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Fluorescence detection by intensity changes for high-performance thin-layer chromatography separation of lipids using automated multiple development

Vicente L. Cebolla[a,*], Carmen Jarne[a], Pilar Domingo[a], Andrés Domínguez[b], Aránzazu Delgado-Camón[a], Rosa Garriga[c], Javier Galbán[b], Luis Membrado[a], Eva M. Gálvez[a], Fernando P. Cossío[d]

[a] Instituto de Carboquímica, Consejo Superior de Investigaciones Cientificas (CSIC), C/Miguel Luesma, 4, 50018 Zaragoza, Spain
[b] Departamento de Química Analítica, Universidad de Zaragoza, 50009 Zaragoza, Spain
[c] Departamento de Química Orgánica y Química-Física, Universidad de Zaragoza, 50009 Spain
[d] Kimika Fakultatea, Euskal Herriko Unibertsitatea, P.K. 1072, 20080 San Sebastián-Donostia, Spain

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ABSTRACT

Changes in emission of berberine cation, induced by non-covalent interactions with lipids on silica gel plates, can be used for detecting and quantifying lipids using fluorescence scanning densitometry in HPTLC analysis. This procedure, referred to as fluorescence detection by intensity changes (FDIC) has been used here in combination with automated multiple development (HPTLC/AMD), a gradient-based separation HPTLC technique, for separating, detecting and quantifying lipids from different families. Three different HPTLC/AMD gradient schemes have been developed for separating: neutral lipid families and steryl glycosides; different sphingolipids; and sphingosine–sphinganine mixtures. Fluorescent molar responses of studied lipids, and differences in response among different lipid families have been rationalized in the light of a previously proposed model of FDIC response, which is based on ion-induced dipole interactions between the fluorophore and the analyte. Likewise, computational calculations using molecular mechanics have also been a complementary useful tool to explain high FDIC responses of cholesteryl and steryl-derivatives, and moderate responses of sphingolipids. An explanation for the high FDIC response of cholesterol, whose limit of detection (LOD) is 5 ng, has been proposed. Advantages and limitations of FDIC application have also been discussed.

1. Introduction

A number of phenomena giving increases in fluorescence emission intensity involving no apparent chemical reaction have long been in use as physical, non-destructive thin-layer chromatography (TLC) detection methods. Thus, increases in emission of fluorophores in the presence of a broad variety of compounds have been extensively used through indirect detection in TLC, either for detecting non-fluorescent lipophylic compounds [1–12] or for improving sensitivity of the fluorophores themselves when they are the target analytes [13–22].

In previous papers it could be demonstrated that ionic fluorophores, e.g., berberine or coralyne cations, have been useful for sensitively detecting and quantifying by TLC chromophore-free molecules or compounds that have poor absorption properties [23–26]. As an example, a TLC-method has been developed for separating and determining saturated hydrocarbons in fossil-fuel products by fluorescence scanning densitometry, through pre- or post-impregnation of silica gel plates using berberine or coralyne salts [27].

Likewise, it has been shown that these cationic fluorophores give changes in emission in the presence of virtually any compound [25,26]. They experience fluorescence increases in the presence of lipophylic compounds, and fluorescence quenching in the case of molecules with high polarity.

We studied the nature of this fluorescent emission as, for a long time, no thorough explanation had been proposed for indirect fluorescent detection in TLC.

Molecular simulation and analysis of molecular orbitals demonstrated that these phenomena are governed by weak, non-covalent interactions [23–25]. Thus, berberine or coralyne cations behave as a probe that experiences changes in its emission (enhancement or quenching) in the presence of analytes through the non-covalent interactions established in its microenvironment.

Computational results suggested that enhancements in fluorescent signal are consequence of the interaction between the cationic fluorophore and the hydrocarbon chain of analytes which isolates the fluorophore in an apolar microenvironment. This protects it from polar nonradiative decays. A model was proposed for this

* Corresponding author. Tel.: +34 976733977; fax: +34 976733318.
E-mail address: vcebolla@icb.csic.es (V.L. Cebolla).

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ion-induced dipole interaction, which accounted for experimental results [23,24].

These changes in emission used in silica gel medium are referred here as FDIC, i.e. fluorescence detection by intensity changes, as a general detection procedure for thin-layer chromatography.

Previous works pointed out that lipids can also be detected using berberine and coralyne-FDIC [23,26]. The aim of this work is to develop original HPTLC-based methods for separating lipids, using FDIC for detection. FDIC is used here in combination with different automated multiple development (HPTLC/AMD) strategies for separating lipids of interest: (a) neutral lipid families and steryl-glycosides; (b) different sphingolipids which are directly involved in the diagnosis of human diseases resulting from abnormal accumulations of membrane lipids related with human diseases [28]; and (c) mixtures of sphingosine and sphinganine, whose ratio is considered as a biomarker to evaluate exposure to toxic fumonisins [29].

An in-depth study of FDIC response of saturated and unsaturated lipids belonging to mentioned lipid families has been intended in this work. Fluorescent molar responses of lipids and difference in response among different lipid families have been rationalized in the light of the previously proposed model and also using molecular mechanics-based computational calculations. Finally, advantages and limitations of FDIC application are discussed.

2. Experimental

2.1. Fluorophores

Berberine sulfate (>95+%; CAS number: 633-66-9) was from Sigma–Aldrich, Steinheim, Germany). Its chemical structure is depicted in Fig. 1 (**1**).

2.2. Standards and mixtures

The following standards were purchased from Sigma–Aldrich, Inc. Saint Louis, USA, unless otherwise stated. Structures of steryl glycosides and sphingolipids standards and their corresponding names are given in Fig. 1. Bold numbers in text refer to corresponding structures in Fig. 1.

