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Rhodamine B as ligand for affinity chromatography. Fixation studies onto cellulose by a curing method

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Abstract

Rhodamine B was post-grafted onto cellulose in order to be lately used for affinity chromatography as a biomimetic ligand. A curing method has been developed to bound rhodamine onto cellulose via esterification, by heating an intimate mixture of both, at different temperatures above 180 °C. The lactone form of rhodamine B has been proposed as the effective reactant generated in situ during the experimental conditions. Pursuing further improvements of the amount of rhodamine bound onto cellulose, identical experiments were carried out in the presence of catalytic amounts of zinc chloride and sodium hypophosphite. The grafted materials obtained were quantitatively and qualitatively characterized using SEM, tlc, GPC, EA, FT-IR and Vis spectroscopy.

Keywords: Cellulose; Curing of polymers; Chromatography; Dyes; Rhodamine B

1. Introduction

Rhodamine B (RB) is a xanthene dye with a large variety of technical applications due to its high fluorescence and photostability. Generally, rhodamines have mainly been used as laser dyes, although other important applications have been developed in the last years such as biological stains, water tracing agents, electrochemical luminescence sensitizer, molecular probes, chromoionophores in optical chemical sensors, solar collectors and many others [1–4].

Nevertheless this wide scope of applications, the use of this cationic dye as a ligand in affinity chromatography (AC) has never been experimented. Due to the presence of hydrophobic, hydrophilic, ionic and π–π interactions in different moieties of its structure, a desirable selectivity between this dye and the biomolecules to be purified can be expected.

The first task aiming at the use of any dye as a ligand for AC is to assure a suitable functionality against the chromatography support together with a method of fixation. Unfortunately, whereas simple molecules can be easily and totally linked to substrates like cellulose [5], the fixation of bulky molecules like dyes are not so easily accomplished. A literature survey revealed that there are few

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examples of bound rhodamines onto materials which are traditionally used as chromatographic supports, namely cellulose [6], silica and other polymeric materials [7–10], nevertheless, the aim of these studies was quite different.

There are different ways to prepare cellulose derivatives with different functional groups and patterns of functionalization. In general, the fixation onto macromolecular materials is almost exclusively performed in homogeneous medium conditions. In this regard, different homogeneous reactions such as both aqueous and non-aqueous and derivatizing and non-derivatizing ones, use different solvents as the reaction media [5].

Regarding the carboxylic group moiety present in the RB dye, we looked for a simple but effective way to bind this particular dye onto cellulose via esterification. Therefore, herein we report the post-grafted of RB onto cellulose in order to be used for AC as a biomimetic ligand. A curing method has been developed to bind rhodamine onto cellulose via esterification, at different temperatures above 180 °C, in the absence or in the presence of a basic or an acid catalytic agent. The number of experiments (cycles) for each sample was also diverse. The lactone form of RB has been proposed as the effective reactant generated in situ during the experimental conditions. The grafted materials obtained were quantitatively and qualitatively characterized by different analytical techniques.

The chromatographic behaviour of cellulose in beads derivatized with RB using the conditions optimized here is currently the subject of a separate study. A selective interaction between some standard proteins and the immobilized RB were observed opening an interesting and promising approach for protein purification.

2. Experimental part

2.1. Materials

Commercial microcrystalline cellulose powder (Fluka DS-0) was treated in vacuum at 100 °C for 16 hours in the presence of phosphorus pentoxide prior to use. RB, RB base (lactone form), lithium chloride, zinc chloride and sodium hypophosphite hydrate of the highest purity available were purchased from Aldrich and used as received. Solvents were of analytical grade. Stock solutions of LiCl/*N*,*N*-dimethylacetamide (DMA) 8.5 wt.% were prepared in accordance with the procedure of McCormick and Dawsey [11]. Ethylic and butylic esters of RB have been prepared as previously described [12].

2.2. Methods

All syntheses and cellulose derivatizations were carried out using a Büchi Glass Oven B-580 as a reaction vessel under vacuum with variable temperatures in the presence of phosphorus pentoxide inserted in the drying accessory.

Cellulose samples were qualitatively and quantitatively characterized by using Scanning Electron Microscope (SEM), thin-layer chromatography (tlc), Gel Permeation Chromatography (GPC), Elemental Analysis (EA), Fourier Transformed Infrared (FT-IR) and Visible (Vis) spectroscopy. Control samples were also conducted where the same samples were submitted to the same conditions apart from the final heating (control no. 2 as representative sample) or identical samples without the dye were submitted to the same derivatization process (control no. 1 as representative sample).

The microanalyses were performed in triplicate on a Carlo-Erba, CHNS-O EA-1108 Elemental Analyser. Melting points (m.p.) were determined in open capillary tubes in a Büchi 530 melting point apparatus and are uncorrected.

2.2.1. Cellulose derivatization

RB (0.10–3.00 g, 0.21–6.26 mmol) was well mixed with cellulose (1.00 g, 18.39 eq. OH) and zinc chloride or sodium hyphophosphite hydrate (0–5 wt.%) in an agate mortar in order to obtain an intimate mixture. This mixture was transferred to a silicate specimen tube, covered with a holed aluminium foil and was wetted with 2.5 ml of *N*,*N*-dimethylformamide (DMF). This mix so prepared

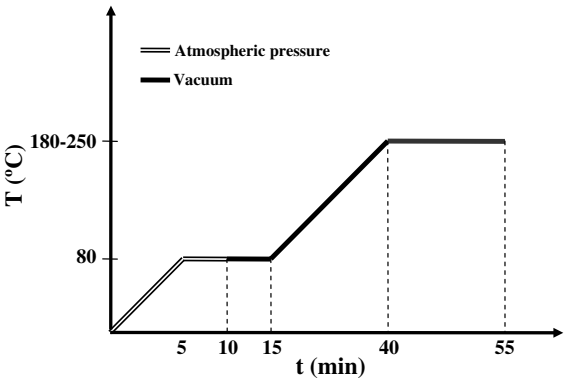

Fig. 1. Cellulose derivatization process.

was inserted into the reaction vessel and was submitted to heating under vacuum as described in Fig. 1 by varying the maximum temperature used and the number of cycles for the same sample.

The dyed cellulose so obtained was sequentially washed-off with several portions of DMF, water and ethanol and was continuously submitted to Soxhlet extraction process with ethanol until no leaking was observed.

2.2.2. Synthesis of rhodamine B base (lactone form)

1.00 g (2.09 mmol) of RB in a porcelain crucible inserted in the reaction vessel, under vacuum pressure and in the presence of phosphorus pentoxide, was submitted to the same cellulose derivatization programme temperature until 225 °C and was left for an additional 15 min at this temperature. After cooling to room temperature, the product so formed was removed from the crucible to yield 91% of almost pure sample, m.p. 164–166 °C (165 °C) [13], with ^{1}H NMR, ^{13}C NMR ($CDCl_3$) and IR (KBr) spectra superposable with a commercial sample, which was recristalized from xylene to give an analytical sample.

