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Analytical Methods

Micelles mediated separation fluorimetric methodology for Rhodamine B determination in condiments, snacks and candies

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ABSTRACT

Dye Rhodamine B (RhB) has been widely used in textile industry and foodstuffs but due to its proved carcinogenicity and toxicity on human, many countries have forbidden its use in foods. A new methodology is proposed for Rhodamine B (RhB) fluorimetric determination applying separation with a surfactant. Experimental parameters that affect the separation and determination steps like surfactant concentration, time and temperature of equilibration, pH of extraction, nature and composition of diluting agent of surfactant rich phase have been studied and optimised. The implementation of separation step permitted to remove successfully the interferences of matrix components, improving selectivity and sensitivity. Developed methodology presents a linearity range between 4.67×10^{-2} and 100 $\mu g\,L^{-1}$ with a correlation coefficient of 0.999. At optimal experimental conditions, a LOD of 1.40×10^{-2} $\mu g\,L^{-1}$ and LOQ of 4.67×10^{-2} $\mu g\,L^{-1}$ were obtained, allowing RhB determination in a variety of commercial condiments, snacks and bubble gums.

1. Introduction

Rhodamine B (RhB) is a highly water-soluble (50 g L^{-1}) compound belonging to methyl-xanthene family. It is presented as a green solid or red powder and has been widely used as colourant in textile industry and foodstuffs. Due to its fluorescent properties, it is useful as a pathological marker in lab testing and as a fluorescent tracer in water courses. Some of RhB derivatives have also been used as fluorescent chemosensors for metal ions (Dujols, Ford, & Czarnik, 1997; Hojo et al., 2002; Thorn & Fultz, 1989).

The carcinogenicity and toxicity of RhB towards human and animals have been experimentally proved (Jain, Mittal, Mathur, & Sikarwar, 2007). Its effects in gastric and intestinal tracts have shown multiple problems like inflammation and irritation, causing nauseas, vomits and affecting the skin, eyes and respiratory zone. As a result of its multiple effects in the human health, some countries had legislated and forbidden its employ. In USA, the "Colours in Food Regulations" has classified RhB as *illegal colourant*. In Argentine, there is no regulation on RhB use as colourant in foods.

Analytical methods for RhB dye determination involve microscopies (Choudhry, 1991), chromatographies (Chuanxian et al., 2008; Desiderio, Marra, & Fanali, 1998; Franke, Westerholm, & Niessner, 1997; Gagliardi, De Orsi, Cavazzutti, Multari, & Tonelli, 1996) and spectroscopies (Pourreza, Rastegarzadeh, & Larki, 2008; Wang, Masi, & Fernandez, 2008). Some of actual techniques have presented inadequate sensitivity or require a sample pretreatment to isolate the dye from matrix.

Cloud point extraction (CPE) has shown to be an advantageous separation strategy and has been used for the extractive preconcentration, separation and purification of metal ions, metal chelates, biomaterials and organic compounds present in different complex matrixes (Akita & Takeuchi, 1995; Akita, Rovira, Sastre, & Takeuchi, 1998; Bai, Li, Chen, & Chen, 2001; Liang, Wang, Xu, Li, & Qi, 2009; Madrakian, Afkhami, & Mousavi, 2007; Pourreza, Rastegarzadeh, & Larki, 2011; Talio, Luconi, Masi, & Fernández, 2009; Wang, Luconi, Masi, & Fernández, 2007).

At certain temperature, aqueous solution of a nonionic surfactant separates into two phases. The first one is a surfactant-rich phase containing a high concentration of surfactant, which has small volume compared to the solution and the second one is the aqueous phase containing a low concentration of surfactant, near to critical micellar concentration (cmc). This separation temperature is known as cloud point temperature (CPT) of the surfactant (Hinze & Pramauro, 1993). When an sparingly water soluble analyte is put in contact with an aqueous solution of non-ionic surfactant and the system is heated at temperature above the surfactant cloud point temperature, then the analyte is distributed between the two phases, choosing of preferential mode the surfactant rich phase (Wang, Zhao, & Li, 2003). This procedure is known as cloud point extraction (CPE).

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A sensitive and selective spectrofluorimetric methodology has been developed for RhB quantification, employing CPE as previous step to fluorescent detection. CPE brings the possibility of analyte preconcentration, improving the compound signal and allowing the RhB trace determination.

