for lipid analysis in microbore-LC

E. Caudron, J.Y. Zhou, P. Chaminade, A. Baillet, P. Prognon *

Laboratoire de Chimie analytique EA 3343, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, 92290 Châtenay-Malabry, France

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Abstract

A general approach, still few exploited so far and never associated with microbore-LC, consisting of detection of various lipid classes (i.e. phospholipids, triglycerides, ceramides and glycosphingolipids) by non-covalent association with 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence probe is developed. This mode of detection was coupled with non-aqueous reversed-phase microbore-LC (C_{18}) by using classical post-column fluorescence detection. The classical LC system was first adapted to microbore-chromatography (internal diameter 1 mm) without apparatus miniaturization of the solvent delivery system and the detection cell. For this purpose, the detection parameters (probe concentration, post-column flow rate, post-column reactor length and post-column system temperature) were optimized by a central composite design (CCD) using a mixture of phosphatidylcholine (PC) species as a lipid model and DPH (λ_{ex} = 350 nm, λ_{em} = 430 nm) as a fluorescence probe. The optimal conditions of detection for the various molecular species of PC were determined for a DPH concentration of 3.35 μmol/L, a post-column flow rate of 0.5 mL/min, a reactor length of 1.4 m and a temperature of 35 °C. The fluorescence response was linear over a wide range of PC species from 5 μg/mL to 100 μg/mL and the lower limit of detection (signal/noise = 3) was about 1 μg/mL, that is equivalent to evaporative light scattering detection (ELSD). Others molecular species of various classes of lipids, i.e. triglycerides, ceramides and glycosphingolipids were also easily detected. Thus, this study demonstrated the versatility of the proposed system of detection which was shown to be sensitive, easy to perform, non-destructive and allowed, in contrast to ELSD, for a linear response with various polarity lipid classes.

Keywords: Microbore-LC; Fluorescence; Post-column detection; 1,6-Diphenyl-1,3,5-hexatriene; Lipid species; Lipid classes

1. Introduction

Originally, lipid class analyse evolved from one-dimensional, to two-dimensional thin layer chromatography [1,2]. Since these techniques are tedious and time consuming, high performance liquid chromatography (HPLC) techniques for the separation of lipids have been widely developed.

The choice of the mode of detection is decisive in developing lipid analysis by HPLC. Direct lipid detection is possible by ultraviolet (UV) for phospholipids [3–6] or the glycolipids [7], by mass spectroscopy (MS) for phospholipids [8,9], ceramides [10] or glycolipids [11,12]. However, UV detection is well known for its insufficient sensitivity because of the lipids' weak chromophoric functionality which is only located in the 200–210 nm region [7]. MS detection makes it possible to identify the lipid structure and to obtain a lower limit of detection than that the other direct modes of detection, but it remains an expensive technique for routine analysis especially in quality control. Chemical derivatization for lipid analysis widely developed which enabled efficient detection using UV or fluorescence [13]. The drawback of these derivatization methods is the time needed for the sample preparation. Evaporative light scattering detection (ELSD) has been described as one of the most suitable detection methods for lipid analysis [14–22], but ELSD needs careful calibration because of its nonlinear response. All these features prompted us to consider another mode of detection.

An alternative detection technique is presented in this paper based on a post-column fluorescence derivatization

* Corresponding author. Tel.: +33 146835476.
E-mail address: patrice.prognon@cep.u-psud.fr (P. Prognon).

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using a supramolecular combination of a fluorescence probe and eluted lipid. Fluorescence probes are small molecules which undergo changes in their fluorescence characteristics due to their interaction with their environment (i.e. solvent, macromolecules, fluidity, pH, . . .) as a result of non-covalent interaction [23]. In this work, the use of the rotational fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH) in presence of some classes of lipids of various polarities was studied. Among the probes tested, DPH appears to be one of the most suitable probes for fluorimetric assays of lipids because it exhibits a very weak fluorescence in water and a high quantum yield of fluorescence in organic solvent or in a hydrophobic environment (i.e. lipid). Therefore, this characteristic was exploited in order to enhance the emission of the probes by association with the apolar moiety of lipids after convenient post-column mixing [24].

This supramolecular detection technique has been proposed for analysis of phospholipids with 2,5-bis-[5-*t*-butyl-2′-benzoxazoly]-thiophene as a molecular probe [25] but this probe appeared to be specific of phospholipids [26]. DPH has been proposed for analysis of phospholipids eluted by an isocratic HPLC system [4,27–29]. More recently, post-column fluorescence detection with DPH was proposed by our group as an alternative to evaporative light scattering detection (ELSD) for ceramide (Cer) analysis with gradient elution [22].

