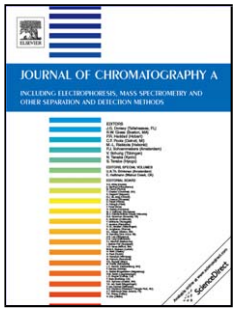

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Coralyne cation, a fluorescent probe for general detection in planar chromatography

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Abstract

A large number of analytes, including non-fluorescent ones, can be sensitively detected by fluorescence scanning densitometry using silica gel HPTLC plates impregnated with a solution of coralyne cation. This is carried out by the variation, increase or decrease, that the corresponding analyte induces on native coralyne emission at a given excitation wavelength. A similar phenomenon was previously described for berberine cation, and Reichardt's dye probes. However, the sensitivity of coralyne in HPTLC detection of non-fluorescent, structurally different analytes (e.g., long-chain alkanes, alcohols, alkylbromides, neutral lipids) is superior to that of the above-mentioned probes.

In this work, the analytical viability of this phenomenon for HPTLC detection using coralyne as a probe is explored, and fluorescent responses of a number of analytes on the coralyne system are rationalized in the light of a previously proposed model. This establishes that the resulting intensity for a probe in the presence of a given compound can be explained as a balance between radiative (contribution of non-specific interactions) and non-radiative processes (specific interactions), the latter producing fluorescence quenching. Experimental results and proposed model suggest that this phenomenon may be general for practically all kind of analytes.

1. INTRODUCTION

Derivatization agents used in HPTLC and HPLC are usually efficient for single analytes or groups of analytes. Derivatization reactions normally involve formation of covalent bonds [1]. Development of universal detection procedures based on fluorescence would be of interest in chromatography.

In a previous work [2], it was experimentally proven that a large number of analytes, regardless of their chemical structure, alter the native emission of berberine cation and Reichardt's dye without significant variation in the emission wavelength of these probes. They can consequently be quantitatively detected. These analytes include non-fluorescent compounds.

This phenomenon, which occurs in presence or absence of solvent, was first evidenced in the case of saturated hydrocarbons [3, 4], compounds that have neither fluorescence nor UV spectra under the usual conditions of analytical work. Saturated hydrocarbons produce increases in berberine or Reichardt's dye emission [2]. It has been possible to use this phenomenon in HPTLC-scanning fluorescence mode, by impregnating silica gel plates with the above-mentioned probes for determining saturated hydrocarbons in different types of fuels [5, 6].

Fluorescent responses in these systems depend on analyte structure through the interactions that they establish with the corresponding probe. Thus, a model has been proposed that accounts for the experimental data corresponding to the increment of emission intensity of berberine cation and Reichardt's dye for different molecules in absence of solvent. Accordingly, the resulting intensity for a probe in the presence of a

given compound can be explained as a balance between radiative (non-specific) and non-radiative (specific) processes.

Coralyne cation, an ionic fluorophore, is more aromatic and fluorescent than berberine and can be considered "a priori" a potential candidate for operating according to the proposed model. Therefore, in this work, the viability of coralyne cation as a probe in HPTLC-fluorescence scanning densitometry is evaluated for detecting structurally different analytes, particularly non-fluorescent ones. Analyte responses in coralyne system have been rationalized in the light of the proposed model and compared with those obtained using berberine-impregnated plates.

Our purpose is not to give finished and specific analytical protocols, but to present the basis of a general detection procedure, thus giving analysts tools with which to develop their particular analytical procedures.

2. EXPERIMENTAL

2.1. Fluorescent probes

Coralyne chloride (>98 %; CAS number: 38989-38-7; from Across Chimica, Geel, Belgium), and berberine sulfate (>95+%; CAS number: 633-66-9; from Sigma-Aldrich, Steinheim, Germany) were used. Their chemical structures are depicted in Figure 1.