2.2.3. Thin layer chromatography

Tlc monitoring the synthesis of RB base as well as tlc analysis of cellulose derivatives together with its sample controls, were performed on aluminium plates pre-coated with silica gel Macherey–Nagel G/UV_{254} (0.2 mm) using dichloromethane as eluent. A dichloromethane solution of RB was also analyzed by tlc on aluminium plates pre-coated with cellulose Fluka G/UV_{254} (0.1 mm) prior and after submission to cellulose derivatization conditions using methanol/dichloromethane (10.0 vol.%) or acetic acid/methanol (10.0 vol.%) as the eluent. The spots were examined under 254, 312 and 365 nm UV light.

2.2.4. FT-IR spectroscopy

Infrared spectra (IR) were performed on a Mattson 5000-FTS FTIR spectrometer. All samples were prepared by mixing FTIR-grade KBr (Aldrich Chemicals) with 1.0 wt.% of the dye or cellulose derivative and grinding to a fine powder. Spectra were recorded at 4 cm^{-1} (128 scans) over the 400–4000 cm^{-1} range without baseline corrections. Bands are given in cm^{-1}. The ratios between the area of the axial deformation of aromatic C=C bond band between 1593 and 1595 cm^{-1} related to RB and the area for the axial deformation of cellulose C–H bond at 2899 cm^{-1} (A_{dye}/A_{cel}) for all cellulose samples were calculated. The calibration plots were obtained from the relative A_{dye}/A_{cel} ratio of known concentrations mechanical mixtures of ethylic and butylic esters of RB with cellulose.

2.2.5. Vis spectroscopy

Vis spectra of RB, ethylic and butylic esters of RB and dyed samples in acidified (1.0 vol.% of HCl) solutions of LiCl/DMA 8.5 wt.% were recorded on a Perkin–Elmer Lambda 6 spectrophotometer. The wavelength of maximum absorption is reported in nm.

2.2.6. Thermogravimetric analysis

Thermogravimetric analysis (TGA) studies were performed using a Rheometric Scientific TG 1000 thermobalance. Samples of RB used for thermogravimetric studies were transferred to open platinum crucibles and analysed at a heating rate of 10 °C min^{-1} using dried flowing argon or air, and were heated from 25 to 400 °C.

2.2.7. Scanning electron microscopy

SEM images were acquired in a Hitachi apparatus, model S-2700 with a UHV Dewar detector (Rontec EDX). Cellulose and cellulose derivatized samples were magnified 100, 250, 500 and 5000×.

2.2.8. Gel permeation chromatography

GPC analyses were performed on homogeneous and clear solutions of 80 mg of the sample (cellulose, cellulose derivatized or RB) or mixture of samples, in 10 ml of acidified (1.0 vol.% of HCl) solutions of LiCl/DMA 8.5 wt.%. The GPC system consisted of a Perkin Elmer GPC system equipped with a PLgel 5 μm Pre-column followed by a PLgel 5 μm Mixed 300 × 7.5 mm ID Volumes (Perkin–Elmer 0258-2145) connected in serial. The column was pumped with LiCl/DMA (Aldrich, spectrometric grade) 0.5 wt.% at a flow rate of 0.8 ml/min. The mobile phase was sonnicated for a period of 15 min and filtered through a 0.2 μm PTFE inline filter. The 100 μl of sample injected into a loop of 50 μl was previously filtered through a 0.2 μm Anotop 25 (Whatman) inline filter. The columns, guard column and the injection system were maintained at 80 °C. Detection was performed with a Refraction Index (RI) and Vis (565 nm) detectors in serial. The collected data were treated using Excel application software (Windows).

3. Results and discussion

Following our previous work [14,15] and pursuing the immobilization of dyes possessing a carboxylic acid group to be used as ligands in AC, we developed a curing method to bind RB onto cellulose via esterification by heating a heterogeneous, but intimate, mixture of both, in the presence of vacuum at different temperatures above 180 °C. Even so there are some examples in the literature for curing methods based on the carboxylic acid group in order to crosslink materials [16], or to prepare starch esters [17], the immobilization of dyes onto cellulose regarding the use of the resulting materials in AC has never been used.

The RB was chosen as a model for these studies, regarding its very high solubility in water, ethanol [18] and in a variety of usual organic solvents like dichloromethane, DMF and others which allows the easy removal of the remaining unbound dye from cellulose after the derivatization process.

Having in mind the RB melting point of 210–211 °C with decomposition [19], which could prevent its fixation onto cellulose by methods using temperatures over this range, RB was thermogravimetrically analyzed in a temperature range from 25 to 400 °C, under air and argon atmosphere (Fig. 2). In both analyses, the calculated higher onset-value indicates a temperature near to 180 °C of the starting loss of weight.

The quantitative transformation of RB into its lactone form was observed when it was submitted to the cellulose derivatization programme temperature up to the temperature of 225 °C. The identity of the compound so formed is supported by superposable ^{1}H and ^{13}C RMN and IR spectra when compared to a commercial sample of RB base. This observation clearly indicates that a conversion, rather than decomposition, is the right word to describe this process. In fact, the TGA calculated value near 250 °C corresponds to a 7.61% loss of weight due to a total interconversation between this two forms with the loss of HCl molecule to indicate that both forms could coexist between 180 and 250 °C and probably the lactone form being the principal responsible for the esterification of the dye onto cellulose (Fig. 3). Nevertheless, the direct esterification of the carboxylic group of RB cannot be disregarded.

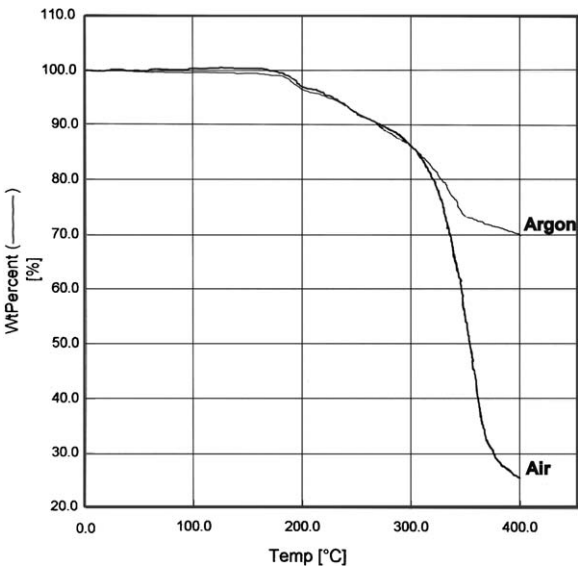

Fig. 2. TGA of RB under air and argon atmosphere.