The aim of the present study was to generalize this concept for post-column detection using a microbore-LC device and to extend the technique to the analysis of species of different lipid classes: non-polar lipids (triglycerides (TG)), amphiphilic neutral lipids (Cer and glycosphingolipides (Gb)) and charged amphiphilic lipids (phosphatidylcholine (PC)). Another goal is to demonstrate the usefulness of our approach in the field of microbore separation techniques by coupling the post-column detection device to a 1 mm I.D. column.

2. Experimental

2.1. Chemicals and solutions

All lipids tested were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France) and used without further purification. Their structure was presented in Fig. 1 (PC and TG nomenclature: 12:0, laureoyl; 14:0, myristoyl; 16:0, palmitoyl; 18:0, stearoyl; 18:1, oleoyl; 18:2, linoleoyl).

LC grade methanol and acetone were purchased from Prolabo (Fontenay-sous-Bois, France). Brij 35 (30%, w/v) was obtained from Sigma–Aldrich and choline chloride from Labosi (Elancourt, France). Ultra pure water was provided by an UHQ PS MK3 system (USF Elga, Wycombe Bucks, England). DPH was produced by Molecular Probes (Eugene, USA) and purchased from Interchim (Montluçon, France).

1 mmol/L DPH stock solution in tetrahydrofuran was prepared monthly and stored in the dark at −22 °C. 1 mg/mL stock solutions of studied lipids were prepared in tetrahydrofuran for TG, PC and Cer and in methanol/chloroform (2:1, v/v) for Gb. Methanolic working solutions of the lipids were prepared as needed.

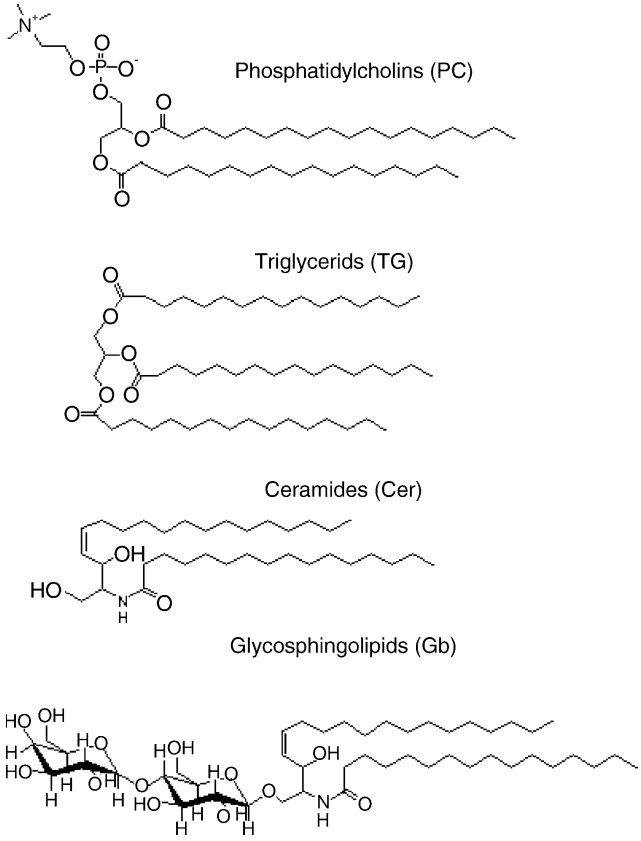

Fig. 1. General chemical structure of the four classes of lipids studied: PC, TG, Cer and Gb.

2.2. Equipment and methods

The chromatographic equipment consisted of a SP 8800 gradient pump used at room temperature with a SMC 400 vacuum membrane degasser (all Spectra Physics, San José, California, USA) connected to an AS 300 auto sampler (Thermo Separation Products, San Jose, California, USA) with a 20 μL sample loop injection valve. The flow rate of the mobile phase was set at 0.1 mL/min. The analytical column, a Nucleosil C_{18} (5 μm, 150 mm × 1 mm I.D.) (Macherey-Nagel, Düren, Germany) was used for all separations. The data monitoring was performed with a Kroma system 2000, version 1.60 software (Kontron instruments, Milan, Italy). The software computed the peak areas, the number of theoretical plates and the asymmetry factors (A_S). A_S was determined by using the US pharmacopoeia definition, where *a* and *b* are the front and back half-widths, respectively at 5% of the peak height:

$$A_S = \frac{a+b}{2a}$$

The post-column fluorescence detection system consisted of a Shimadzu LC-10 AS pump (Shimadzu, Kyoto, Japan)

connected via a LP 21 pulse damper (Scientifics systems, State college, PA, USA) to a Valco Tee piece on line with a 1400 × 0.5 mm knitted PTFE tubing (Supelco, Bellafonte, USA) and leading to a Shimadzu Model RF-551 fluorescence spectrometer equipped with a 12 μL detection cell. The column, the tee piece and the tubing were thermostatically controlled at 35 ± 0.5 °C with a Shimadzu CTO-6A temperature controller. The excitation and emission wavelengths were set to 350 nm and 430 nm, respectively. The post-column reagent composition was 3.35 μmol/L DPH and 57.5 μmol/L Brij 35 in ultra-pure water. This solution was prepared daily, sonicated in a bath sonicator (Bransco 2200, Danbury, USA) and was thermostatically controlled at 35 °C with a boiler TS2 (IKA, Staufer, Germany) before being mixed with the chromatographic eluent pumped at the flow rate of 0.5 mL/min.