- *Neutral lipids and steryl glycosides*: cholesteryl oleate (C18:1, cis-9; 98%; [303-43-5] CAS); oleic acid methyl ester (C18:1, cis-9; ≥99%; [112-62-9] CAS); cholesterol (≥99%; [57-88-5] CAS; glyceryl tristearate (99%; [555-43-1] CAS); 1,3-distearoyl-rac-glycerol, (≥99%; [504-40-5] CAS); stearic acid (C18:0); oleic acid (C18:1, cis-9; ≥99% [9000-69-5] CAS; palmitic acid (C16:0; ≥99%; [57-10-3] CAS); linoleic acid (C18:2, cis-9, cis-12; ≥99%; [60-33-3] CAS); 1-oleoyl-rac-glycerol (99%; [111-03-5] CAS); 1-stearoyl-rac-glycerol (≥99%; [123-94-4] CAS); **2**, steryl glycosides (98+%; from Matreya, PA, USA); **3**, esterified (palmitoyl) steryl glycosides (98+%; from Matreya, PA, USA).
- *Sphingolipids*: **4**, ceramide, with mostly non-hydroxy acyl groups (stearoyl, 98+%; [2304-81-6] CAS from Matreya); **5**, glycosyl ceramide (98%); **6**, lactosyl ceramide (98+%; [4682-48-8] CAS from Matreya); **7**, ceramide trihexosides (CTH; 98+%; [71965-57-6] CAS from Matreya); **8**, D-sphingosine (99%; [123-78-4] CAS); **9**, *DL*-erythro-dihydrosphingosine (sphinganine) (>99%; [3102-56-5] CAS; from Fluka, Stenheim, Germany); **10**, sphingomyelin (≥97%;[85187-10-6] CAS).

Table 1
AMD solvent gradient conditions for separating neutral lipids and steryl glycosides.

	MeOH (vol%)	*t*-Butyl methyl ether (vol%)	*n*-Heptane (vol%)	Migration (mm)
Step 1	20	80	0	20.0
Step 2	10	90	0	30.0
Step 3	0	100	0	60.0
Step 4	0	60	40	70.0
Step 5	0	50	50	80.0

2.3. Planar chromatography experiments

2.3.1. Plates

High-performance silica gel HPTLC plates, on glass, 10 × 20 cm; 3–10 μm particle size; 60 Å pore size; 0.2 mm thick layer), from Merck (Darmstadt, Germany) were used. Before using, plates were developed (9 cm) with tetrahydrofuran (THF). In the case of sphingolipid analysis, a second development (9 cm) with methanol (MeOH) was performed.

2.3.2. Sample application

Samples were dissolved in a 1:1, v/v mixture of HPLC-grade dichloromethane (DCM, 99.5%) and methanol (MeOH, 99.9%), both from Scharlau, Barcelona, Spain). They were applied using the Automatic TLC Sampler 4 (Camag, Muttenz, Switzerland), as 4 mm bands. Typically, up to 28 samples were applied on the same plate with a distance of 2.5 mm between tracks. Two tracks were always kept free of application, as blank runs.

The first application position was 20 mm (*x* coordinate), and the distance from lower edge of plate was 10 mm (*y* coordinate).

Typical application volumes were between 0.1 and 1 μL, and concentrations range from 1 to 4 mg mL^{-1}.

2.3.3. Chromatographic development

An Automatic Multiple Development (AMD2) system (Camag, Muttenz, Switzerland) was used. This equipment operates as follows: before introduction into a vacuum-tight chamber, the mobile phase for each development is automatically prepared by mixing appropriate portions from up to five different solvents. Chromatography is monitored, and the run stops when the selected developing distance is reached. The remaining solvent is withdrawn from the chamber by vacuum, and the plate is completely dried. While vacuum is released the plate can be preconditioned via the gas phase, leaving the system ready for the next development step.

An HPTLC/AMD run is defined by four parameters: number of steps; distance per step (mm $step^{-1}$) gradient (initial mobile phase composition: final mobile phase composition; or mobile phase composition of each step); and total developing distance (in mm). Operating conditions for mixtures were:

- *for neutral lipids and steryl glucosides*: see Table 1 for conditions. HPLC-grade *tert*-butyl methyl ether (99.9%), *n*-heptane (99%), and MeOH were purchased from Panreac (Barcelona, Spain) and Scharlau (Barcelona, Spain), respectively;
- *for sphingolipids*: a linear gradient of MeOH–DCM, from 80:20 to 0:100, v:v, in 17 steps (3 mm $step^{-1}$), over 76 mm total developing distance;
- *for mixtures of sphingosine and sphinganine*: MeOH–DCM, from 70:30 to 60:40, v:v, in 24 steps (12 steps at 70:30 and 12 steps at 60:40; 2 mm $step^{-1}$), over 65 mm total developing distance.

2.3.4. UV and fluorescence scanning densitometry

A TLC Scanner 3 (Camag, Mttenz, Switzerland) was used in UV and fluorescence modes. The plates were post-impregnated by dipping using a Camag Chromatogram Immersion Device III. Impregnation was carried out using solutions of berberine cation in