2. Experimental

2.1. Instrumentation

Shimadzu RF-5301PC spectrofluorometer (Shimadzu Corporation, Analytical Instrument Division, Kyoto Japan) equipped with a discharged Xenon lamp was used for recording fluorimetric measurements.

Beckman DU 520 UV–visible spectrometer (Beckman Instruments Inc., CA, USA) with quartz cells of 10-mm path length for absorptiometric measurements was used.

HPLC experiments were realised with Beckman System Gold High Performance Liquid Chromatograph (CA, USA) using a C_{18} column (250 mm × 4.0 mm), with programmable Solvent Module 126 HPLC pump equipped with a Rheodyne injection valve fitted with a 10 μL loop. Detection was carried out with a Beckman System Gold 168 diode array detector. System Gold software was used for data acquisition.

Adjustments of pH were carried out using Orion Expandable Ion Analyser pH-meter (Orion Research, MA, USA) Model EA 940 with a combined glass electrode.

A centrifuge (ROLCO SRL, Argentina) equipped with an angle rotor (6-place, 3500 rpm) was used to accelerate the phase's separation process.

2.2. Reagents

Stock solution of 100 μg L^{-1} of RhB was prepared by dissolving RhB (Fluka AG, Chemische Fabrik, Buchs SG, Switzerland) in ultrapure water. Afterwards it was daily diluted obtaining a working standard solution 1 μg L^{-1}.

Surfactant polyethyleneglycolmono-*p*-nonylphenylether (PONPE 7.5, Tokyo Kasei Industries, Chuo-Ku, Tokyo, Japan) 10% (v/v) in ethanol was the employed as extracting solution.

Buffer Tris 0.1 mol L^{-1} (Mallinckrodt Chemical Works, NY, USA) and sodium tetraborate (Mallinckrodt Chemical Works) 0.1 mol L^{-1} were used, obtaining the desired pH by addition of dilute HCl (Merck, Darmstadt, Germany) or NaOH (Mallinckrodt Chemical Works). The pH values were adjusted by the addition of solutions of NaOH 0.01 mol L^{-1}, NaOH (c), HCl 0.01 mol L^{-1} or HCl (c) until the target pH value was reached.

Mobile phase composition by HPLC experiences was (A) acetonitrile (Merck) and (B) 0.1 mol L^{-1} sodium perchlorate (Mallinckrodt Chemical Works) solution in water, adjusted to pH 4.0 with perchloric acid (Mallinckrodt Chemical Works).

All used chemicals were of analytical grade and ultrapure water was throughout used.

2.3. General procedure

Aliquot of sample containing between 4.67×10^{-2} and 100 μg L^{-1} of RhB, 20 μL HCl (0.05 mol L^{-1}) and 0.750 mL of extracting solution, were placed in a 10 mL graduated centrifuge tube. The whole mixture was diluted to 10 mL with ultrapure water. The prepared solution was kept at 313 K for 5 min in a thermostatic bath for equilibrating and then centrifuged for 7 min at 3500 rpm (approximately 1000 g). After being cooled at 255 K during 10 min, the surfactant rich phase became a viscous gel at the bottom of tube and the aqueous phase could be poured off. The surfactant rich-phase in the tube was then made up to 4 mL by adding 2 mL of buffer sodium tetraborate pH 9.2, 1.5 mL of absolute ethanol and ultrapure water. RhB concentration was determined measuring fluorescent emission at λ_{em} = 576 nm (λ_{exc} = 555 nm).

2.4. Sampling procedure and sample preparation

Samples were acquired in local shops, choosing products manufactured in Argentine. In order to guarantee representative samples, a randomise strategy sampling was used; a total of three recipients of the same brand for each product were acquired. The whole of the contents of each product was homogenised and reserved for sample preparation.