PC, Cer and Gb were eluted in an isocratic mode with 40 mmol/L choline chloride in pure methanol. TG were eluted with acetone/methanol with 40 mmol/L choline chloride (40:60, v/v).

Minitab® 12.2 software (Minitab Inc. 1998) was used for central composite design (CCD) data analysis, contour diagrams, regression coefficients, *t*-statistics and response optimization. CCD was used to fit data into a second order polynomial model that relates the variables in the experiments. CCD is a response surface methodology used to study variables independently for their interactions and quadratic effects. The behaviour of the system was explained by the following second order polynomial Eq. (1):

$$Y = B_0 + \sum B_i X_i + \sum B_{ii} X_i^2 + \sum B_{ij} X_i X_j \quad (1)$$

in which the dependant variable Y is the predicted response, i.e. peak surface area (A), number of theoretical plates (N) or peak asymmetry factor (A_S); and in which as independent variables, B_0 is an off set term (constant); B_i a linear effect; B_{ij} a quadratic effect when $i = j$ and a interaction when $i < j$ (i and j are the studied factors, i.e. DPH concentration, post-column flow, length of mixture tubing and temperature); B_{ii} is a squared term; X_i is the ith variable.

3. Results and discussion

3.1. Coupling microbore-LC with post-column device

The advantages of micro-LC are well established [30]. Briefly compared to conventional LC, micro-LC makes it possible to increase the sensitivity and reduce solvent consumption [31,32]. The main problem lies with the necessity of miniaturizing the equipment and limiting extra-column band broadening due to the contribution of (1) the detection cell; (2) the tubing connections and (3) the injection system [33–37].

The variance associated with the detection cell (σ^2_{det}) is given by Eq. (2):

$$\sigma^2_{det} = \frac{V^2_{det}}{F^2} \quad (2)$$

V_{det} was the cell detection volume and F was the mobile phase flow rate (the sum of the chromatographic and the post-column flow rate). As seen, reducing V_{det} implies reducing the flow rate passing through the cell. This means that reducing the inner diameter tubing of the post-column pump is mandatory. In order to perform an efficient post-column detection with microbore-LC without sophisticated adaptation (see Section 2), the post-column flow rate five times higher than the chromatographic flow rate was chosen in order to use a non-reduced detection cell in spite of the dilution effect. This makes, according to the Eq. (2), the variance due to the detection cell decreased by a theoretical factor of 36. With classical LC device (about 1 mL/min chromatographic flow rate and 5 mL/min post-column flow rate), this post-column detection system would imply a high solvent consumption and would apply a detrimental pressure on the detection cell windows. To avoid high flow rates and solvent consumption, a 1 mm microbore column was used at a flow rate of 0.1 mL/min. The resulting post-column flow rate of 0.5 mL/min is compatible with conventional chromatography equipment and makes it possible to use of the standard 12 μL detection cell of our fluorimeter. As the band broadening due to the dead volume of the connections is proportional to the tubing length, the flow rate and to the fourth power of their diameter [33], the length and the inner diameter of the connections were reduced before the mixing Tee from 0.5 mm to 0.13 mm (the flow rate was 0.1 mL/min). After the mixing Tee, the total flow rate was 0.6 mL/min and the tubing diameter was restored to 0.5 mm.

Concerning the injection contribution, it was shown that a 20 μL injected volume, did not contribute to the enlargement of the peak profile provided a stacking injection [38] was used. The lipid samples were prepared in a weaker eluting solvent than the mobile phase to obtain a concentrated sample onto the top of the analytical column. Hence, stacking allowed 20 μL injection of the 1 mm inner diameter column. To verify the influence of these adjustments, an increased water content was added to the injected mixture of PC 12:0/12:0 and PC 16:0/16:0; 50 μg/mL for each compound. Each sample (20 μL) containing from 0% to 17.5% (v/v) of water was injected in triplicate. The DPH concentration was fixed at 3.35 μmol/L, the post-column flow rate was 0.5 mL/min, the tubing post-column length was 1.4 m and the temperature fixed at 35 °C. The mobile phase was pure methanol. Fig. 2 shows the influence on the efficiency measurement (N) of the column as a function in the water content of the injected sample. It was seen that the efficiency increased about three-fold for the two lipids studied when the water content of the injected sample increased from 0% to 12.5% (Fig. 3). Whereas the peak area remained constant, their respective asymmetry factor (A_S) was significantly decreased from 2.0 ± 0.1 to 1.6 ± 0.1 and from 2.3 ± 0.3 to 1.7 ± 0.1 for PC C12:0/12:0 and for PC C16:0/16:0, respectively. However, as the tested