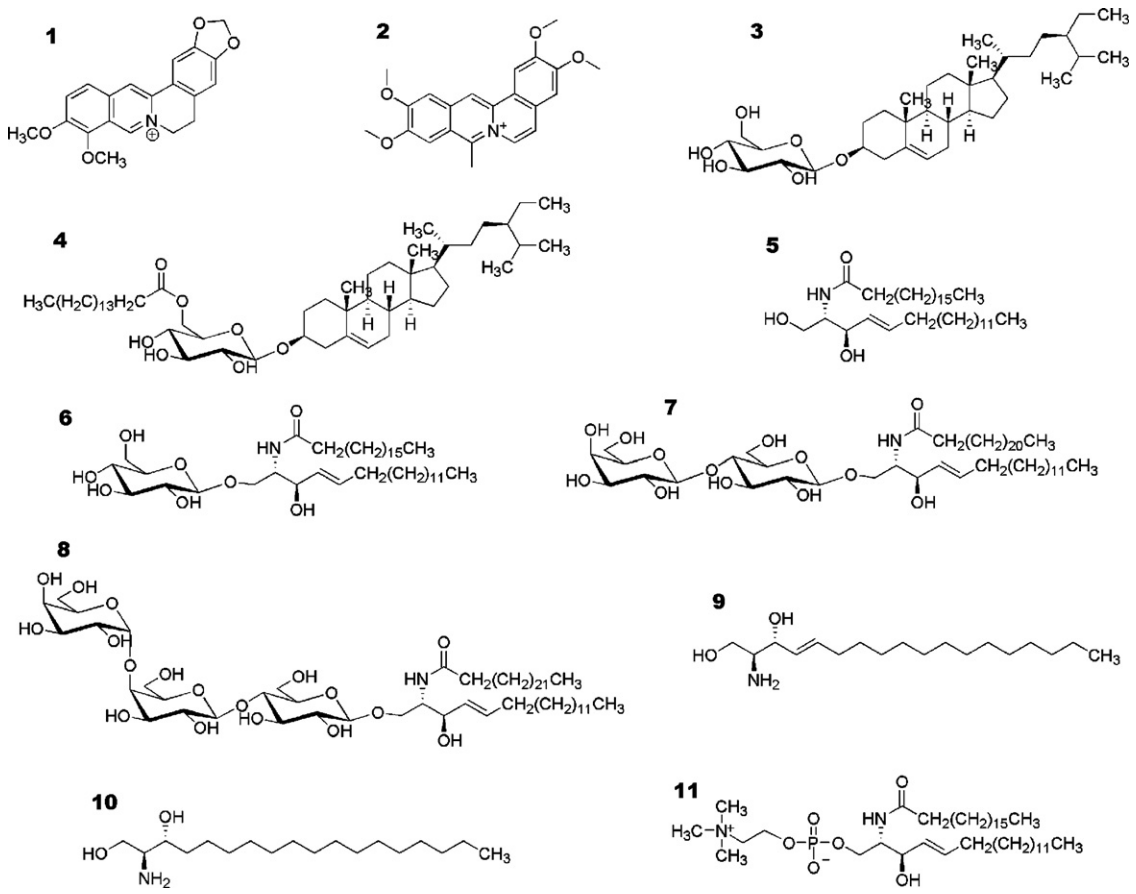

Fig. 1. Chemical structures of berberine cation (**1**) and lipids (**2**–**10**; see bold numbers in text).

MeOH (60–240 mg L^{-1}). Excitation wavelength was 365 nm. Emission was collected at wavelengths longer than 400 nm.

2.4. Computational studies

Geometries of complexes formed by berberine cation and some lipids have been optimized by molecular mechanics (MM) calculations. They were carried out with the OPLS_2005 force field as implemented in the MacroModel package [30], which is integrated through the Maestro v 9.0 graphical interface (Schrödinger 2009 suite for molecular model, Schrödinger, New York).

Given the size of the structures, the optimal conformations were located using Monte Carlo simulations. Since FDIC also occurs in solution, silica–berberine or silica–lipid interactions were not included at this stage of our computational analysis.

3. Results

3.1. Separation and detection of neutral lipids and steryl glucosides

HPTLC/AMD is a gradient elution technique for thin-layer chromatography in which successive runs are performed with decreasing solvent strength and increasing developing distance [31]. HPTLC/AMD, under the conditions reported in Table 1, has been a useful tool for separating the main families of neutral lipids and steryl glycosides.

Fig. 2 shows FDIC-berberine (120 mg L^{-1}, λ_{exc} = 365 nm) and UV (at 190 nm) HPTLC chromatograms resulting of separation of lipids. They include representatives of different classes of neutral lipids: cholesteryl and fatty acid methyl esters, cholesterol, triglycerides, diglycerides, fatty acids, monoglycerides, esterified steryl glycosides, and steryl glycosides, which are cited in order of decreasing migration distance under the chosen HPTLC/AMD conditions.

In Fig. 2A, it can be observed that FDIC-berberine allows the detection of saturated fatty acids, mono-, di-, and triglycerides to be carried out. Saturated lipids cannot be detected by UV (see Fig. 2B). All studied neutral lipids and steryl-glycosides have been detected by FDIC.

Fig. 2 includes peaks corresponding to individual applications of standards under the described HPTLC/AMD conditions. This illustrates the different FDIC responses for different saturated lipids of a given family, and differences in detection between FDIC and UV.

Separation of these lipid families has also been carried out from mixtures of these lipids. In HPTLC/AMD runs, the distance that a given compound migrates before stopping is largely independent of the sample matrix and repeatability of migration distances has been ±0.45 mm.

As shown in the calibration curves depicted in Fig. 3A, FDIC-response for cholesterol is much higher than UV response. FDIC-

Table 2
LOD[a] of several lipids (μg)[b] using UV (190 nm) and FDIC-berberine.

	FDIC-berberine	UV
1-Oleoyl-rac-glycerol	0.15	0.15
1-Stearoyl-rac-glycerol	0.05	Not detected
1-Palmitoyl-rac-glycerol	0.05	Not detected
1-Monopalmitoleoyl-rac-glycerol	0.10	0.10
Steryl glycoside	0.005	0.10
Cholesterol	0.005	0.10

[a] LOD was calculated from S/N ≥ 3 (S is Area in A.U.).
[b] AMD solvent gradient conditions as specified in Table 1.