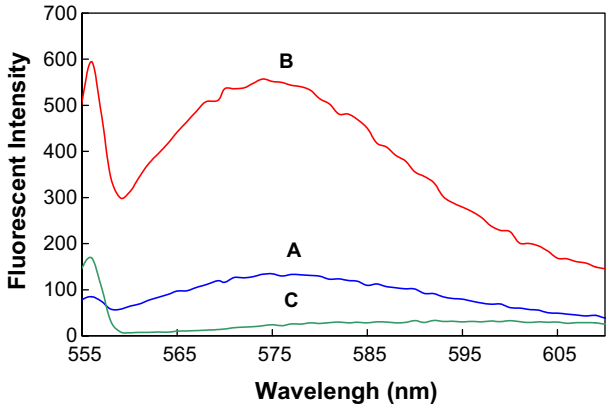
About 1.00 g of each sample (condiment, snacks or bubble gums) was dissolved in ultrapure water, filtered and diluted to 50 mL in a volumetric flask. Aliquot of 5 mL of sample solutions were poured in graduated centrifuge tube, and variable volumes from 0 to 250 μL of RhB (40 μg L^{-1}) was added in each one and treated by general procedure.

3. Results and discussion

Dye RhB in aqueous solution exists as many different forms characterised by typical absorption and fluorescent spectra (Fig. 1A). The structural variation includes neutral forms, molecular aggregates and ionised species depending of pH, solvent composition, temperature and concentration.

CPE efficiency as perceptual extraction is influenced by analyte polarity, capacity of solubilisation into the micelle (nonpolar core or polar micelle–water interface) and additives presence. With the aim of improving the performance of the methodology, some experimental parameters like equilibration pH, surfactant concentration, nature solvent, temperature and equilibrium time, were investigated.

A successful CPE should maximise the extraction efficiency by minimising the phase's volume ratio. Nonionic surfactant PONPE 7.5 presents a CPT near room temperature, offering operative advantages and being especially adequate for organic species extracting (Luconi, Silva, Olsina, & Fernandez, 2001; Shariati, Yamini, & Zanjani, 2008; Sombra, Luconi, Silva, Olsina, & Fernandez, 2001) (Fig. 1B). An additional advantage is related to low fluorescent emission of PONPE 7.5 at emission wavelength of RhB (see Fig. 1C). Then, this surfactant was chosen for CPE experiences.



RhB concentration = 4.67 x 10^{-2} μg L^{-1}
A- Emission spectrum of RhB in aqueous solution.
B- Emission spectrum of RhB after general procedure.
C- Emission spectrum of blank solution after general procedure.

Fig. 1. Fluorescent spectra for RhB in aqueous solution and treated as in general procedure.

3.1. Effect of equilibrium pH

Ionisable organic species have maximum extraction efficiency at pH values which their uncharged form prevails, and therefore, analyte will be partitioned into the surfactant rich phase. In order to locate the optimal pH range for the quantitative RhB extraction, experiments were realised varying this parameter. Each desired pH value was obtained by the addition of HCl(d) and/or NaOH (d). RhB extraction begins at pH 1.0 and starts to decrease at pH 8.0, offering a wide range for quantitative extraction. Maxima RhB extractions were exhibited between 4 and 7 pH values. A pH value of 4.0 was chosen for following experiences between 4 and 7 pH values. A pH value of 4.0 was chosen for following experiences.

3.2. Volume of extracting solution

The phase separation phenomenon in dilute solutions of surfactant organised media is associated with the attractive interaction between micelles; on the other hand, the increase of cloud point temperature at higher surfactant concentrations is due to the more ordered water/surfactant system (Koshy, Saiyad, & Rakshit, 1996). To clarify these phenomena, diverse mechanisms have been proposed:

- The rapid micellar growth, i.e., an increase in aggregation number of the micelles, in response to heat (Schick, 1987).
- The attractive inter-micellar interactions, which become more important in the vicinity of the cloud point temperature (Kumar, Sharma, Khan, & Kabir ud, 2002).
- The diminished solubility of micelles in water due to increasing temperature (Hinze & Armstrong, 1987).

In order to establish the adequate volume of extracting solution that guarantees quantitative RhB extraction, assays were carried out varying this parameter from 0.25 to 1500 mL, increasing the volume of extracting solution 0.25 mL each time. Extraction was checked by RhB fluorescent signal and it was close to 100% for all studied volume range, using simple step extraction procedure. Nevertheless, the quantitative RhB extraction was obtain using 0.75 mL of extracting solution, confirmed by double extraction process; this volume was selected as optimal for further studies.

3.3. Temperature and equilibration time

The shortest equilibration time and the lowest equilibration temperature compromise the efficient separation of phases. The phase volume ratio decreases as the equilibration temperature increases improving the analyte preconcentration. The temperature which the separation phase occurs is function not only of surfactant concentration but also the presence and concentration of organic and/or inorganic additives.