2.2. Standards and mixtures

The *n*-alkanes (Fluka, Buchs, Switzerland) used were: *n*-decane (*n*-C_{10}, 98+%); *n*-dodecane (*n*-C_{12}, 99+%); *n*-hexadecane (*n*-C_{16}, 99+%); *n*-octadecane (*n*-C_{18}, 98+%); *n*-docosane (*n*-C_{22}, 98+%); *n*-tetracosane (*n*-C_{24}, 99+%). The alcohols (Sigma-Aldrich) used were: 1-decanol (1-C_{10}OH, 97+%); 2-dodecanol (2-C_{12}OH, 99+%); 1-hexadecanol (1-C_{16}OH, 99+%); 1-octadecanol (1-C_{18}OH, 99+%); 1-docosanol (1-C_{22}OH, 97+%). The alkylbromides (Fluka) used were: 1-bromohexadecane (1-C_{16}Br, 97+%), and 1-bromodocosane (1-C_{22}Br, 97+%). An antibiotic, rifampicine (Sigma-Aldrich, 95+%), was also used as a standard.

A mixture of neutral lipids (Sigma-Aldrich, 95+%) was used consisting of cholesterol, cholesteryl oleate (C18:1, cis-9), oleic acid (C18:1, cis-9), oleic acid methyl ester (C18:1, cis-9), and triolein (C18:1, cis-9).

2.3. Planar chromatography experiments

2.3.1. Plates

High-performance silica gel TLC plates (HPTLC plates, on glass, 10 x 10 cm; 3-10 µm particle size; 60 Å pore size; 0.2 mm thick layer), from Merck (Darmstadt, Germany), and Macherey-Nagel (Düren, Germany), were used.

2.3.2. Incorporation of fluorescent probes

Incorporation to the HPTLC plates was usually carried out by pre-impregnation before chromatographic development. A solution of either 60 mg l^{-1} berberine sulfate in methanol, or 6 mg l^{-1} coralyne chloride in methanol was used during 20 s. Plates were subsequently dried overnight at 40º C. Alternatively, plates can be post-impregnated after chromatographic development by spraying the corresponding solution of coralyne or berberine. In this case, overnignt drying of the plate is not necessary.

2.3.3. Sample application

Samples were dissolved in dichloromethane or methanol, and applied, at least in triplicate, onto the impregnated TLC plates using a band-sprayer Linomat IV sample applicator (from Camag, Muttenz, Switzerland), as 2-mm bands. Sample application point is at 85 mm in HPTLC plates. On each plate, one track was left blank (without applying sample).

2.3.4. Chromatographic development

Samples were developed using a horizontal development chamber (Camag) by placing the plates downward. When either *n*-alkanes or alkylbromides were applied on the plate, development was performed using *n*-hexane (HPLC grade, Scharlau, Barcelona, Spain) during 5 min; when alcohols were applied, development was performed using dichloromethane (HPLC-grade, Lab Scan, Dublin, Ireland) during 5 min; when *n*-alkanes, alcohols, and alkylbromides were applied together, development was performed first using

5 min n-hexane; and then, 2 min 40 s dichloromethane. Rifampicine was developed using methanol (5 min). Development of lipid mixture was carried out using light petroleum (b.p.= 50-70 ºC)-diethyl ether-acetic acid (80:20:1) as eluent (20 min), as reported elsewhere [7].

2.3.5. Fluorescent detection

A CS9301 TLC scanning densitometer (Shimadzu, Kyoto, Japan) was used in the fluorescence mode. In berberine experiments, excitation wavelengths (λ_{ex}) 365 nm or 410 nm and detection in the >450 nm zone were used, while in coralyne experiments, conditions changed to either λ_{ex} = 410 nm or 430 nm, and detection was in the zone >450 nm. Linear scanning was used in both cases, with a 1.0 x 1.0 mm beam size. Peak area data were collected, displayed and stored using Shimadzu CS9310 personal computer software. Some probe displacement by the solvent front may occur during development. This can be detected and, subsequently, substracted with the above-mentioned software. Further details on equipment used have been reported elsewhere [5].