Looking for further evidence for this proposed mechanism, we performed identical derivatization experiments using RB lactone. Characterization studies performed on the resulting dyed cellulose materials revealed indistinguishable behaviour and identical amounts of dye bound into cellulose.

The thermal solvent free interconversation between RB into its lactone form presents an innovative and expeditious method to lactonize rhodamines in quantitative yields alternative to the normal reaction in the presence of an inorganic base like sodium hydroxide [20–23] or catalyzed in presence of an enzyme like erythromycin [24,25].

Several experiments were carried out based on cure methods described in the literature [16,17] in which the maximum temperature and the number of cycles were varied. The influence of acid ($ZnCl_2$) and basic ($NaHPO_2$) catalysts which already proved their efficiency in the cure process [16,17] was also studied (Table 1).

In all experiments, the first 40 min of the programme until the maximum temperature was reached were kept constant. During this period of time the whole DMF was removed. Furthermore, the simultaneous use of vacuum and the presence of phosphorus pentoxide ensure the removal of HCl and water formed during the condensation reaction between cellulose and the dye.

The resulting dyed cellulose was sequentially washed-off with DMF, water and ethanol and was submitted to Soxhlet extraction with the latter solvent to ensure the removal of the unfixed dye. The samples obtained were then well dried at 50 °C under vacuum in the presence of phosphorus pentoxide. The resulting materials were then characterized in

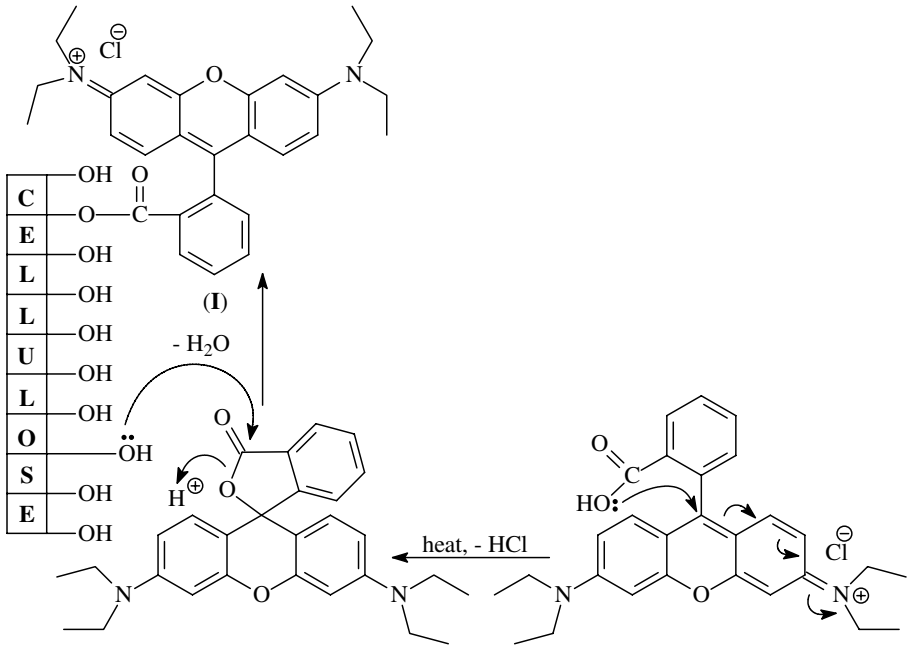

Fig. 3. RB lactone formation and fixation onto cellulose as cellulose derivative I.

Table 1
Cure conditions used

Sample	Rhodamine/cellulose (wt.%)	Catalyst	Temperature (°C)	Number of cycles
Control 1	0	$ZnCl_2$	240	1
Control 2	300	$ZnCl_2$	rt	0
1	300	–	240	1
2	300	NaH_2PO_2	240	1
3	300	$ZnCl_2$	240	1
4	300	–	215	1
5	300	NaH_2PO_2	215	1
6	300	$ZnCl_2$	215	1
7	300	$ZnCl_2$	250	1
8	200	$ZnCl_2$	250	1
9	100	$ZnCl_2$	250	1
10	50	$ZnCl_2$	250	1
11	33	$ZnCl_2$	250	1
12	25	$ZnCl_2$	250	1
13	10	$ZnCl_2$	250	1
14	33	$ZnCl_2$	180	1
15	33	$ZnCl_2$	190	1
16	33	$ZnCl_2$	200	1
17	33	$ZnCl_2$	210	1
18	33	$ZnCl_2$	220	1
19	33	$ZnCl_2$	230	1
20	33	$ZnCl_2$	240	1
21	33	$ZnCl_2$	240	2
22	33	$ZnCl_2$	240	3
23	33	$ZnCl_2$	240	4
24	33	$ZnCl_2$	240	5

order to prove the effective establishment of a dye-cellulose covalent bond and to quantify the amount of dye bound onto cellulose.

The control experiment no. 2 afforded the expected uncoloured cellulose whereas the cellulose derivatized samples gave different pink to dark-red hues according to the amount of dye determined by FT-IR, Vis and EA techniques.

The SEM of a 100, 250, 500 and 5000× magnification, performed on cellulose samples before and after the dyeing process did not show any perceptive differences between them (Fig. 4). This observation demonstrates the preservation of the cellulose physical properties after the dyeing process, which is crucial to its application in AC.

Tlc on aluminium plates pre-coated with silica gel of dissolved derivatized cellulose samples showed that the colour of the dyed cellulose remained at the starting point whereas the colour of the control experiment no. 2, before wash off, migrated readily at the same rate of flow (Rf) of RB in both forms. On the other hand, tlc on aluminium plates pre-coated with cellulose of a RB dichloromethane solution, prior and after being submitted to cellulose derivatization conditions, showed Rfs of 0.9 (equal to both rhodamine forms) and zero, respectively. According to the literature [26,27], both tlc analyses

