Bull. Chem. Soc. Ethiop. **2023**, 37(6), 1315-1323.
DOI: https://dx.doi.org/10.4314/bcse.v37i6.2

ISSN 1011-3924
Printed in Ethiopia
Online ISSN 1726-801X

HIGHLY SENSITIVE ZERO-ORDER EMISSION SYSTEM - FLUORESCENCE DETECTION-LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF TWO BANNED PHENYL XANTHENE DYES IN BOTTLED CHILI SAUCE

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(Received April 13, 2023; Revised August 28, 2023; Accepted August 31, 2023)

ABSTRACT. A zero-order emission system was applied to address the variation in emission wavelengths between analytes. Also, it assists to compensate for the drop in emission intensity resulted from the changes in analyte excitation wavelength. So, a high performance liquid chromatography-fluorescence detector (HPLC-FLD) with zero order emission mode was used for the simultaneous estimation of rhodamine B (RhB) and eosin Y (EsY) based on their native fluorescence. The presented method is considered the first fluorescence method for simultaneous determination of RhB and EsY. The efficiency of the developed method was tested by determining the two dyes in a high matrix sample, chili sauce. Under the optimum condition, a linear calibration graphs for RhB and EsY in range of 0.5 -300 ng/mL with r = 0.9994 and 0.9991, respectively, were acquired. The limits of detection for RhB and EsY were 0.344 ng/mL (0.355 ng/g) and 0.215 ng/mL (0.222 ng/g), respectively. The recovery percentage and relative standard deviation of two analytes were obtained in the range of 98.38-101.86% and 0.83-1.66%, respectively. RhB was detected only in one chili sauce sample at a concentration of 0.388 ng/mL while EsY was not found in any sample.

KEY WORDS: Rhodamine B, Eosin Y, HPLC-FLD, Zero-order emission mode, Solid phase extraction

INTRODUCTION

Food dyes and additives are commonly used to make food more appealing, compensate for color loss during processing or storage, and maintain food uniformity. Rhodamine B (RhB) and eosin Y (EsY) are used as coloring agents in food, cosmetics, pharmaceuticals, and textiles owing to their bright fluorescent colors [1–3]. These are basic and acidic phenyl xanthene dyes, respectively, as shown in Figure 1. Based on safety evaluation, there is sufficient evidence to suggest that RhB and EsY have potentially deleterious effects on humans and animals, including toxicity and carcinogenicity [4–9]; consequently, the US authorities, European Food Safety Authority (EFSA), and other regions and countries have prohibited their use in foods [10, 11]. The toxicity impacts of RhB and EsY provide a stimulus for researchers to design a highly sensitive and efficient method for their identification in foods and drinks.

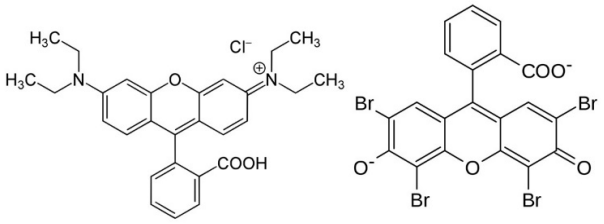

Figure 1. Chemical structure of RhB and EsY.

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Various analytical techniques, such as spectrophotometry [12–19], spectrofluorimetry [20, 21], HPLC-optical detection [2, 22–26], LC-MS [27–30], UHPLC-HRMS [31], and voltammetric methods [32, 33] have been used to estimate individual RhB and EsY dyes. Only one LC-DAD approach has been reported for the simultaneous estimation of RhB, EsY, and other dyes in foodstuffs [34]. The limits of detection of the two dyes using this method were 0.14 µg/g.

Solid phase microextraction (SPME) has become one of the most preferred approaches for the preconcentration/removal or separation of the dyes from water, foods, cosmetics and personal care products, thanks to its advantages such as low cost, use of low amount, selectivity, and time saving [17, 22, 23, 31].