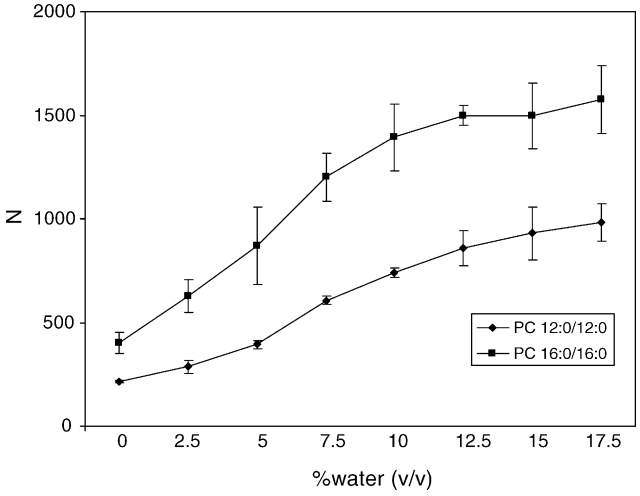

Fig. 2. Evolution of the measured efficiency (N) according to the water content of the injected sample (mixture of PC 12:0/12:0 and PC 16:0/16:0, 50 μg/mL, $n = 3$).

lipids were poorly soluble in water, it was not possible to further increase the water content. The percentage of water to be added was therefore fixed at 12.5% (v/v). To conclude, it should be stressed that the post-column detection, described here, allowed the use of microbore-LC without any particular adaptation of the equipment.

3.2. Chemometric optimisation of the post-column fluorescence detection parameters

3.2.1. DPH post-column detection optimisation using central composite design

A systematic approach was required for the detection optimisation. A central composite design was used to evaluate the significance of each factor and to study the influence of the interaction between the variables and the optimum conditions. In general, a four variable CCD is constructed in such way that 25 experimental conditions are tested. In order to estimate the pure experimental uncertainty of a CCD, it is important to access the repeatability of the response function in the conditions determined by the central point (five repetitions) and by each measure (duplicate). Thus, in our case, 53 experiments were performed.

Response surface methodologies graphically illustrate relationships between optimisation parameters and responses and are the most appropriate means of obtaining a global optimum. Here, the optimization was performed with a lipid test mixture consisting of four saturated PC (12:0/12:0; 14:0/14:0; 16:0/16:0; 18:0/18:0) and one unsaturated (18:1/18:1), each set at a concentration of 50 μg/mL. The flow rate of the mobile phase (pure methanol) was set at 0.1 mL/min.

Before specific limits for the individual CCD factors were selected, five factors influencing the post-column detection had to be determined. These factors were probe concentration, post-column flow rate, Brij 35 concentration [22], post-column tubing length and post-column system temperature.

Brij 35 was necessary to limit build-up background fluorescence due to the lipids and fluorescence probe adsorption into the flow cell [29], but it also facilitates lipid and probe solubilisation, as well as the molecular assembly probe-lipid. A spectroscopic study showed that the increase of the surfactant concentration lead to a significant increase fluorescence in the absence of lipids and this increase occurred around the surfactant's critical micellar concentration [24]. This was consolidated by Zhou's results [22] which reported a significant increase of the fluorescence when the Brij 35 concentration reached 60 μmol/L, which was close to the micellar critical concentration (about 90 μmol/L) reported in literature [39] for this surfactant. When the Brij 35 concentration was superior to its critical micellar concentration, the fluorescence due to the probe increased due to the insertion of the probe into the micelle. Consequently, the background noise increased and the reported signal to noise ratio decreased. Thus, the concentration in the post-column mixture must be maintained lower than its critical micellar concentration. This justifies that the Brij 35 concentration was not a detection parameter to optimize and was fixed throughout the study at 57.5 μmol/L, that is just below the CMC, but sufficiently high to maintain a convenient lipid solubility.

In preliminary experiments, CCD limits have been determined by studying the effect of the probe concentration, post-column flow rate, tubing post-column length and post-column system temperature.