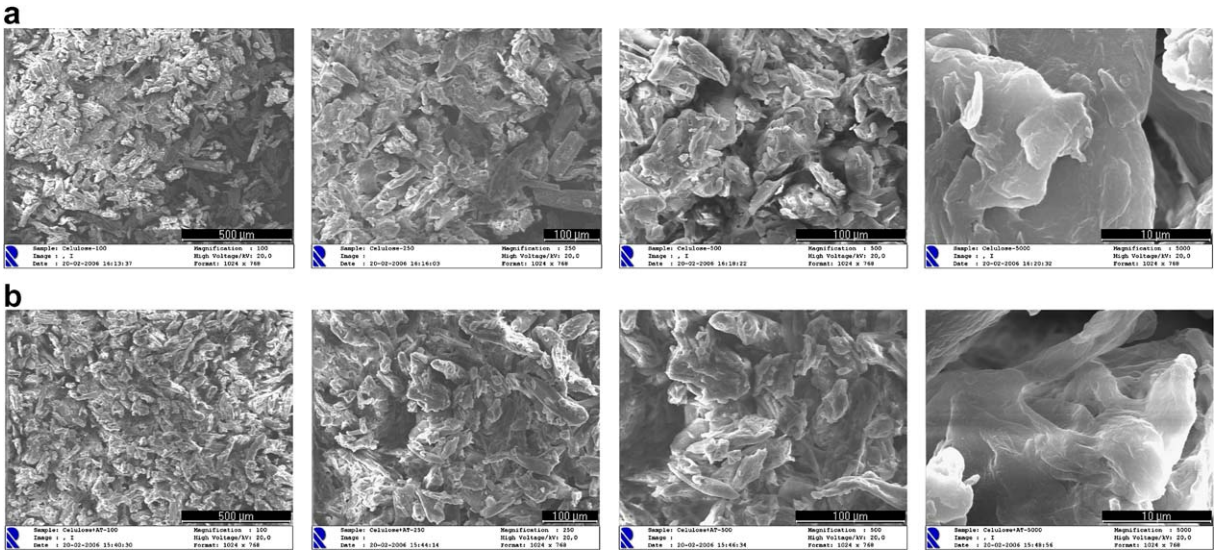

Fig. 4. Representative SEM micrographs of cellulose fibres with 100, 250, 500 and 5000× magnification: (a) prior to use; (b) dyed with RB.

proved the establishment of a covalent bond between the cellulose and the dye.

GPC chromatograms of homogeneous and clear solutions of cellulose, representative derivatized cellulose (sample no. 19) and control samples are present in Fig. 5.

The analysis of these results shows that both cellulose (Fig. 5c) and derivatized cellulose (Fig. 5a) present a similar polydispersity expected for a macromolecular material. Nevertheless, where the cellulose just presents a sign to the IR detector, the derivatized cellulose shows a very clear and well defined distribution curve (Fig. 5a) at 565 nm near the maximum absorbance expected for a rhodamine derivative (Table 2). This band cannot be assigned to any form of free RB, which comes from the column at longer times together with the solvent, LiCl and other types of low molecular molecules (Fig. 5b). The observation of a distribution curve with less intensity at longer times in the Vis detector present in the derivatized cellulose (Fig. 5a) could suggest the presence of a residual amount of unbound form of RB trapped inside the cellulose macromolecules. This could also be the result of a partial hydrolysis occurred during the dissolution by heating on the acidified solution of DMA/LiCl, together with some solvent residual signal, also observed on the cellulose prior to the use as shown in Fig. 5c. This set of GPC experiments has been, for the best of our knowledge, used for the first time to explore the viability of this technique in proving

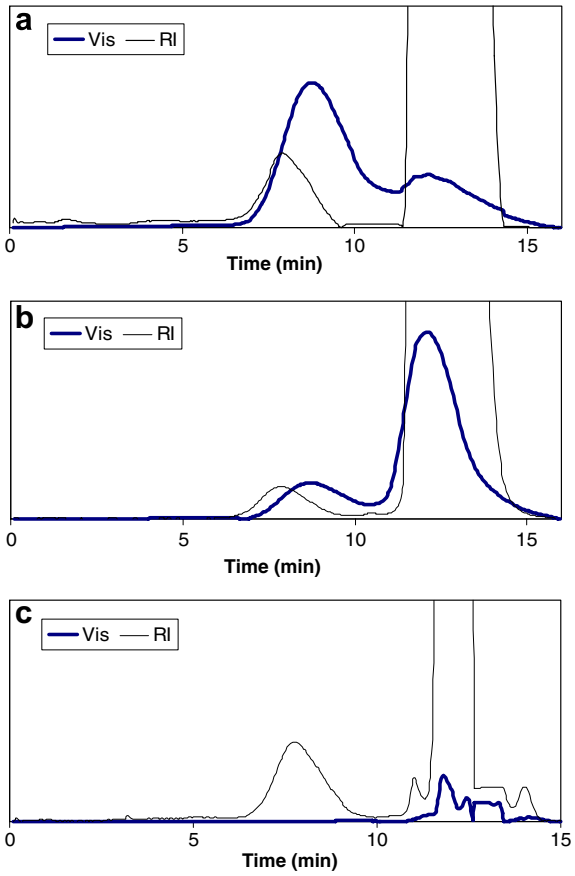

Fig. 5. Representative GPC chromatograms of derivatized cellulose sample (no. 19) (a), derivatized cellulose sample (no. 19) mixed with RB (b) and cellulose prior to use (c) analysed by means of visible (565 nm) and refraction index detectors.

Table 2
Maximum wavelength of RB and it esters in acidified (1.0 vol.% of HCl) solution of LiCl/DMA 8.5 wt.%

Rhodamine forms	λ_{max} (nm)
Rhodamine B	564.0
Ester ethylic of Rhodamine B	568.5
Ester butylic of Rhodamine B	568.5
Derivatized cellulose	560.5–571.5

the existence of a covalent bond between a dye and a macromolecule.

Maximum wavelengths of RB and its esters were measured in acidified (1.0 vol.% of HCl) solutions of LiCl/DMA 8.5 wt.% (Table 2). The addition of HCl was necessary since solvents like DMA or DMF afford colourless solutions of RB, unless acidic medium is guaranteed [28]. Unfortunately, the