3. RESULTS

3.1. Detection of saturated hydrocarbons by TLC-coralyne-induced fluorescence

When a silica gel TLC plate is impregnated with a solution of coralyne cation, the application onto the layer of a saturated hydrocarbon produces an increase in the fluorescence signal (>450 nm) when the system is irradiated with UV light (e.g., at 410, 430 nm). This emission is exclusively due to the sample in the presence of coralyne, and its intensity is in proportion to sample load (Figure 2) and alkylic chain length (Figure 3), for the given wavelength. It is to be noted that saturated hydrocarbons do not exhibit fluorescence or ultraviolet spectra within the usual range of analytical work.

Emission spectra obtained on the layer have not shown changes in emission wavelength: only intensity (quantum yield) is affected by alkane addition. This also occurs in liquid media when using polar solvents [8]. A similar phenomenon was reported using berberine cation as impregnating agent [1,2], which was used to develop sensitive analytical procedures for detecting saturated hydrocarbons in diesel fuels and other fossil fuel-derived products [5,6,9]. Figure 3 shows that coralyne gives higher increase in fluorescent responses (area counts) than berberine for the studied alkanes, with regard to their respective baselines, even where the coralyne concentration (6 mg l^{-1}) is much lower than that of berberine (60 mg l^{-1}).

For a given chromatographic system, fluorescent response depends on different variables coming from probe, sample and chromatographic conditions: impregnation conditions (probe concentration), scanning beam size (the larger the beam size the lower the sensitivity; beam size must cover a substantial zone of the sample), probe nature (λ_{ex} and emission wavelength, λ_{em}), sample structure, sample concentration, and application volume. All this aspects were discussed in the case of berberine [5,6,9]. The choice of conditions for each analytical system is a compromise between all these parameters. Conditions given in Experimental (6 mg l^{-1} of coralyne, 410 or 430 nm as λ_{ex}, λ_{em}> 450 nm, 1 x 1 mm beam size) are of general utilization in the case of coralyne with the analytes studied.

As in the case of berberine cation [9], the sensitivity of detection on the layer can be tailored through variation of coralyne concentration.

3.2. Detection of molecules other than alkanes

A wide number of molecules other than alkanes, including non-fluorescent compounds, have been detected using TLC-coralyne-induced fluorescence. They produce a change, either enhancement or quenching, in coralyne fluorescence-TLC baseline.

Figure 4 shows emission enhancements produced by a series of non-fluorescent, paraffinic alcohols using berberine and coralyne. As in the case of alkanes, the longer the alkylic chain, the greater the area of the chromatographic peak. Likewise, fluorescent responses are lower for the alcohols than for the alkanes for a given number of carbon atoms.

Table 1 gives fluorescent responses, expressed as area mol^{-1}, of non-fluorescent *n*-docosane (*n*-C_{22}), 2-docosanol and 1-bromodocosane, using berberine and coralyne as probes. Likewise, Table 2 gives those of *n*-hexadecane, 1-hexadecanol, and 1-bromohexadecane. These compounds were applied onto coralyne-impregnated silica gel TLC plates under the same conditions. As in the other experiments carried out, increases in emission are given with regard to the baseline which corresponds to native fluorescence of coralyne on the silica layer. They show enhancements in emission in all cases, fluorescent response decreasing in the order: alkane > alcohol > alkylbromide. Likewise, for a given family of compounds (alkanes, alcohols or alkyl-brominated compounds), the longer the aliphatic chain, the higher the fluorescent response.

As can be seen in Figures 4-6, a number of chemically different analytes give fluorescent response in the coralyne-system. This phenomenon seems general from the point of view of analytes. Positive or negative (quenching) increments in emission can be observed depending on the chemical structure of the analyte with regard to coralyne fluorescent baseline. As an example, Figure 5B shows that the application of rifampicine, a very polar compound, onto a coralyne-impregnated silica gel plate produces a net fluorescence quenching on the coralyne-silica gel baseline. In turn, Figure 5A shows a positive UV response (at 254 nm) for this compound.

Figure 6 illustrates the possibility of applying the coralyne probe to the separation and detection of a mixture of neutral lipids from different families on the same HPTLC layer, using the pre-impregnation procedure. A classical development sequence was used for separating these compounds. Pre-impregnation has been used due to the compatibility of development solvents with coralyne and neutral lipids.