Upon temperature increase, molecular (Brownian) motion favors the non-radiative deactivation of the first singlet state resulting in a decrease of the probe fluorescence quantum yield. In contrast, the probe insertion into the supramolecular association will be favored by an increase of diffusion due to temperature as in the case of DPH insertion into lamellar structures [40]. As a consequence, a compromise must be

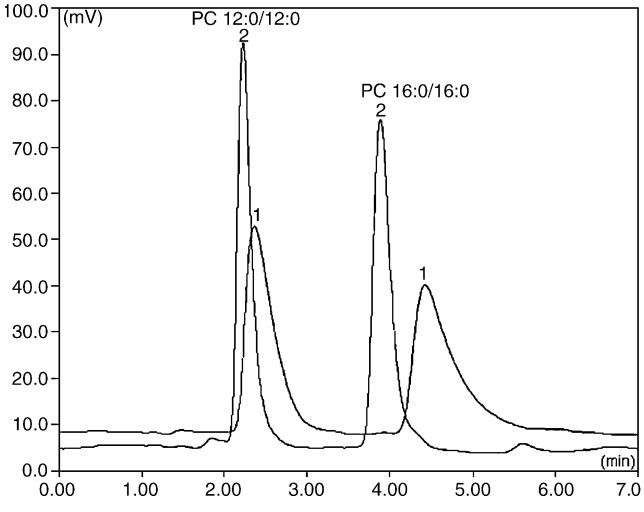

Fig. 3. Chromatogram of a PC mixture (PC 12:0/12:0 and PC 16:0/16:0) 50 μg/mL injected in methanol [1] or in methanol/water (87.5/12.5, v/v) [2]. For chromatographic conditions: see text.

Table 1
Factors and levels used in the CDD

	Start point (lower)	Factorial point (lower)	Centre point	Factorial point (upper)	Start point (upper)
DPH concentration (μmol/L)	1	2	3	4	5
Temperature (°C)	25	30	35	40	45
Tubing length (m)	0.2	0.7	1.2	1.7	2.2
Post-column flow (mL/min)	0.3	0.6	0.9	1.2	1.5

found taking into account temperature increase that favors insertion into the supramolecular association but decreases the fluorescence emission of the probe. So, the post-column temperature system was programmed to allow for 25 °C and 45 °C.

The contact time between the fluorescence probe and the lipids depends on the tubing length. This time was necessary to allow the formation of the supramolecular assembly and to achieve the insertion of the probe within to this structure. On the other hand, as excessively long tubing should increase the extra-column band broadening effects by diffusion, the post-column reactor length varied from 0.2 m to 2.2 m for a fixed inner diameter of 0.5 mm.

The post-column flow rate determined the dilution factor and the aqueous phase/organic phase ratio. The chromatographic flow rate was fixed to 0.1 mL/min due to the 1 mm column inner diameter and the low viscosity chromatographic eluent.

Since DPH is fluorescent in organic environment, it was important to add a sufficient amount of aqueous solution, so that the background fluorescence, in absence of lipid species, remains as weak as possible. This was obtained with a high post-column reagent flow rate and so a high sample dilution. The post-column flow rate varied from 0.3 mL/min to 1.5 mL/min: 0.3 mL/min was the minimal flow rate that could be achieved with a suitable precision.

In order to keep the residual fluorescence of the post-column mixture as weak as possible, the probe concentration in the post-column phase had to be limited. As DPH is only slightly soluble in water, tested DPH concentrations ranged from 1 μmol/L to 5 μmol/L.

The experimental parameter values tested for the CCD are reported in Table 1. From the chromatographic settings determined by the CCD design, the response factors: A (peak area), N (number of theoretical plates) and A_S (peak asymmetry factor) were calculated.

The analysis was applied to all the optimization parameters but by taking into account only the terms which have a significant influence on the parameter studied (this was determined on the complete analysis of the design). Results are presented in Table 2. In this analysis, all the significant terms made it possible to determine a second order relation between optimization parameters (A, N and A_S) and optimization factors (DPH concentration, flow rate, tubing length and temperature) for every lipid according to the general Eq. (1) (see Section 2.2).

All interpretations of the CCD and the response surface data for a single response illustrated how the equation and the confidence values were represented by the response surfaces. The preponderance of a particular term could be identified. The goals for the optimisation of this method included: maximising A and N, minimising A_S. For example, Fig. 4 shows the effect of the DPH concentration and the tubing length on PC 12:0/12:0 peak area.

A successive analysis of the three response functions with regard to the four optimization factors showed that the behavior of the PC species was nearly identical and that their optima were similar.

- The area A increased with the decreasing post-column flow (reduction of the dilution effect) and the decreasing temperature (reduction of collisional quenching of the probe). The area optimum is reached for 3.35 μmol/L DPH concentration and for a tubing length of 1.4 m.
- N increased as the post-column flow, tubing length and temperature increased (reduction of the extra-column dispersing effect). It was demonstrated that the DPH concentration had no effect on the N calculation.
- A_S increased as the post-column flow increased (as the band broadening increased) and decreased as the tubing length increased (by decreasing extra-column effect) and as the temperature increased (by diffusion effect). The variation of DPH concentration had no effect on S.