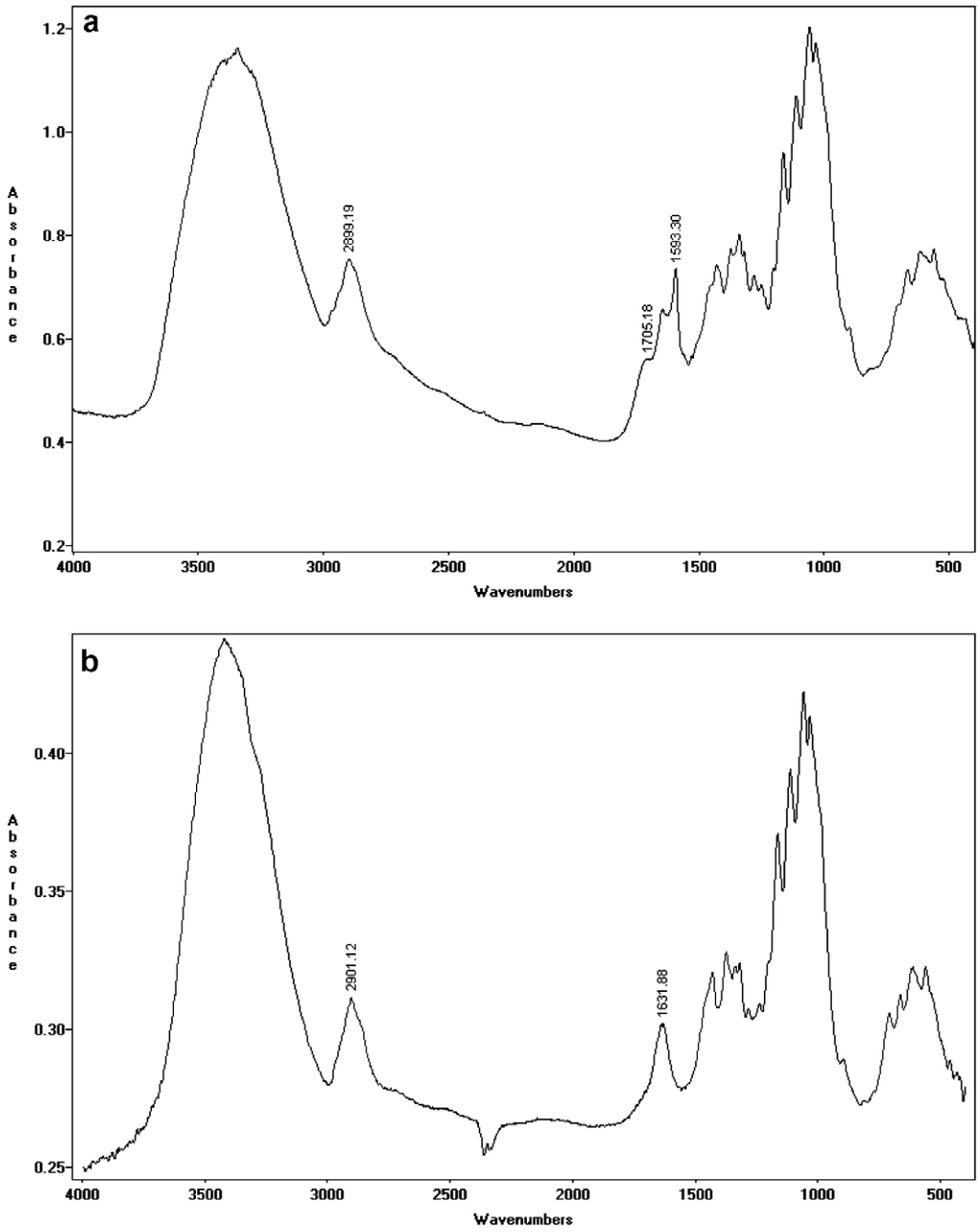

Fig. 6. IR spectra of the derivatized cellulose sample no. 11 (a) and control sample 1 (b).

variability of values obtained for the derivatized cellulose samples, whose values range from 560.5 to 571.5 nm, does not give any additional qualitative confirmation in what concerns the carboxylic or ester nature of the bound rhodamine.

IR spectra of cellulose derivatives reveal the most intense C=C deformation band of rhodamine at 1593–1595 cm^{-1}, together with the expected C=O ester band at 1705–1710 cm^{-1}. These bands are absent in both cellulose and control samples as shown in Fig. 6. Spectra of cellulose and control samples revealed to be superimposable. The values found for the ester band are in agreement with several RB ester bands at 1707–1728 cm^{-1}, as previously described [12].

The amount (Q, mmol dye g^{-1} dyed cel) and fixation yields (FY, %) of RB anchored onto cellulose was determined in all of the well-dried derivative cellulose samples by FT-IR, Vis and EA (Table 3).

Of these three different methods, EA is the unique absolute method, nevertheless Vis and especially FT-IR methods might be more convenient. On the other hand, the FT-IR and Vis are relative methods based on calibration plots of samples where the dye is not bound onto cellulose forcing to assume as true the same behaviour for the bound dye. Fig. 7 presents the calibration plots obtained from the IR spectra of known concentrations of RB ethylic and butylic esters mechanical mixtures with cellulose, together with the calibration plots obtained from the Vis spectra of the same esters in known concentration solutions dissolved in acidified (1.0 vol.% of HCl) solutions of LiCl/DMA 8.5 wt.%.

The Vis calibration plot equations resembled the molar absortivity of the ethylic ($\varepsilon_{max} = 96.700$ mol l^{-1}) and butylic ($\varepsilon_{max} = 108.700$ mol l^{-1}) esters of RB in the same solvent system.

Q was determined by FT-IR directly from the ratio between the area of the axial deformation band of the dye aromatic C=C bond and the cellulose's C–H in the absorption spectra of the dyed samples, based on the calibration plots determined before (Fig. 7). The aromatic C=C bond at 1593–1595 cm^{-1} is the most intense bond of RB, and it is expected to be independent of the environment

Table 3
Amount of RB bounded onto cellulose (Q, mmol dye g^{-1} dyed cel.) and respective fixation yields (FY, %)

Method	FT-IR				Vis				EA		
Standard	Ethylic ester		Butylic ester		Ethylic ester		Butylic ester		–		
Sample	$Q \times 10^2$	FY	$Q \times 10^2$	FY	$Q \times 10^2$	FY	$Q \times 10^2$	FY	%N [a]	$Q \times 10^2$	FY
Control 1	0.27	–	0.78	–	0.00	–	0.00	–	0.05	1.79	–
Control 2	0.27	0.04	0.78	0.12	0.00	0.00	0.00	0.00	<0.01	<0.36	<0.06
1	1.26	0.20	1.58	0.25	1.57	0.25	1.33	0.21	0.207	7.39	1.18
2	4.29	0.70	4.01	0.66	5.47	0.90	4.81	0.79	0.330	11.79	1.94
3	6.26	1.00	5.61	0.90	5.86	0.94	5.15	0.83	0.420	15.00	2.40
4	0.58	0.09	1.03	0.16	0.67	0.11	0.58	0.09	0.120	4.29	0.68
5	1.26	0.27	1.58	0.34	1.40	0.30	1.23	0.27	0.140	5.00	1.09
6	1.32	0.21	1.63	0.26	1.60	0.26	1.40	0.23	0.200	7.14	1.15
7	7.06	1.13	6.25	1.00	4.83	0.77	4.24	0.68	0.707	25.25	4.04
8	8.05	1.95	7.05	1.71	6.06	1.47	5.33	1.29	0.930	33.21	8.04
9	8.67	4.19	7.54	3.64	6.57	3.17	5.79	2.79	0.943	33.68	16.26
10	9.90	9.53	8.54	8.21	8.18	7.87	7.22	6.94	0.807	28.82	27.72
11	9.41	13.71	8.14	11.87	6.02	8.78	5.30	7.72	0.547	19.54	28.47
12	6.63	12.71	5.90	11.31	3.88	7.43	3.39	6.50	0.360	12.86	24.64
13	6.45	30.52	5.75	27.24	2.27	10.76	1.96	9.28	0.323	11.54	54.61
14	0.27	0.40	0.78	1.14	0.00	0.00	0.00	0.00	0.150	5.36	7.81
15	0.27	0.39	0.78	1.12	0.00	0.00	0.00	0.00	0.220	7.86	11.25
16	0.58	0.83	1.03	1.48	0.11	0.15	0.04	0.05	0.210	7.50	10.79
17	1.14	1.64	1.48	2.13	0.45	0.65	0.34	0.49	0.143	5.11	7.36
18	1.75	2.53	1.97	2.85	1.25	1.80	1.05	1.51	0.360	12.86	18.53
19	3.05	4.42	3.02	4.38	2.24	3.25	1.93	2.80	0.367	13.11	19.00
20	4.47	6.43	4.16	5.99	3.50	5.04	3.05	4.39	0.453	16.18	23.29
21	8.17	11.77	7.15	10.29	6.78	9.76	5.97	8.59	0.543	19.39	27.92
22	8.79	12.67	7.64	11.01	5.81	8.37	5.11	7.36	0.650	23.21	33.44
23	9.22	13.28	7.99	11.50	5.91	8.51	5.20	7.48	0.733	26.18	37.68
24	7.99	11.53	7.00	10.10	6.96	10.05	6.13	8.85	0.830	29.64	42.78