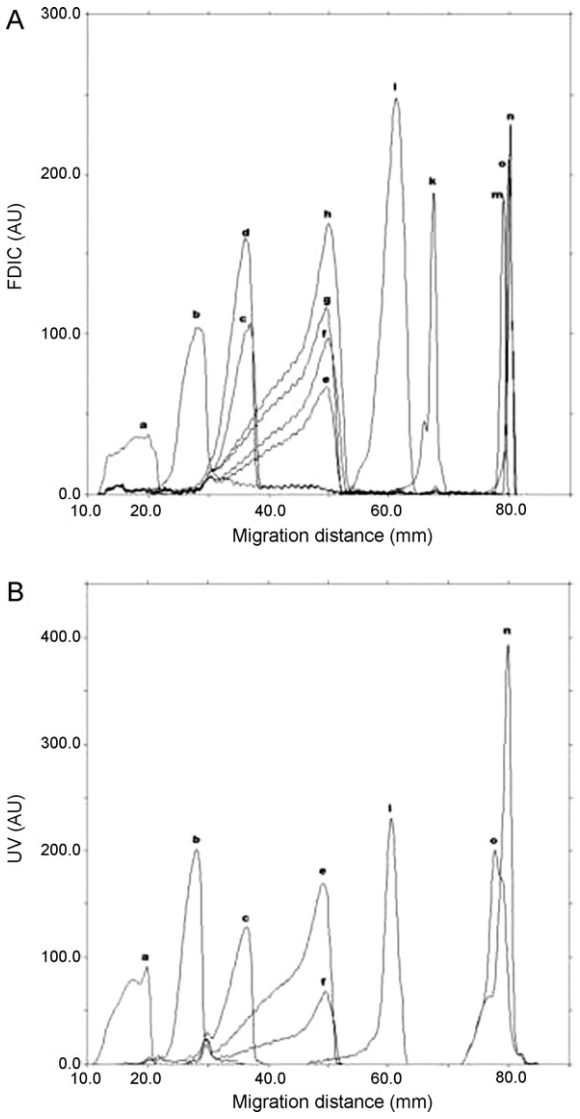

Fig. 2. FDIC-berberine (A) and UV (B). HPTLC chromatograms of: steryl glycosides (a), esterified steryl glycosides (b), 1-oleoyl-rac-glycerol (c), 1-stearoyl-rac-glycerol (d), linoleic acid (e), oleic acid (f), palmitic acid (g), stearic acid (h), cholesterol (i), disteroyl-rac-glycerol (k), glyceryl tristearate (m), cholesteryl oleate (n), and methyl oleate (o). Sample load of each: 3 μg. Application point at 10 nm; AMD conditions in Table 1; UV at 190 nm; FDIC-berberine (120 mg L^{-1}) at λ_{exc} = 365 nm; λ_{em} > 450 nm.

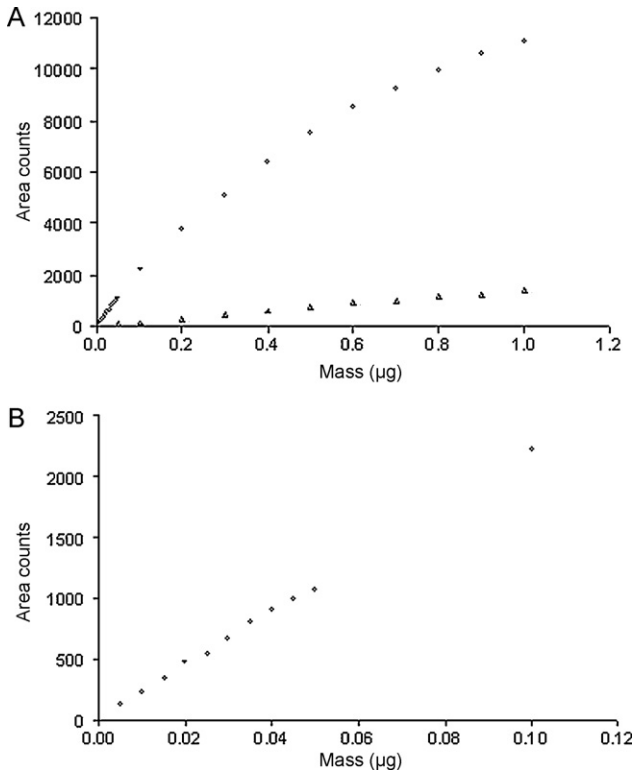

Fig. 3. (A) UV (△) and FDIC-berberine (○) calibration curves for cholesterol. (B) Detail of the 0.0–0.1 μg zone of FDIC-berberine curve (r^2 = 0.9986). Conditions as in Fig. 2.

berberine allows cholesterol to be detected with high sensitivity, with a limit of detection (LOD) of 0.005 μg as reported in Table 2. Fig. 3B shows a detail of calibration curve at low cholesterol loads.

In addition to cholesterol, LOD of several lipids, obtained from both UV and FDIC-berberine, are also given in Table 2. Values have been obtained considering a signal-to-noise ratio equal or higher than 3.

LOD of cholesterol, steryl glycoside and unsaturated monoglycerides obtained using UV are in the range 0.10–0.15 μg (as effective mass applied). Saturated monoglycerides are not detected by UV.

LOD values obtained from FDIC-berberine detection can be classified in three ranges: those of the unsaturated monoglycerides are between 0.10 and 0.15 μg, a limit of detection similar to that obtained in the UV case; those of saturated monoglycerides are 0.05 μg; and those of cholesterol and steryl glycoside are 0.005 μg.

Fig. 4 shows the corresponding berberine-FDIC calibration curves for these lipids. Table 3 shows the analytical responses (Area $mass^{-1}$), molar responses (Area mol^{-1}), and polarizabilities (α, in $Å^3$) of several cholesterol derivatives and monoglycerides. Polarizability measures the ease with which the electron cloud of a molecular entity is distorted by an electric field, such as that created owing to the proximity of a charged reagent. It is experimentally measured as the ratio of induced dipole moment (μ_{ind}) to the field E that induces it ($\alpha = \mu_{ind}/E$).