The optimization of the multiple responses with each tested PC, made a compromise possible between A, N and A_S factors. As an example, Fig. 5 shows the optimization of the multiple responses for PC 12:0/12:0. It allowed to see simultaneously the three optimization factors for each optimization parameter and to choose the values to be attributed to each optimization parameter for this PC. As seen, the DPH

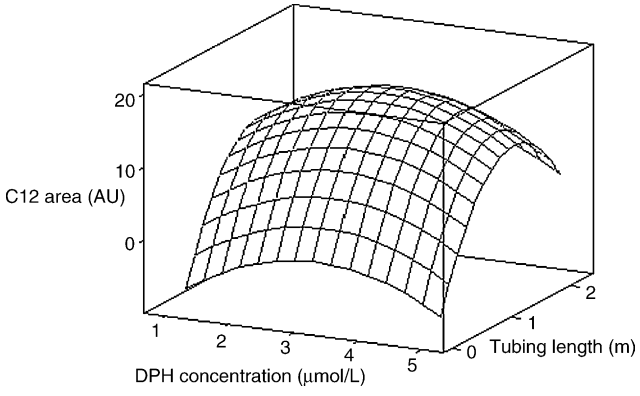

Fig. 4. Response surface showing the effect of the DPH concentration and the tubing length on the PC 12:0/12:0 peak area.

Table 2
Summary of the regression coefficients and their confidence values p (with $p < 0.05$) for the various parameters of optimization

PC	Linear effect					Quadratic effect					Interaction				
	B_0	B_A	B_B	B_C	B_D	B_{AA}	B_{BB}	B_{CC}	B_{DD}	B_{AB}	B_{AC}	B_{AD}	B_{BC}	B_{BD}	B_{CD}
Peak area (A)															
12:0	23.5*	0.73**	−5.07*	2.02*	−3.16*	−1.43*		−3.04*	−0.821*	−0.565***				1.64*	0.77*
14:0	23.5*		−8.95*	3.14*	−2.98*		4.09*	−3.46*						2.18***	
16:0	15.2*		−4.99*	2.29*	−2.12*		2.08*	−1.89*							
18:1	24.6*	1.03***	−6.79*	1.66*	−3.69*	−1.50**	1.44**	−3.31*						2.04*	1.27***
18:0	5.0*			1.65*	−1.42*				0.451**						−1.17*
Number of theoretical plates (N)															
12:0	608*		66.4*	109*	333*										
14:0	640*		107.0*	108*	24.3***			19.4***							
16:0	913*		60.9*	70*	86.2*		−48.9*								35.5***
18:1	778*		84.9*	110*	54.3*										
18:0	913*			−81***	63.8***										113*
Peak asymmetry factor (A_S)															
12:0	1.59*		0.033*	−0.026**	−0.086*		0.025**								
14:0	2.01*			−0.060**	−0.122*										
16:0	1.90*		0.039**		−0.151*										
18:1	2.04*		0.051**		−0.209*										
18:0															

With A: DPH concentration (μmol/L), B: post-column flow (mL/min), C: length of mixture tubing (m), D: temperature (°C).
* $p < 0.0005$.
** $0.0005 \leq p < 0.005$.
*** $0.005 \leq p < 0.05$.