TLC-berberine has been also applied to detection of phospholipids [2]. Coralyne may be also used for this purpose. However, methanol and water used for phospholipids development elute berberine and coralyne. In this case, pre-impregnation cannot be used, and scanning of the HPTLC plate may alternatively be carried out after post-impregnation with the corresponding berberine or coralyne solution.

In short, coralyne cation behaves as a general and sensitive detecting agent for planar chromatography detection.

4. DISCUSSION

4.1. Explanation of increases in emission for coralyne in the presence of alkanes

A model for explaining increases of fluorescent emission of apolar compounds in the berberine-silica gel system has been proposed elsewhere [4]. Here, we present data that demonstrate that increases in emission in the coralyne-silica gel system can also be rationalized using this model, summarized as follows. According to this model, other ionic fluorophores may behave in a similar way.

Fluorescence intensity depends on quantum yield, given by

$$\Phi = \Gamma / \Gamma + K_{nr}$$

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

The intensity enhancement was explained by taking into account the fact that non-specific, electrostatic, alkane-probe interactions (ion-induced dipole) can create a microenvironment that isolates the fluorescent probe from other non-fluorescent decay mechanisms [4].

This can be explained by considering two factors:

(1) the Einstein coefficient of spontaneous emission, which depends inversely on the dielectric constant of the medium, will be increased by the corresponding alkane. In effect, the alkane that interacts with the corresponding probe, coralyne in this case, and surrounds it, creates an apolar microenvironment that, in turn, lowers the dielectric constant of the system silica gel-probe, thus enhancing the intensity of the fluorescence

signal.

(2) the quantum yield (Φ) must be proportional to the interaction energy between, in general, a fluorescent ion P and the alkane i, according to:

$$U_{P,i} = -\frac{1}{(4\pi\varepsilon_0)^2}\frac{Z_P^2 e^2 \alpha_i}{2r^4}$$

where $U_{P,i}$ is the proportionality constant at a given temperature, Z_p is the charge of the fluorescent ion (In the case of berberine and coralyne, Z_p=1), r is the distance from the charge to the center of the induced dipole, ε_0 is the dielectric constant of the medium and α_i is the intrisic polarizability of the analyte. This expression models an ion-induced dipole interaction. According to this, for a given molecule *i,* the value of $\Phi_{P,i}$ depends only upon the value of α_i at a given temperature, since the remaining magnitudes must be practically constant on passing from one alkane to another (or from one apolar compound to another in a homologous series). Therefore, under these conditions and at a given concentration, the intensity enhancement is linearly dependent on the polarizability of the neutral molecule surrounding the probe:

$$\Delta I = A_p + B_P \alpha_i$$

where A_P and B_P are constants for a given probe and α_i is the polarizability of the analyte.

This ion-induced dipole model allows the influence of alkane chain length on fluorescence intensity to also be explained in the case of coralyne cation. Our experimental results (Figure 7A, Table 3) for the coralyne-alkane system show that a linear regression exists between the fluorescent response and the polarizability of *n*-alkanes in this case. This explanation, protection from quenching through a hydrocarbon skeleton, can also explain experimental fluorescence enhancements obtained in the cases of long-chain alcohols (Figure 7B, Table 3), and alkylbromides (Tables 1 and 2).

Other preliminary results obtained in our laboratory [8] show that saturated hydrocarbons produce increases in emission when applied on other ionic fluorophores impregnated on silica gel plates, using appropriate excitation wavelengths. This occurs for ionic fluorophores (e.g., coptisine, ethidium bromide, indocyanine green), regardless of probe chemical composition, as equation 2 suggests (through Z_P).

We believe that there is experimental evidence and theoretical support to suggest that this phenomenon is general from the analyte point of view, and for a wide number of ionic probes.