[a] Standard deviation between 0.00 and 0.02.

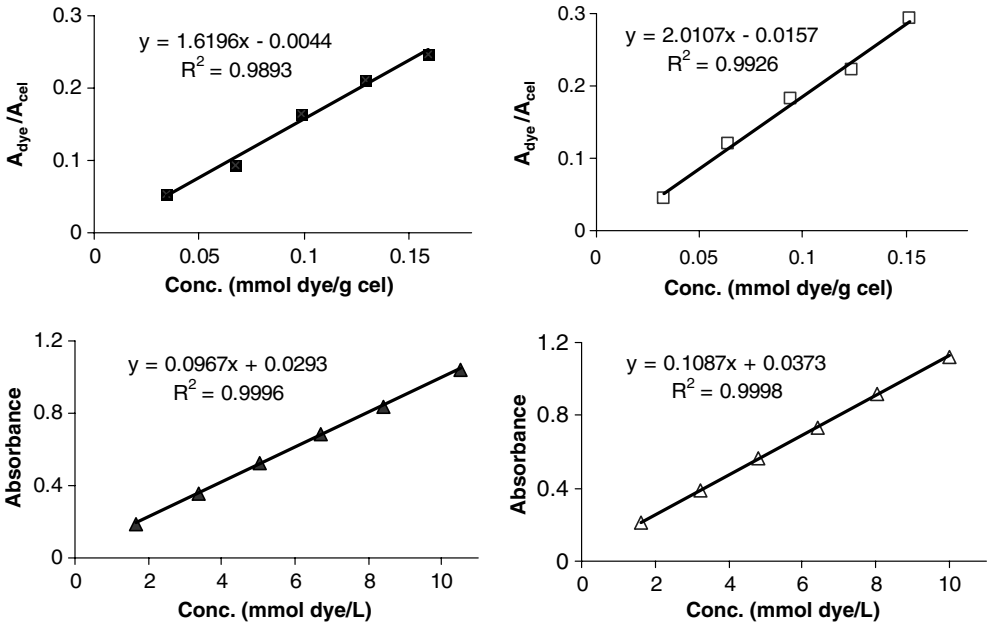

Fig. 7. FT-IR (■,□) and Vis (▲,△) calibration plots using ethylic (■,▲) and butylic (□,△) esters of RB as standards.

since the aromaticy of the xanthene dye is preserved, which allows us to relate the intensity with the amount of dye even when bound onto cellulose.

Q was also determined from the absorbance of known quantities of dyed samples after being dissolved in acidified (1.0 vol.% of HCl) solution of LiCl/DMA 8.5 wt.%, following the calibration plots shown in Fig. 7 and using Eq. (1), where V is the volume of the sample solution (l), C is the concentration of the dye in the sample solution (mol l^{-1}), and M is the weight of the sample used for the solution (g).

$$Q = \frac{VC}{M} \times 10^3 \tag{1}$$

This method also assumes that the molar absortivity of the cellulose ester of rhodamine is identical with its analogues ethylic and butylic ones, since the colour and the concomitant aromaticy of the xanthene dye bound onto cellulose is preserved.

Alternatively, EA furnished a direct method to determine Q based on the %N present in the dyed cellulose, assuming that all nitrogen present in the sample comes exclusively from the dye, as confirmed by the negligible residual amount of nitrogen determined in the control sample no. 1. The quantity of the dye immobilized can be easily determined by using Eq. (2), where %N is the percentage of nitrogen determined by EA.

$$Q = \frac{\%\mathrm{N}}{2.8} \tag{2}$$

The fixation yields (FY) can also be calculated by using Eq. (3), where Q is the amount of rhodamine bound onto cellulose (mmol g^{-1}) determined by FT-IR, Vis or EA and D is the amount of RB (mmol) used for dyeing 1 g of cellulose.

$$\mathrm{FY} = \frac{Q}{D} \times 100(\%) \tag{3}$$

Figs. 8–11 present the influence of the catalyst effect (sodium hypophosphite and zinc chloride), temperature (180–250 °C), number of dyeing cycles used (1–5 cycles), and weight ratio between cellulose and dye (0.33–10.0 w/w) in the amount of the dye bound onto cellulose (Q) by using FT-IR (■,□) Vis (▲,△) and EA (○) as quantification methods.

Generally speaking, these results reveal that the amount of the dye bound onto cellulose determined by FT-IR and Vis, using either ethylic or butylic ester as standards, are in fairly good agreement. This result is not a surprise regarding that both methods are sensible to the presence of rhodamine, since the chromophore moiety of the dye is preserved. Unexpectedly, EA reveals 3–4 times more quantity of Q than those calculated by the two other methods, although the main source of nitrogen in the dyed sample is RB regardless the negligible presence of nitrogen in the control samples 1 and 2.

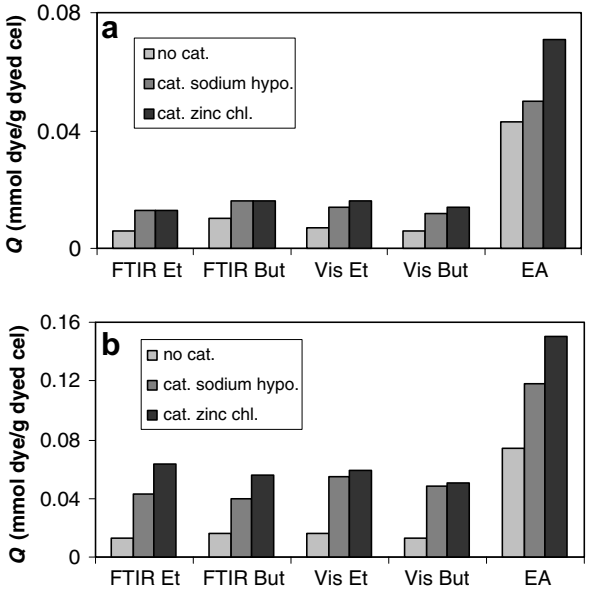

Fig. 8. Influence of basic and acid catalyst in the amount of dye bound onto cellulose (Q) with temperatures of (a) 215 °C and (b) 240 °C using FT-IR, Vis and EA as method of quantification.