Fluorescence detection (FLD) is a highly sensitive, specific, and selective optical technique. In this study, the first high performance liquid chromatography-fluorescence detection (HPLC-FLD) method for the simultaneous estimation of RhB and EsY at the nanogram level in bottled chili sauce is introduced based on its native fluorescence. This study aimed to design and validate a simple, rapid, sensitive, and cost-effective method for its determination. In addition, the effective removal of the interference of macromolecules in the sample matrix and the extraction and preconcentration of the two dyes in bottled chili sauce samples were achieved using the solid-phase extraction method.

EXPERIMENTAL

Chemicals and materials

RhB, EsY, acetonitrile, methanol (HPLC grade), and formic acid were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Ultrapure water was obtained using a Barnstead™ Smart2Pure™ water purification system. Sep-Pak® C18 plus short cartridges (360 mg) were purchased from the Waters Corporation (Milford, Mass, USA). Eight bottled chili sauce samples were collected from a local Saudi Arabia market and stored at room temperature (25 °C) until use.

Standard solution

Stock solutions of RhB and EsY were separately prepared in a mixture of ultrapure water and methanol (80:20% v/v) at a concentration of 100 mg/L. RhB and EsY were further diluted with the same mixture to obtain appropriate concentration.

Chili sauce sample preparation and solid phase extraction

Chili sauce (10 g) and 40 mL of a mixture of water and methanol (80:20% v/v) were poured into a 50 mL glass tube, stirred for 5 min, and then sonicated for 5 min. The resulting mixture was transferred to a centrifuge tube and centrifuged at 6000 rpm for 10 min. The supernatant was collected using a 20 mL disposable syringe and directly injected into a Sep-Pak® C_{18} cartridge that was prewashed with 5 mL of water and 5 mL of acetonitrile. RhB and EsY were eluted from the cartridge with 10 mL of a mixture of water and acetonitrile (80:20% v/v). The filtrate (2 mL) was poured into 2 mL HPLC vials and injected directly into the HPLC instrument for the determination of RhB and EsY.

Apparatus

RhB and EsY were analyzed using a Thermo Scientific Dionex Ultimate 3000 UHPLC$^+$ focused standard system coupled with an FLD-3000 fluorescence detector. The analysis data were recorded using a Chromeleon™ 7.2 Chromatography Data System. The mixture of RhB and EsY was separated on a ZORBAX Eclipse plus C18 column (100 mm length, 4 mm inner diameter,

and 5 μm particle size). The RhB and EsY fluorescence spectra were recorded using a Cary Eclipse spectrofluorometer (Agilent Technologies, USA).

Chromatographic conditions

An isocratic elution mode was carried out using a mobile phase composed of 0.1% formic acid in water (A) and methanol (B) at ratio of 30:70 v/v at flow rate 0.8 mL/min. Standard solutions (10 μL) and samples were injected into an HPLC apparatus. The fluorescence detection of RhB and EsY was achieved at 530 nm for excitation and the zero-order mode for emission.

RESULTS AND DISCUSSION

Optimization of HPLC condition

The excitation and emission wavelengths of RhB and EsY were measured using a spectrofluorimeter, and applied to HPLC-FLD. Several trials have been conducted to identify the best excitation and emission wavelengths for the simultaneous quantification of RhB and EsY. RhB and EsY exhibited native fluorescence characterized by excitation wavelengths of 555 nm and 535 nm with emission wavelengths of 578 nm and 545 nm, respectively. The obtained values of the excitation and emission wavelengths of RhB and EsY show a wide difference in excitation and emission wavelengths between the two dyes, which clearly appears in the difference in peak area and intensity values, as shown in Figure 2.