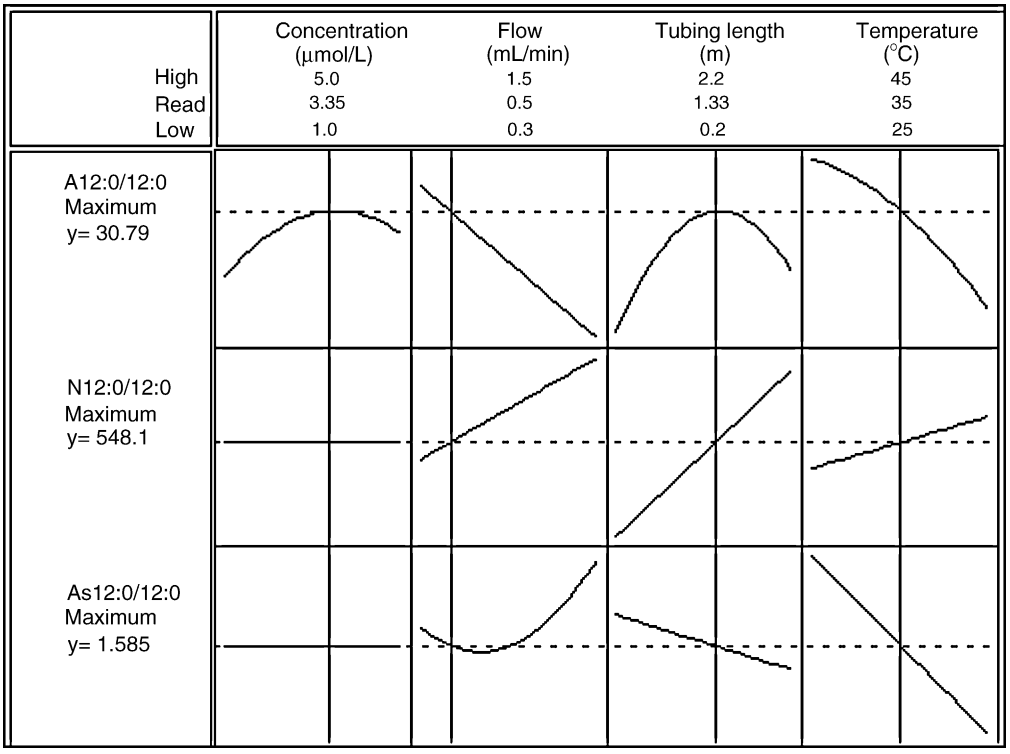

Fig. 5. Optimization of the factors from the responses of area (*A*), number of theoretical plates (*N*) and asymmetry factor (A_S) using PC 12:0/12:0 as a illustration.

concentration had an influence on the PC 12:0/12:0 peak area and presented an optimum at 3.35 μmol/L. This concentration was then used at this optimum. The area decreased quickly when the post-column flow rate increased (dilution effect). A_S was minimal at 0.6 mL/min. On the other hand, *N* increased when the flow rate increased. Finally the selected post-column flow rate was 0.5 mL/min. Since the area was considered as the main criterion in this study and the flow rate had a very strong influence on this parameter, 0.5 mL/min flow was selected to optimize the supra-molecular association in order to obtain a maximal peak area. A lower flow rate was not considered because it involved peak deformations, i.e. increases A_S. The tubing length was fixed to 1.4 m. This length corresponded to the optimal area for PC with *N* and A_S. An increase of the temperature significantly decreased the peak areas but was very favorable to A_S and *N*. We decided to work at 35 °C because the decrease of the area induced by the increase in temperature was slight in comparison to its positive influence on A_S and *N*.

To summarize, chromatography was performed on a Nucleosil® column 100-5 C_{18} with a reduced diameter (150 × 1 mm) at 35 °C. The mobile phase was composed of 100% methanol containing 40 mmol/L choline chloride acting as ionic interaction suppressor between the choline group of PC and the stationary phase [3,28], the chromatographic flow rate was fixed at 0.1 mL/min. The selected optimal post-column conditions were a DPH concentration of 3.35 μmol/L, a post-column flow rate of 0.5 mL/min, a tubing length of 1.4 m and a temperature of 35 °C.

The linearity and the limit of detection were carried out in optimal conditions determined by the CCD. The limit of detection was established for a signal/noise ratio equal to 3. The limit of detection concentrations were 0.5 μg/mL for PC 14:0/14:0 and PC 18:0/18:0; 1 μg/mL for PC 12:0/12:0; PC 16:0/16:0 and PC 18:0/18:1 and 2 μg/mL for PC 18:0/18:2; which corresponded to injected amounts ranging from 10 pmol to 50 pmol. The fluorescence response was linear over a wide range of PC species between 5 μg/mL to 100 μg/mL.

3.2.2. Others lipid class applications

Attempts were made to test the feasibility of this post-column fluorescence detection mode for other lipid classes all characterized by hydrocarbon chains. The post-column conditions used were shown to be the same that optimal conditions determined by the CCD for PC. The mobile phase was methanol with choline chloride 40 mmol/L for all the lipids studied, except for the TG for which the mobile phase was a mixture of 40 mmol/L choline chloride in methanol/acetone (60:40, v/v).

Saturated TG with hydrocarbon chains from 12 to 18 atoms of carbons and unsaturated C18 TG (3 μg/mL each) were tested. As an example, a typical chromatogram of the unsaturated C18 TG mixture is presented in Fig. 6. Every chain possessed 1 (TG 18:1/18:1/18:1), 2 (TG 18:2/18:2/18:2) or 3 (TG 18:3/18:3/18:3) insaturations. Experiments were also made on mixtures of Cer (Cer extracted from bovine brain)