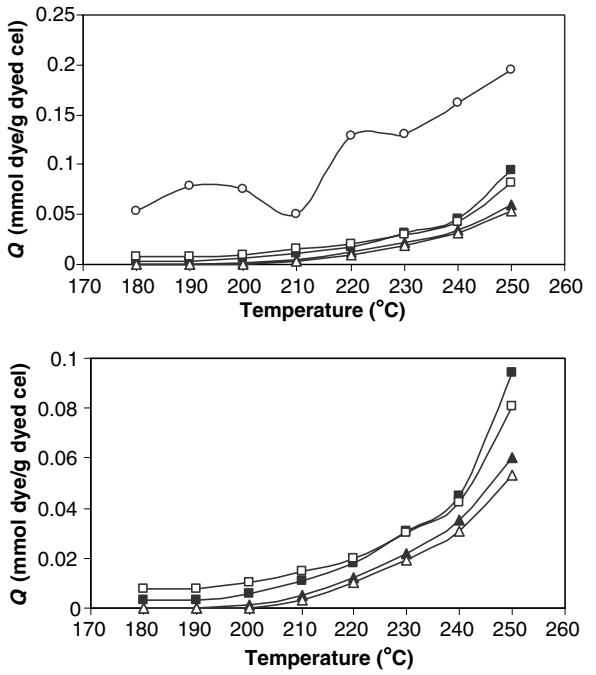

Fig. 9. Influence of temperature (180–250 °C) in the amount of dye bound onto cellulose (Q), using FT-IR, Vis and EA as method of quantification.

This difference could be explained by an immobilization of the dye onto cellulose in other ways than esterification. The derivatives II and III are two

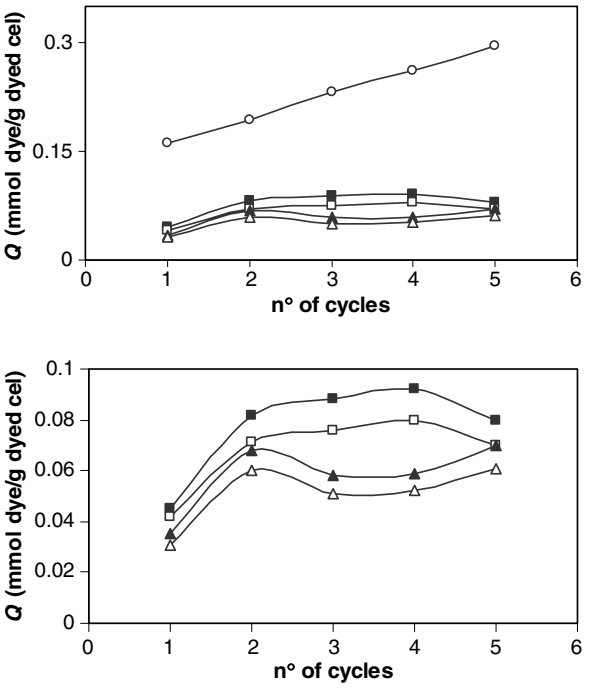

Fig. 10. Influence of number of cycles (1-5) in the amount of dye bound onto cellulose (Q), using FT-IR, Vis and EA as method of quantification.

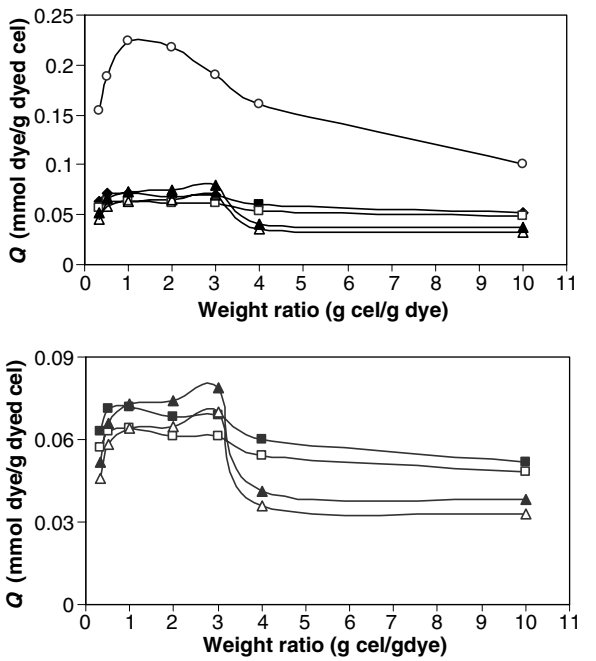

Fig. 11. Influence of weight ratio between cellulose and dye (w/w) in the amount of dye bound onto cellulose (Q) at 250 °C, using FT-IR, Vis and EA as method of quantification.

possible hypotheses for colourless forms (Fig. 12). The formation of the derivatives II and III outcomes from the addition of cellulose to a *meta* position with respect to the lactone moiety of the rhodamine dye. Although, this reaction is quite improbable to occur in normal conditions, the high temperatures and the closest position of the two substrates makes this hypothesis reasonable. The interruption of the conjugated systems with no possibility of rearomatization together with a partial distortion of the π system conjugation explained the discoloration of the dye making both derivatives invisible for Vis quantification methods. This explanation could also be responsible for the attenuation or shift of the C=C band in IR.

To sum up, the EA together with FT-IR or Vis methods of quantification are complementary methods allowing us to determine the amount of rhodamine bound onto cellulose in both forms, colour and colourless ones.

Increasing of rhodamine fixation has been observed in the presence of sodium hypophosphite and zinc chloride in catalytic amounts (samples 1–6), been more effective at the higher temperature of 240 °C than at 210 °C (Fig. 8). Depending on the method of quantification used, a roughly four times more of the amount of dye bound was observed in the case of zinc chloride compared to its absence. The acid catalysis proves to be more effective than the basic one, being used generally in the remaining set of experiments.