4.2. Explanation of the decrease in fluorescence intensity produced by molecules other than alkanes in TLC-coralyne

In the case of analytes other than alkanes, we should also take into account that specific donor-acceptor interactions result in the well-known phenomenon of fluorescence quenching [10, 13]. The resulting intensity for a given analyte on the coralyne-impregnated layer can be explained as a balance between radiative (protection from quenching; non-specific interactions) and non-radiative processes (quenching). This can be illustrated with some examples.

4.2.1. Decrease in positive fluorescence

n-Hexadecane, 1-hexadecanol, and 1-bromohexadecane have the same hydrocarbon skeleton, and their corresponding peaks show positive fluorescence with regard to the baseline because of the influence of this hydrocarbon chain, as previously explained. However, there is a gradual decrease in emission when passing from n-hexadecane to 1-hexadecanol, and when passing from the latter to 1-bromohexadecane, in a medium (silica gel-coralyne) having the same initial ε_0 for the studied molecules. The signal decrease when passing from n-hexadecane to 1-hexadecanol may be attributed mostly to the increase in ε. However, the signal decrease for 1-bromohexadecane with regard to 1-

hexadecanol cannot be explained in terms of variation in ε*. Specific interactions between -Br group and coralyne may also be invoked. The effect of -Br in quenching has been described [10]. Experiments on berberine-impregnated plates using the same compounds have provided similar results.

4.2.2. Quenching of fluorescence

Negative peaks with regard to baseline can be attributed to net specific interactions between polar compounds and coralyne, as in the case of rifampicine (Figure 5). Negative responses have also been obtained for histidine and albumin, among others examples (not shown here). The balance between non-specific and specific interactions depends on the characteristics of the interacting molecule, as well as on the coralyne concentration on the layer.

5. CONCLUSIONS

The use of coralyne-impregnated silica gel plates allows a sensitive detection of a wide number of analytes, including non-fluorescent ones, to be carried out in HPLC-fluorescence scanning densitometry mode. Coralyne system has been found to be more sensitive than that of other probes such as berberine cation or Reichardt's dye. Separation of compound mixtures in HPTLC are viable using pre-impregnated coralyne plates. Fluorescent responses of analytes can be positive or negative depending on the particular analyte-coralyne interactions involved. Coralyne works according to the proposed model.

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* Dielectric constants at the same temperature (50ºC) have been obtained from refs. [11, 12].

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Captions for figures

Figure 1. Chemical structures of coralyne (A) and berberine (B) cations

Figure 2. Fluorescent response (area counts) vs. mass of *n*-C_{22} (A), and *n*-C_{16} (B) on a coralyne-impregnated (6 mg l^{-1}) silica gel HPTLC plate. Application volume: 2 µl; development: *n*-hexane (5 min); λ_{ex} = 430 nm; emission > 450 nm.

Figure 3. (A) HPTLC-scanning fluorescence chromatograms of *n*-C_{12} (• • •), *n*-C_{16} (**----**), *n*-C_{18} (——), *n*-C_{22} (• • •), *n*-C_{24} (**----**), and blank (——) on a coralyne-impregnated (6 mg l^{-1}) silica gel HPTLC plate. (B) Fluorescent responses (area counts) vs. number of C atoms for the above-mentioned alkanes on coralyne (a) (6 mg l^{-1}) and berberine (b) (60 mg l^{-1})-impregnated silica gel HPTLC plates. Application volume: 1 µl; applied mass: 0.8 µg; development: *n*-hexane (5 min); λ_{ex} = 410 nm; emission > 450 nm

Figure 4. (A) HPTLC-scanning fluorescence chromatograms of *n*-C_{10}OH (• • •), *n*-C_{12}OH (**----**), *n*-C_{16}OH (——), *n*-C_{18}OH (• • •), *n*-C_{22}OH (**----**), and blank (——) on a coralyne-impregnated (6 mg l^{-1}) silica gel HPTLC plate. (B) Fluorescent responses (area counts) vs. number of C atoms for the above-mentioned alcohols on coralyne (a) (6 mg l^{-1}) and berberine (b) (60 mg l^{-1})-impregnated silica gel HPTLC plates. Application volume: 1 µl; applied mass: 0.8 µg; development: dichloromethane (5 min); λ_{ex} = 430 nm; emission > 450 nm

Figure 5. (A) HPTLC chromatogram of rifampicine (dotted line:1 µg; continuous line: 3 µg) on a coralyne-impregnated (6 mg l^{-1}) silica gel HPTLC plate, and detected by UV at 254 nm. (B) corresponding HPTLC chromatogram, under the same conditions, detected by fluorescence scanning (λ_{ex} = 430 nm; emission > 450 nm). Application volume: 1 µl; development: methanol (5 min).