Responses reported in Table 3 have been obtained from the same sample load (0.1 μg), which is in the linear range for the studied compounds.

Studied cholesteryl- and steryl (**2**,**3**)-derivatives share the same hydrocarbon ring although have a 8 and 10 C atom-hydrocarbon tails, respectively. These compounds and pure cholesterol have the highest FDIC responses among the molecules studied in this work. Their responses are higher than those of previously studied *n*-alkanes. It was previously reported that long-chain alkanes have

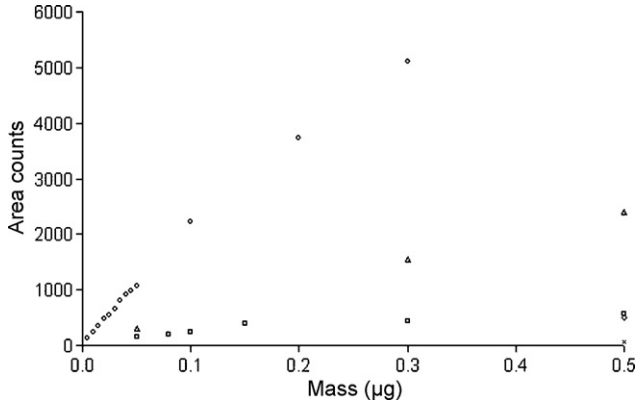

Fig. 4. FDIC-berberine calibration curves for cholesterol (○), 1-oleoyl-rac-glycerol (◊), 1-stearoyl-rac-glycerol (△), 1-palmitoyl-rac-glycerol (□), 1-palmitoleoyl-rac-glycerol (×). Detail of the 0.0–0.5 μg zone of calibration curves (r^2 = 0.998). Conditions as in Fig. 2.

Table 3
Analytical responses (Area mass^{-1}), molar responses (Area mol^{-1}), and polarizabilities (α, in Å^3) and molecular weight (MW) of several cholesteryl, steryl derivatives, and monoglycerides using FDIC-berberine.[a]

	Area mass^{-1} (A.U. μg^{-1})	Area mol^{-1} (A.U.)	α (Å^3)	MW
Cholesteryl oleate	16,074	10.5	80.69	651.10
Esterified (palmitoyl)-steryl glucoside	8140	6.6	94.09	815.26
Cholesterol	16,500	6.4	47.56	386.65
Steryl-glycoside	5760	3.3	64.59	576.85
1-Stearoyl-rac-glycerol	6563	2.35	41.28	358.56
1-Palmitoyl-rac-glycerol	3616	1.19	37.61	330.50
1-Oleoyl-rac-glycerol	3026	1.08	41.30	356.54
1-Monopalmitoleoyl-rac-glycerol	1172	0.38	37.64	328.49

[a] Conditions: 0.1 μg sample load; AMD: 1 step, MeOH–tert-butyl methyl ether (20:80, v/v), migration distance: 20 mm; detection: FDIC-berberine 120 mg L^{-1}.

a sensitive FDIC response. Cholesterol gives a higher FDIC response (6.4 Area mol^{-1} units) than, for example, *n*-octadecane (1.2 Area mol^{-1} units) although the latter has one carbon atom more (28 vs. 27).

Concerning cholesteryl and steryl-derivatives, analytical FDIC responses, expressed as Area mass^{-1}, are higher for cholesterol and cholesteryl oleate than for steryl glycosides. However, molar responses rather than analytical ones should be considered to understand the mechanism of FDIC response. Molar response of these derivatives decreases in the order: cholesteryl oleate > esterified steryl glycoside > cholesterol > steryl glycoside.

Monoglycerides have lower FDIC responses than cholesteryl and steryl derivatives. Responses for monoglycerides are in the order: C18:0 > C18:1 ∼ C16:0 > C16:1.

Relationships between lipid structure and properties, and FDIC response are discussed below.

3.2. Separation and detection of sphingolipids

Fig. 5 shows the corresponding UV and berberine-FDIC chromatograms of an HPTLC/AMD separation of sphingolipids. Separation of sphingomyelin (a), sphingosine (b), CTH (c), lactosyl ceramide (d), glycosyl ceramide (e), and ceramide (f) has been carried out using a 17-step, universal gradient scheme (MeOH–DCM, from 80:20 to 0:100) as described in Section 2.3.3. These conditions have been applied to individual standards and standard mixtures. Application to test its general performance regarding real samples (e.g., urine) is in progress.

Separation on silica gel plates has been carried out according lipid polarity. Under the conditions used, ceramides containing different number and nature of sugar units have been separated: CTH (3 units), lactosyl ceramide (2 units) and glycosyl ceramide (1 unit).

Fig. 5 shows that FDIC-berberine also provides positive peaks for sphingolipids under the studied berberine concentrations although responses are lower than in the case of neutral lipids. In general, FDIC for polar lipids seems to be less sensitive than for apolar ones. Fig. 5 shows, for comparative purposes, the chromatographic peak of cholesterol together with those of sphingolipids for the same sample load.

It has been reported [25] that highly polar compounds produce loss in FDIC signal and even a fluorescent quenching with regard to the baseline. Response loss for polar lipids is particularly remarkable in our case for CTH with regard to ceramide.

As in the case of saturated neutral lipids, non-absorbing sphingolipids can also be detected by FDIC. An example of this is presented in Fig. 6 where UV and FDIC-berberine chromatograms of a mixture containing sphingosine (**8**) and sphinganine (**9**) are presented.

In sphingolipid-related samples, sphingosine is usually accompanied by sphinganine which only differs from it by the absence of the only double bond in its structure. Therefore sphinganine is not detected by UV under HPTLC detection conditions but it is using FDIC-berberine.