Taking into account that preconcentration is affected for temperature and equilibration time, a systematic study was carried out in order to optimising both parameters.

An equilibration time up to 10 min has been suggested as sufficient for the completion of the physicochemical process and quantitative extraction of organic species into micellar aggregations (Biparva & Hadjmohammadi, 2007; Manzoori & Karim-Nezhad, 2004; Tabrizi, 2006). However, for RhB, the dependence of extraction efficiency upon equilibration time was studied within a range of 5–30 min. It was found that the equilibration time affects scarcely the extraction efficiency. In order to minimise the sampling time, an equilibration time of 5 min was used in following assays.

Afterward equilibration temperature was varied from 293 to 323 K. Cloud point was promptly achieved for temperatures of 313 K and superior. Nevertheless, systems which were submitted to equilibration temperatures above 313 K, they showed a decreased RhB fluorescence signal. It is well known that excessive heating can cause partial degradation of organic reagent.

An equilibration temperature of 313 K was chosen as optimal for following experiences.

3.4. Centrifugation time

Centrifugation accelerates phase separation in the same sense as in conventional separations of a precipitate from its original aqueous medium. Therefore, the effect of this parameter upon extraction efficiency was studied. The complete phase separation was achieved for centrifugation times of 7 min; this time was chosen as optimal, with good efficiency for separating both phases and experimental convenience. No appreciable improvements were observed for long time of centrifugation.

3.5. Influence of surfactant rich phase's diluent

Although the very high viscosity of the surfactant rich phase (20 cP approximately) facilitates the phase separation, its manipulation for the following measuring step is complicated. Besides, the small volume (approximately 300 μL) of this phase after centrifuging was insufficient to allow the fluorescence measurement, being necessary an adequate dilution.

Different solvents for the surfactant-rich phase were tried so as to select the one producing the optimal results regarding sensitivity and decreasing the viscosity; HCl was used for pH low ranges, obtaining low fluorescent signals. In order to attain alkaline dissolutions, sodium tetraborate 0.1 mol L^{-1} was used. The best results

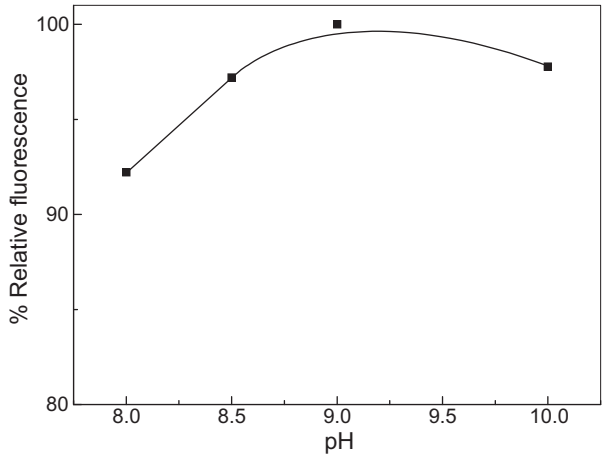


Fig. 2. Influence of diluting agent pH of the surfactant rich phase on RhB fluorescence.

Table 1
Studied experimental conditions and analytical parameters for RhB determination.

Parameters	Studied range	Optimal conditions
Equilibrium pH	1.0–8.0	4
PONPE 7.5 volume	0.25–1.5 mL	0.750 mL
Equilibration time	5–30 min	5 min
Equilibration temperature	293–323 K	313 K
Time centrifugation	1–20 min	7 min
pH of diluent solution	6.0–10.00	9.2
Buffer sodium tetraborate	4×10^{-2} –0.5 mol L^{-1}	0.1 mol L^{-1}
LOD	–	1.40×10^{-2} μg L^{-1}
LOQ	–	4.67×10^{-2} μg L^{-1}
LOL	–	4.67×10^{-2}–100 μg L^{-1}
R^2	–	0.999

Table 2
Determination of RhB in condiments. Recovery study.