Fig. 9 illustrates the temperature effect on the amount of rhodamine bound onto cellulose (samples 11, 14–20), where three ranges of temperature can be identified. In the first range, between 180 and 210 °C, the amount of rhodamine fixed is fairly low, which can be easily explained since this range of temperature is coincident to the formation of the rhodamine lactone form and to the melting of both rhodamine forms. From our experience with other carboxylic dyes, it seems that for this cure process take place, the substrate needs to melt before reacting with cellulose to ensure some mobility, since at these temperatures there are no solvents to assure the reaction

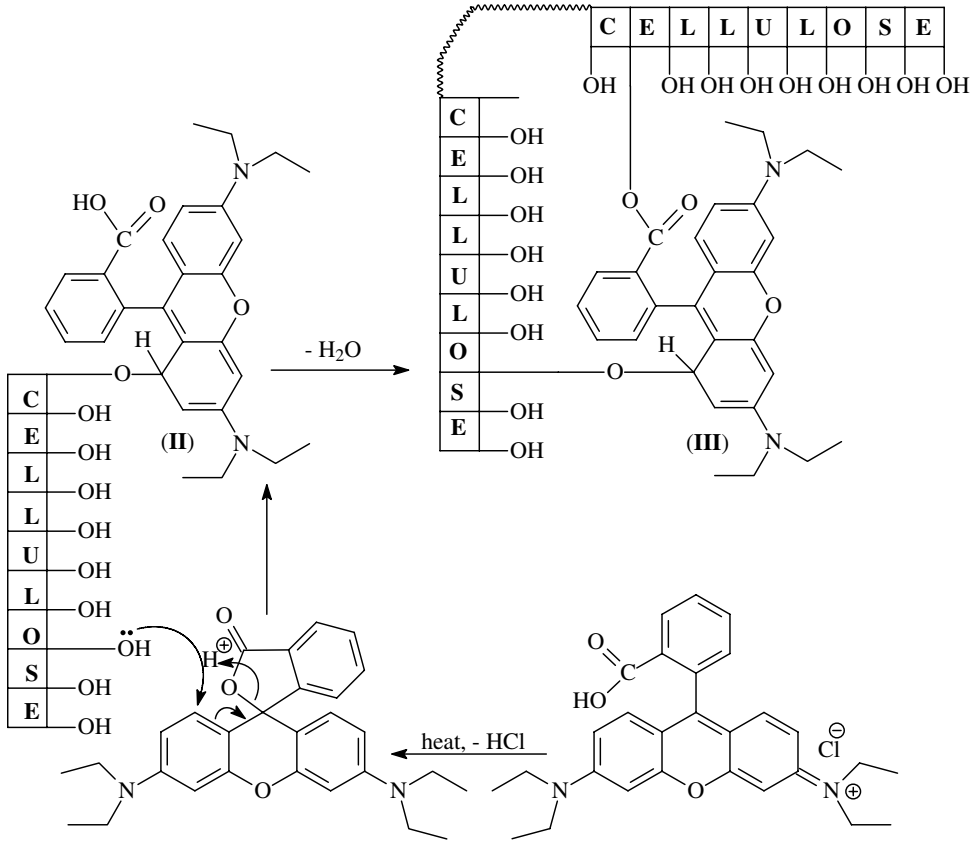

Fig. 12. Alternatively pathway for the fixation of RB onto cellulose via addition into lactone *meta* position as cellulose derivative II and III.

media. Between 210 and 240 °C the increase of the amount of dye fixed is linear with temperature, almost doubled between 210 and 230 °C and near quadrupled between 230 and 240 °C, which is easily rationalized since at this temperature the two premises which had not been achieved before, are now satisfied. Finally, an increase of 10 °C, until reach the temperature of 250 °C, duplicates the amount of dye bound onto cellulose, which makes this temperature the most efficient one.

Fig. 10 illustrate the influence of repeated cycles of derivatization (samples 20–24) in the amount of dye immobilized onto cellulose. EA method suggests a regular and linear increase of the fixation over the five cycles whereas the FT-IR and Vis methods reveal just a duplication after the second cycle where a plateau is attained. According to our previous rationalization, it seems that after the second cycle, the formation of II and III derivatives come from the emerging rhodamine or from the already bound one, are the most favourable processes.

The influence of the weight ratio between cellulose and the dye was also analysed (samples 7–13). As expected, the higher amounts of fixed dye are obtained in cases where the dye has more possibilities to bind onto cellulose (Fig. 11), namely, where the weight ratio cellulose/dye is higher presenting a remarkable FY, near 54% for a bulky molecule as RB. Regarding the amount of bound dye, the maximum value obtained is at a weigh ratio of 0.5 and 1.0 for colourless derivative forms (II, III) or 2.0 for the ester derivative one (I). At lower weight ratios, where the dye is in a great excess in comparison to cellulose, the amount of dye bound onto cellulose is lower which suggests a crow into effect by the dye molecules themselves.

Finally, it can be said that both RB base synthesis and cellulose derivatization processes are two more additional cases of successful solvent-free organic reactions. This effectiveness could be partially explained because solid-state reactions might occur, in certain cases, more efficiently and more selectively than those occurred in solution counterpart [29]. Furthermore, the solid-state reactions have many other advantages such as reduced pollution, low costs, and simplicity in processing and handling [29], which is totally fair applied to our case. Since water can be the only solvent required to process the dyed cellulose regarding the excellent solubility of RB and its lactone in this solvent, we can indeed, classify this reaction as a good example of green chemistry.

In the scope of AC, the use of this curing method to immobilize carboxylic dyes or other type of ligands onto matrices presents additional advantages. Besides the novelty of the method regarding AC and the general good characteristics previously mentioned, this method allows the direct linkage of the substrate onto the chromatographic support without the need of any activating agent. This feature is especially important since the activating agent could present some interaction with the macromolecules to be separated, making the matrix not inert and thus masking the affinity interaction with the immobilized ligand.

4. Conclusions

An innovative, expeditious and green cure method presented here enables us to bind bulky molecules like dyes onto cellulose in a heterogeneous process. It seems that the most important requisites to make this reaction possible are the presence of a carboxylic acid group in the dye with a lower melting point related to the operation temperature, and without decomposition.

The mechanism of binding the rhodamine B onto cellulose seems to involve the lactone form of RB formed in situ, affording not only the ester derivative, but also other colourless forms resulting from an addition to the meta position of the lactone. However, and from our experience with other type of carboxylic dyes, the direct esterification of the carboxylic acid could be the acting process in cases where the lactone form is not possible.

The temperature of 250 °C in the presence of zinc chloride as acid catalyst, at a weight ratio between cellulose and RB between 1.0 and 2.0 seems to be the ideal condition to achieve a higher amount of rhodamine immobilized onto cellulose at the general dyeing process. One to two cycles should be used, unless a great amount of immobilized colourless form is pursued.

All together, the three methods of quantification used showed to be complementary as they allow to determine and to discriminate the amount of rhodamine bound onto cellulose, not only as the colour derivative form but also as the colourless derivative case.

Regarding the use of RB as ligands for AC, and independently of the used conditions, the amount of rhodamine immobilized must be determined by EA and Vis or FT-IR methods simultaneously in order to rationalize the results obtained in AC with the density and the nature of the bound ligand.

Besides the advantages previously mentioned, the use of this curing method to immobilize ligands onto matrices envisioning AC reveals to be new, expeditious and has the advantage to allow the direct linkage without the need of activating agents.

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