Despite their structural similarity, these compounds migrate with different speed and hence the mixture has been separated on silica gel plates under the HPTLC/AMD conditions described in Fig. 6, i.e. using a MeOH–DCM gradient from 70:30 to 60:40, v:v, in

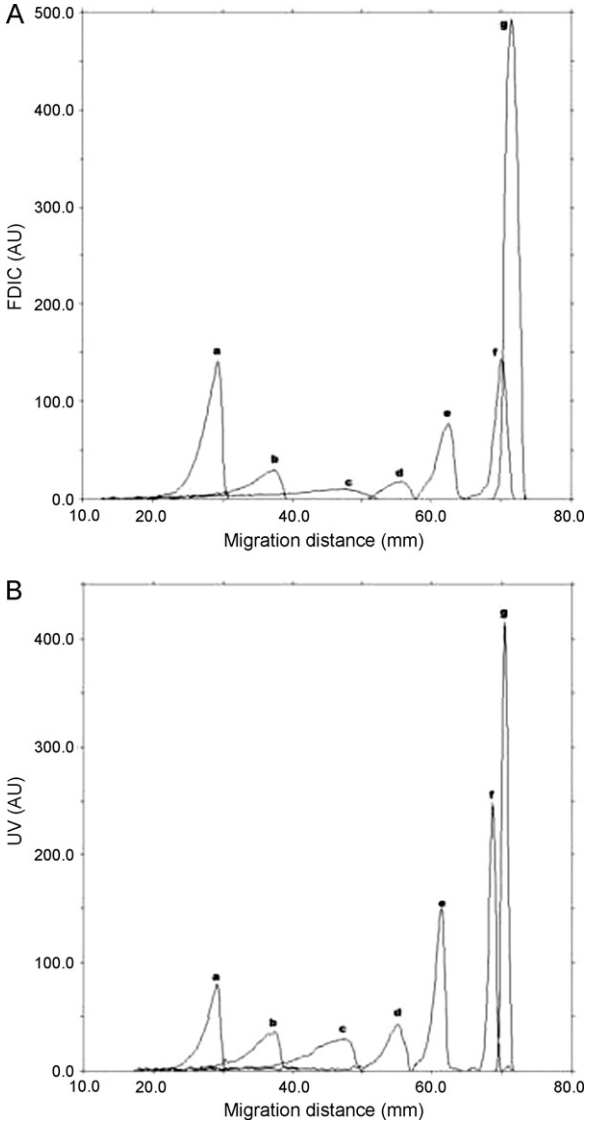

Fig. 5. FDIC-berberine (A) and UV (B). HPTLC chromatograms of: sphingomyelin (a), D-sphingosine (b), CHT (c), lactosyl ceramide (d), glycosyl ceramide (e), ceramide (f), cholesterol (g). Sample load of each: 1.7 μg). See AMD conditions in Section 2. FDIC-berberine (200 mg L^{-1}). All other conditions are as in Fig. 2.

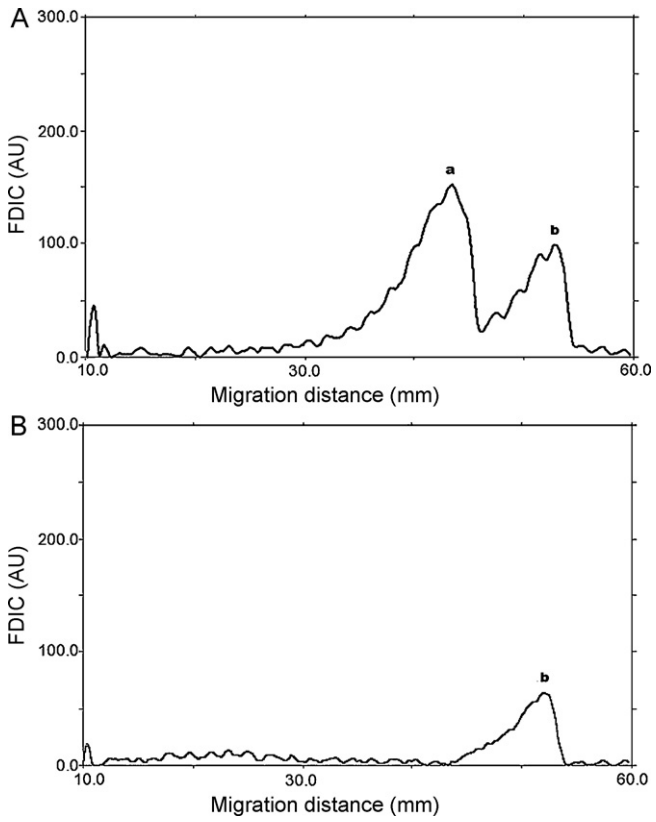

Fig. 6. FDIC-berberine (A) and UV (B). HPTLC chromatograms of a mixture containing: sphinganine (a), 6.96 μg, and sphingosine (b) 13.84 μg. See AMD conditions in Section 2. All other conditions are as in Fig. 2.

24 steps (2 mm step^{-1}), over 65 mm total developing distance (see Section 2.3.3).

There exist other pairs of lipids that, although having a similar structural analogy to that of sphingolipids mentioned, i.e., a polar head and a double bond, however show no differences in their migration on silica gel and hence cannot be separated (e.g., oleic and stearic acid). However in the case of sphingosine and sphinganine, the observed differences in migration may be due to conjugation of the double bond with the lipid polar head. These differences are evidenced under the used AMD gradient conditions and the effect of successive focalization steps.

4. Discussion

4.1. General explanation of increases in emission of berberine cation in the presence of lipids

It was proposed in previous papers that non-specific, electrostatic interactions between ionic fluorophores and polarizable hydrocarbon chain of analytes are responsible of emission increases.