Sample	RhB added ($\mu g\ L^{-1}$)	RhB found ± CV ($\mu g\ L^{-1}$)	Recovery (%, $n = 3$)	Rh B found (ng/g)*
Paprika	–	0.44 ± 0.01	–	22
	1.25	1.68 ± 0.05	97.72	
	2.5	2.93 ± 0.39	97.72	
Chimi churri	–	0.65 ± 0.64	–	32.5
	1.25	1.93 ± 0.46	104.61	
	2.5	3.14 ± 0.32	98.46	
Rice spice	–	0.53 ± 0.13	–	26.5
	1	1.52 ± 0.08	98.11	
Pizza spice	2	2.54 ± 0.03	101.66	
	–	0.21 ± 0.19	–	10.2
	1.25	1.45 ± 0.45	95.24	
Puflitos	2.5	2.72 ± 0.33	104.76	
	–	0.09 ± 0.22	–	4.72
	1	1.09 ± 0.47	100	
Flavored potato chips	2	2.09 ± 0.67	100	
	–	0.06 ± 0.65	–	3.12
	0.44	0.50 ± 0.14	100	
	0.84	0.90 ± 0.32	100	
Krach-itos	–	0.051 ± 0.09	–	2.54
	1.25	1.30 ± 0.51	99.92	
	2.5	2.55 ± 0.40	99.96	
Bubble gum (1)	–	0.00 ± 0.15	–	ND
	1.25	1.26 ± 0.35		
	2.5	2.49 ± 0.80		
Bubble gum (2)	–	0.00 ± 0.13	–	ND
	1	1.01 ± 0.08		
	2	1.99 ± 0.03		

ND, not detectable.
* RhB content in 1 g of sample.

were achieved with ethanol/buffer borax pH 9.20 mixtures, permitting an appropriate viscosity and the best signal for the fluorescence measurement. Results are presented in Fig. 2.

4. Analytical performance: Applications and validation

Table 1 summarises the studied experimental variables and the optimal values for preconcentration/separation of RhB. Limits of detection (LOD) and quantification (LOQ) were calculated in accordance to official compendia methods (Miller & Miller, 1994), using the relation k (SD)/m where $k = 3$ for LOD and 10 for LOQ; SD is the standard deviation from 15 responses of blank replicates and m is the slope of the calibration curve.

Despite the RhB proved toxicity, some cases of food adulteration with this dye have been reported (http://www.emdchemicals.com/rhodamine-b-in-chilli-extract/c_dLOb.s1OJwsAAAEtSOhSU7xq).

Developed methodology was conducted in samples of various products; particular attention was given those they are attractive for children, because of they represent the main risk group for their low body mass index and their immature detoxification system.

Samples were dissolved as it has been described in Item 2.4. Then, they were treated following general procedure. In order to assuring RhB quantitative extraction, pH samples were preconditioned by means of HCl addition.

Accuracy of proposed methodology was performed using the standard addition method. Reproducibility was evaluated by repeating the proposed approach 3 times for each sample. Recoveries and contents of RhB in samples based on the average of replicate measurements are illustrated in Table 2. RhB quantitative extraction was checked by double extraction procedure, repeating general procedure on aqueous phase resulting of CPE process. For all informed samples, RhB fluorescent signal was under LOD value, putting in evidence quantitative extraction of RhB in only one CPE.

The obtained results showed that the proposed methodology was suitable for determination of RhB in such spices samples, snacks and bubble gums, for all range of studied concentrations. Furthermore, the presence of RhB in seven of nine studied samples was validated by HPLC assays with UV–vis detection ($\lambda = 555$ nm, retention time: 9.15 min) using standard addition method.

5. Conclusions

Developed countries have forbidden the utilisation of RhB as colourant in food industry as consequence of the damages on the human health. In Argentine, there is no regulation on RhB use in foodstuffs. Due to health risks derived of RhB consumption, it is considered very important to achieve the quantification of this toxic substance in foods. A simple, sensible, selective, environmentally friend methodology has been developed and applied to traces RhB determination in condiments, using an inexpensive instrumentation. CPE implementation allowed dye analysis removing matrix interferences and improving sensitivity. Likewise, the use of non-ionic surfactant PONPE 7.5 as extractant phase permitted to realise CPE process at room temperature, without risk of analyte decomposition in a short equilibration time. Obtained results confirm that the proposed methodology is useful for RhB control in foodstuffs. Taking into account that the analysed samples have high levels of RhB, they are not suitable for the human consumption. In Argentine, it is necessary the implementation of laws for regulation of RhB as food additive.

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