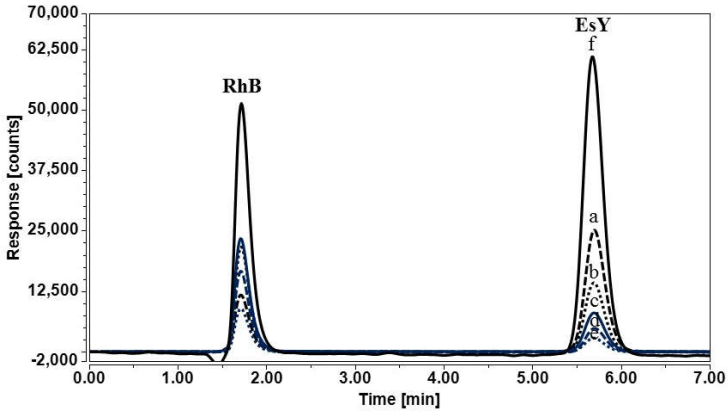

Figure 2. HPLC-FLD chromatogram of RhB and EsY at different emission wavelengths, a) 560 nm, b) 570 nm, c) 580 nm, d) 590 nm, e) 600 nm and f) zero order emission mode after excitation at 535 nm.

To overcome these issues in HPLC-FLD, an excitation wavelength of 535 nm for RhB and EsY was selected as a relatively suitable wavelength. However, the difference in the emission wavelength between RhB and EsY can be overcome by using the zero-order emission mode in the presented FLD (Model Dionex Ultimate 3000) subsequent to excitation at a chosen wavelength of 535 nm. The proposed FLD has two advantages, including the use of dual photomultiplier tube detectors (dual-PMT), which have benefits over the single PMT represented in emission wavelength collection range expansion without any influence on the detection sensitivity. Furthermore, the monochromator in zero order emission mode is adjusted to allow all

wavelengths (not just a single wavelength) emitted from the sample to be reflected onto the detector at a fixed excitation wavelength [35]. Therefore, the advantages of the zero-order emission mode were exploited to achieve a significant peak area and intensity for RhB and EsY after excitation at the chosen wavelength of 535 nm, as depicted in Figure 2.

Table 1. Results of assay validation parameters of the proposed HPLC method for the simultaneous determination of RhB and EsY.

Parameter	RhB	EsY
Range	0.5 - 300 ng/mL	0.5 - 300 ng mL
Slope	288.03	279.23
Intercept	-194.85 ± 33.03	286.1 ± 20.01
r	0.9994	0.9991
LOD	0.344 ng/mL (0.355 ng/g)	0.215 ng/mL (0.222 ng/g)
LOQ	1.145 ng/mL (1.181 ng/g)	0.715 ng/mL (0.737 ng/g)
Recovery	98.38 - 101.9	100.2 - 101.1
RSD%	0.83 - 1.66	1.10 - 1.66

Furthermore, the sensitivity of the proposed method significantly improved, as indicated by the LOD values for RhB and EsY (Table 1). The separation efficiency of RhB and EsY was investigated at three columns included Thermo Scientific ACCLAIM™ 120 C8 (4.6 x 150 mm, 5 µm), Thermo Scientific ACCLAIM™ 120 C18 (4.6 x 150 mm, 5 µm) and Agilent Technologies ZORBAX Eclipse Plus C18 (4 x 100 mm, 5 µm). The last column, maintained at 40 °C, was chosen for further optimization because it resulted in valuable separation of RhB and EsY in a reasonable time. Several mobile phases, comprising of 0.1% acetic acid in water/ acetonitrile and methanol, 0.1% phosphoric acid in water/ acetonitrile and methanol, and 0.1% formic acid in water/acetonitrile and methanol, were tested. A 0.1% formic acid in water (A) and methanol (B) with ratio of 30:70 v/v in an isocratic elution mode at flow rate 0.8 mL/min revealed valuable resolution peaks of RhB and EsY. Under these conditions, the total time required to separate the RhB and EsY mixtures was 7 min, with retention times of 1.78 and 5.67 min, respectively.

Optimization of extracting conditions

Several solvents (water, acetonitrile, methanol, acetone, ethanol, and isopropanol) were examined as extraction solvents for RhB and EsY in bottled chili sauce. A mixture of water and methanol (80:20% v/v) was found to be suitable solvent for the extraction of studied dyes with minimal interference. In addition, the aforementioned solvents were tested as elution solvents for dyes from the Sep-Pak® C18 cartridge. An Effective extraction and good recoveries for the studied dyes were obtained using 10 mL of a solution composed of ultrapure water and acetonitrile (80:20% v/v), without the need for any additional clean up procedures.