In this way, electrostatic interactions between probe and the corresponding hydrocarbon chain of the lipid contribute to the efficiency of the fluorescence emission, creating a microenvironment that isolates the fluorescent probe and prevents non-fluorescent decay mechanisms, decreasing the value of the nonradiative decay rate k_{nr} in quantum yield equation

$$\Phi = \frac{k_r}{k_r + k_{nr}}$$

where k_r is the emissive rate of the fluorophore and k_{nr} stands for the grouped rate constants of all possible non-radiative decay processes.

At a given concentration, the intensity enhancement of fluorescence is linearly dependent on α of the neutral molecule surrounding the probe [23–26].

On the other hand, if the analyte may establish specific donor–acceptor interactions with the fluorophore, a decrease of emission or even a quenching may occur [23–26].

In general, lipids have high values of α and therefore give sensitive FDIC responses. Electrostatic interactions were also evidenced by analysis of molecular orbitals and molecular mechanics [23].

4.2. FDIC responses of neutral lipids and steryl glycosides

For a given family of lipids, the longer and more polarizable the aliphatic chain in the analyte, the larger the electrostatic interaction with cationic fluorophore, the larger the protective effect, and therefore the higher the fluorescent response. As an example, this can be observed for monoglycerides in Table 3. In the case of saturated monoglycerides, α and FDIC response of C18:0 are higher than those of C16:0. The same for unsaturated C18:1 and C16:1 monoglycerides. On the other hand, saturated monoglycerides give higher responses than unsaturated ones.

Within neutral lipids, cholesteryl and steryl-derivatives give the highest responses among the studied compounds.

We optimized at MM level of theory (OPLS_2005 force field, vide supra) the geometries of the complexes formed by berberine and cholesteryl oleate, steryl glycoside and esterified steryl glycoside. The most stable, energetically most favourable geometries found after intensive conformational search (vide supra) are shown in Fig. 7.

Results show a considerable conformational rigidity of berberine, thus indicating that the response for the different derivatives of a given family must not be due to significant changes in the berberine geometry on passing from one compound to another. Likewise, the studied compounds adopt an extended conformation along the main axis of the berberine molecule in order to maximize the ion–molecule interaction.

The averaged complexation energies of lipid–berberine complexes (ΔE values of approx. -50 kcal mol^{-1}) are larger than in the case of previously studied *n*-alkanes (approx. -10 kcal mol^{-1}, [23]), and provide a consistent apolar environment to the berberine molecule.

Fig. 7 shows that the positively charged N atom of berberine interacts with the highly polarizable hydrocarbon chains. As previously mentioned, these chains protect the cation from other polar decays pathways and, as a result of this interaction, k_{nr} decreases. This protective effect gives an increase of the quantum yield.

Fig. 7 shows how the two hydrocarbon chains of these compounds are arranged to maximize the interaction with berberine nitrogen. In the case of cholesteryl oleate, this spatial arrangement is particularly favourable because it allows the interaction of berberine simultaneously with both hydrocarbon chains, protecting berberine cation from polar decays. Moreover, cholesteryl oleate and esterified steryl glycoside have the highest values of α. They give the highest increases of emission per mol. These reasons explain the comparative higher responses of cholesteryl and steryl-derivatives with regard to those of other neutral lipids.

The order in FDIC molar response of these compounds can be justified as follows: the introduction of a long hydrocarbon chain (in cholesteryl oleate and esterified steryl glycoside) induces a higher molar response of both compounds with regard to pure cholesterol. In the case of the esterified compound, the increase in emission due to the acyl group (palmitoyl) compensates the corresponding decrease produced by its glycosyl unit.

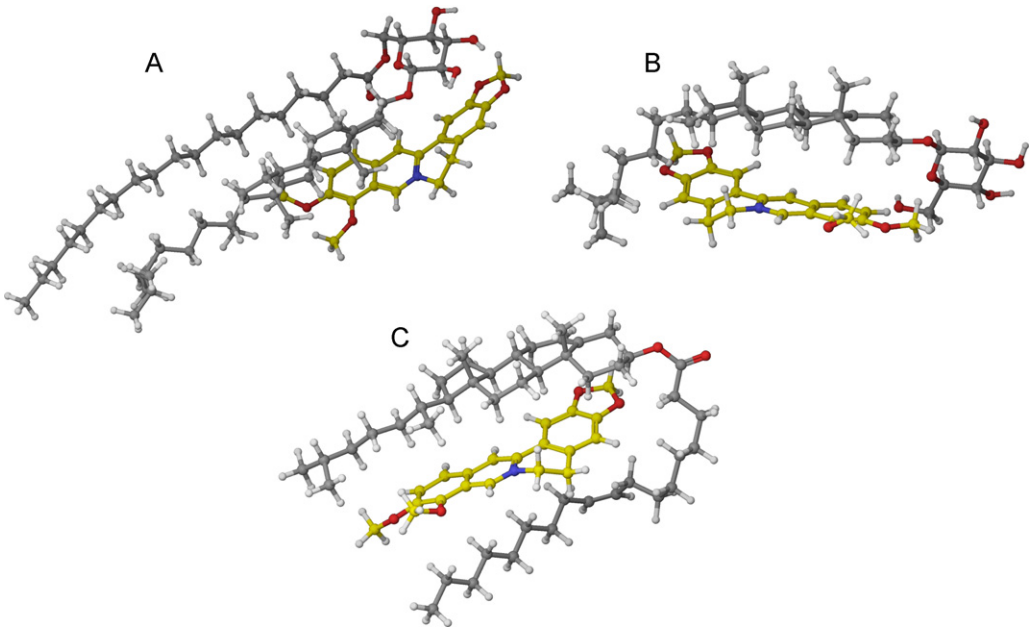

Fig. 7. Lowest energy conformations (see text) of the complexes between berberine and (A), esterified steryl glucoside; (B), steryl glycoside; (C), cholesteryl oleate, computed using OPLC_2005 force field-based MacroModel application. (Yellow: berberine cation; blue: N atom; red: O atoms; grey: C atoms; white: H atoms).