Figure 6. HPTLC-scanning fluorescence chromatograms of a mixture of neutral lipids on a coralyne-impregnated (6 mg l^{-1}) silica gel HPTLC plate: **A**, cholesterol (0.60 µg); **B**, cholesteryloleate (0.60 µg); **C**, oleic acid (0.81 µg); **D**, oleic acid methylester (1.12 µg); **E**, triolein (0.56 µg). Development: see Experimental; application volume: 1 µl; λ_{ex} = 430 nm; emission > 450 nm.

Figure 7.- Fluorescent response (area counts) vs. polarizability (α) of alkanes (A) or alcohols (B) on coralyne (**a**) (6 mg l^{-1}) and berberine (**b**) (60 mg l^{-1})-impregnated silica gel HPTLC plates. Conditions: see Fig. 3 for alkanes, and Fig. 4 for alcohols.

Table 1.- Fluorescent response factors (area mol^{-1}) of *n*-docosane, 1-docosanol, and 1-bromodocosane applied on coralyne (6 mg l^{-1}) and berberine (60 mg l^{-1})-impregnated HPTLC silica gel plates (λ_{ex} = 365 nm for berberine, and 410 nm for coralyne).

	Fluorescence response (area mol^{-1})	
	Berberine	**Coralyne**
***n*-Docosane**	$2.22 \cdot 10^{11}$	$1.10 \cdot 10^{12}$
1-Docosanol	$1.96 \cdot 10^{11}$	$9.22 \cdot 10^{11}$
1-Bromodocosane	$1.81 \cdot 10^{11}$	$5.25 \cdot 10^{11}$

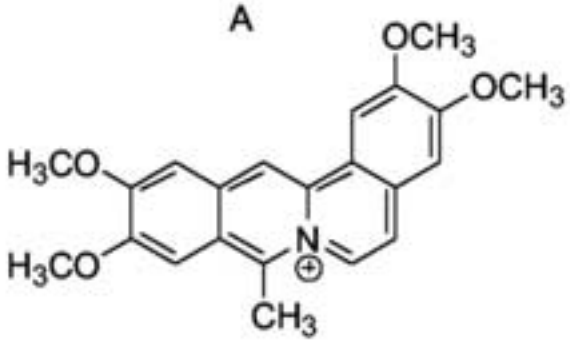
Table 2.- Fluorescent response factors (area mol^{-1}) of *n*-hexadecane, 1-hexadecanol, and 1-bromohexadecane applied on coralyne (6 mg l^{-1})-impregnated HPTLC silica gel plates (λ_{ex} = 410 nm), and values of dielectric constant (ε) for these compounds at the same temperature.

	Fluorescent response (area mol^{-1})	**ε (50ºC)**
***n*-Hexadecane**	$3.62 \cdot 10^{11}$	2.01
1-Hexadecanol	$3.53 \cdot 10^{11}$	3.82
1-Bromohexadecane	$3.06 \cdot 10^{11}$	3.54

Table 3.- Parameters of linearity of ion-induced dipole model, on coralyne (6 mg l^{-1}) and berberine (60 mg l^{-1})-impregnated silica gel HPTLC plates. (Analyte mass: 0.8 μg; λ_{ex}= 410 nm; emission > 450 nm)

Probe	Analytes	A_P	B_P	R^2
Coralyne	Alkanes (n:12-24)	217	-1370	0.99
Coralyne	Alcohols (n:10-22)	169	-1812	0.97
Berberine	Alkanes (n:12-24)	52	-421	0.99
Berberine	Alcohols (n:10-22)	70	-708	0.97

A



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