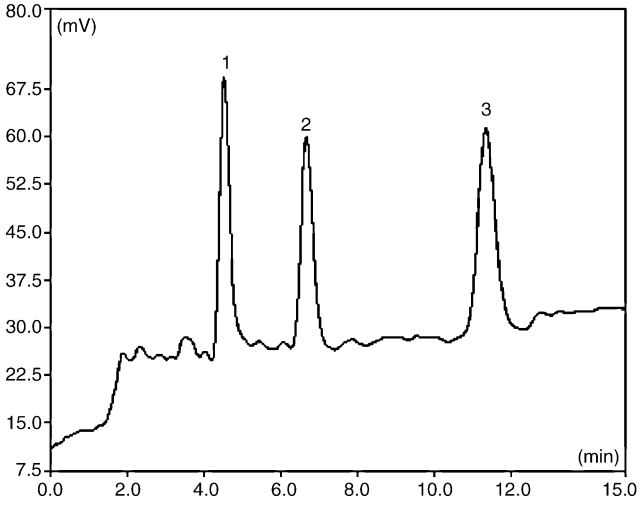

Fig. 6. C18 TG typical chromatogram (1 = 18:3/18:3/18:3; 2 = 18:2/18:2/18:2; 3 = 18:1/18:1/18:1) 3 μg/mL for each compound. Chromatographic conditions: see text.

and of Gb of bovine origin. As an example, a typical chromatogram of a 250 μg/mL lactocerebroside (Gb_2) is presented in Fig. 7. As shown, it was possible to distinguish 12 molecular species at this concentration without any chromatographic optimization.

4. Conclusions

This new microbore-LC technique is able to separate and quantify molecular species of some major lipid classes. The micro-LC is rarely used in spite of the theoretical advantages of this technique; this is certainly due to extra column effects. Coupling micro-LC with a post-column device has, to our knowledge, seldom been reported. On line post-column photochemical derivatization, by placing a microphotoreactor between the micro-column, and the detector were used to detect photochemical unstable products [41]. Chemical post-column derivatization was also described [42,43] but used specific equipment. However, it was presently shown extra-column effects were easily minimized and did not call for particular equipment. Such a post-column detection based on fluorescent supramolecular association makes the quantification of various polarity lipids possible and is also linear over a wider dynamic range. Thus, induced fluorescence detection, with DPH as molecular probe, is an interesting alternative for sensitive lipid detection towards a more classical ELSD. Lastly, the versatility of the LC device described in this paper makes the use of the microbore-LC coupled with post-column detection of interest for many other applications than lipid analysis either in the quality control area or in the bioanalytical field.

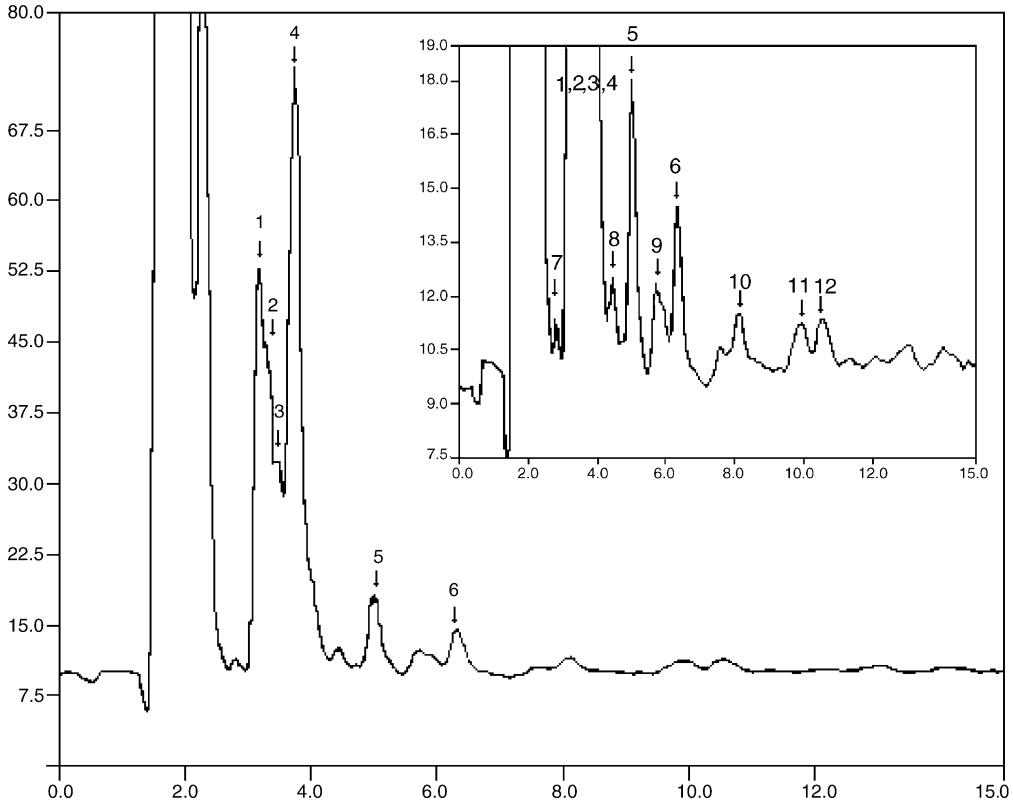

Fig. 7. Typical Gb_2 chromatogram: Gb_2 concentration: 250 μg/mL. Chromatographic conditions: see text.

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