On the other hand, the glycosyl unit of steryl glycoside produces a decrease in its emission with regard to pure cholesterol. In this case, the difference in two C atoms does not compensate the decrease in emission produced by the sugar unit. Finally, the case of cholesterol will be studied in detail in Section 4.4.

4.3. FDIC responses of sphingolipids

In the case of sphingolipids and other polar lipids containing long hydrocarbon chains but also highly polar groups, we should also take into account that specific donor–acceptor interactions result in the well-known phenomenon of fluorescence quenching or in a decrease in emission. Sphingolipids do not show a net quenching in the case of using berberine, but a decrease in fluorescence intensity is obtained with regard to the emission produced by neutral lipids. Likewise, as shown in Fig. 5, response of sphingolipids decrease according to the number of sugar units, giving CTH (with three glycosyl units) gives a weak fluorescent signal under the reported conditions (200 mg L^{-1} berberine).

Computational results are also compatible with the lower FDIC responses for sphingolipids with regard to those of neutral lipids. Sphingolipids were computed in a previous work [23] using a similar computational procedure by means of the AMBER force field. The conclusion was that interaction takes place between the positively charged nitrogen atom of berberine and the polar hydroxy groups in the ceramide moiety, which leads to an increase in k_{nr} and therefore, a decrease in the quantum yield. However, due to the high values of α and the presence of long hydrocarbon chains the resulting intensity is not a net quenching but a decrease of emission with regard to neutral lipids.

4.4. The case of cholesterol

Apparently, the high FDIC response of cholesterol is not completely justified by its physical and interaction properties. Even though its relatively high α could justify a sensitive fluorescent signal, its FDIC response is substantially higher than that corresponding to other compounds with similar values of α, for example, *n*-octacosane. One would expect even greater FDIC response of *n*-octacosane since this compound is not able to establish specific interactions with berberine, and therefore cannot produce an increase of k_{nr} (decrease of signal) in this way. On the contrary, cholesterol molecule has a hydroxyl group in its structure. One may think that hydroxyl, in its interaction with berberine cation, could lead to an increase of k_{nr} via a donor–acceptor interaction and, therefore, a decrease of FDIC signal with regard to *n*-octacosane. The experimental fact is that cholesterol has a greater response than octacosane.

Explanation for this higher-than-expected response may be the formation of cholesterol associated units, through hydrogen bonding. Auto-association of cholesterol to form these units has been reported in protic and non-protic solvents [32,33]. Table 4 shows different properties of linear and cyclic cholesterol dimers, trimer, tetramers and hexamer, which were obtained by other authors [33] using molecular modelling (MM+ and AM1 force fields).

According to the data in Table 4, formation of cholesterol oligomers is, in general, energetically favoured (negative energy interaction).

Given the above, the FDIC response of cholesterol, higher than expected, could be explained by the following reasons:

Table 4
Summary of properties of associated units of cholesterol calculated by Chiosa et al. [33] using MM+ and AM1 force fields.

	Monomer	Dimer	Trimer[a]	Tetramer[a]	Hexamer
Polarizability (α in Å^3)	47.56	95.34	141.73	188.76	282.19
Interaction energy (ΔE in kcal mol^{-1})	–	−5.27	−23.16	−152.24	−19.28

[a] Linear oligomers.

- whether associated units are formed, OH groups would be interacting with each other, not with the fluorophore (decrease in k_{nr});
- α increases with the number of units, as shown in Table 4. Associated cholesterols have high α values and therefore must give a stronger electrostatic interaction with berberine cation than the corresponding "monomeric" cholesterol. This increase in α would have the effect of protecting berberine from polar decays (k_{nr} decrease).

This picture is coherent with a previous description of this phenomenon and the previous results obtained [23,34]. As the corresponding molecule and the fluorophore are supposed to be at a short distance, a bigger molecule may interact with several molecules of the fluorophore at the same time. In the end, we have an approximately constant response per mol of monomer unit.

4.5. Advantages and limitations of FDIC

Although FDIC also occurs in liquid media, the rigid environment provided by silica gel plate favours the direct interaction of the analyte with the fluorophore. It is to be remembered that elution solvent is removed in TLC silica gel experiments. In the case of an alkane and berberine cation, the stoechiometry of direct interaction is 1:1 [23].

FDIC is not restricted to berberine or coralyne cations but variations in fluorescent emission of a probe in the presence of other molecules is a general phenomenon for ionic fluorophores [26]. According to the model developed, it also seems general for all types of analytes. It is particularly useful for detecting molecules that do not absorb, or do it poorly, in UV–vis. Sensitivity for detecting polar molecules is lower and, depending on the particular fluorophore–analyte system, they may be detected through fluorescent quenching.

FDIC can be used by pre- or post-impregnation in combination with development techniques of separation (e.g., HPTLC/AMD) for quantitative analysis of lipids and other compounds with a partially aliphatic structure.

Most of chemical derivatization procedures are usually applied under fixed conditions. In these cases, either the stoichiometry of reaction is not well understood, or it is difficult to modify reaction conditions to obtain a response that is optimized for the detection of analyte. Likewise, heating is usually necessary to complete the corresponding chemical reaction, e.g., complexation, oxidation, thermal aromatization. However, FDIC is not a derivatization as it does not involve any chemical reaction but merely weak, non-covalent interactions between the fluorophore and the analyte on silica gel. Operationally, no heating is required but simply a pre or postimpregnation of the plate, by dipping. Likewise, detection sensitivity can be easily modulated by simply changing the fluorophore concentration.

A mechanism of fluorescence induction for FDIC has been proposed, which reasonably explains the experimental data. Likewise, computational calculations of fluorophore–analyte geometries are a useful tool to understand fluorescent responses and to efficiently design original analyte–fluorophore detection systems based on this technique, as well as to explain other indirect fluorescence-related phenomena previously reported in the literature.

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