Method validation

The linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, and relative standard deviation (RSD) were evaluated to validate the HPLC-FLD method based on preliminary research specificity matrix effects. The external standard method was applied to quantify RhB and EsY, and the concentration of each dye was tested in the range of 0.5-300 ng/mL. Calibration curves were constructed using the direct proportional relationships between the peak areas and dye concentrations. The proposed method has excellent linearity for RhB and EsY in the concentration range of 0.5-300 ng/mL with correlation coefficients (r) ≥ 0.9991, as shown in Table 1. The limits of detection (LOD) and quantification (LOQ) were defined as three and ten times

the intercept standard deviation of a regression line over the average slope of the calibration curve, respectively, repeated in triplicate. The LODs for RhB and EsY were 0.344 and 0.215 ng/mL, respectively. In contrast, the LOQs for RhB and EsY were 1.145 and 0.715 ng/mL, respectively. The recovery % and relative standard deviation (RSD %) are terms referring to accuracy and precision and are calculated using the following formulas: measured value/theoretical value ×100 and SD of measured values/mean measured value × 100, respectively. At three concentration levels, average recoveries of RhB and EsY ranged from 98.38 to 101.9% and 100.2 to 101.1%, respectively, as shown in Table 1. While the average RSD % was in the range of 0.83-1.66 % for RhB and 1.10-1.66 % for EsY, as shown in Table 1). The aforementioned sensitivity, accuracy, and precision values of the method were sufficient for the confirmation and determination of RhB and EsY in the real samples. The specificity of the method was examined by analyzing eight bottled chili sauce samples, in addition to using the spike method. As shown in Figures 3 and 4, there was no interferences in the retention time region in which RhB and EsY were eluted.

Analysis of samples and recovery

The manufactured chili sauce (eight samples) was used as a real sample to verify the performance and efficiency of the HPLC-FLD-zero-order emission mode method and the Sep-Pak® C18 cartridge for solid-phase extraction and preconcentration of RhB and EsY. RhB was detected only in one sample, as shown in Figure 3, at a concentration of 0.388 ng/mL. Where, EsY was not found in any sample. Therefore, the sample was fortified with two concentration levels of RhB and EsY to ensure its positivity, as shown in Figure 3.

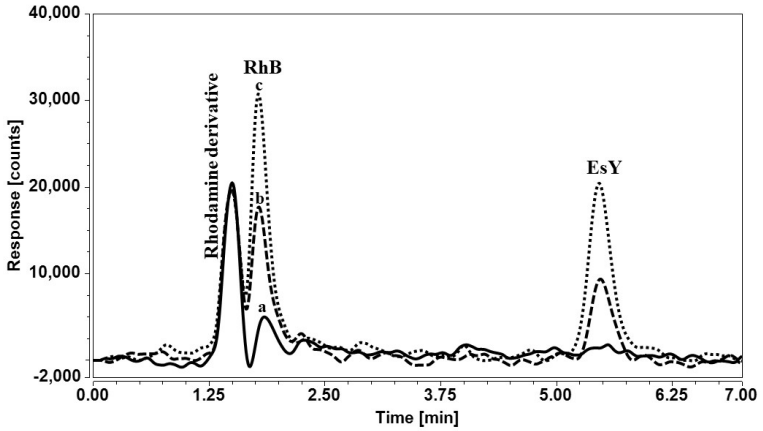

Figure 3. HPLC-FLD chromatogram of a positive chili sauce sample before (a) and after spike (b and c) with 1 and 4 ng/mL of RhB; 0.05 and 0.1 ng/mL of EsY.

Spiked manufactured chili sauce samples with RhB and EsY at three different levels were prepared for additional tests to evaluate the accuracy of the proposed HPLC-FLD-zero-order emission mode method and the efficiency of the extraction cartridge. Figure 4 shows the chromatograms of the blank and spiked chili sauce samples. The amount of RhB and EsY in the spiked samples was measured. The recovery percentage and RSD values for two dyes varied from 85.04 to 96.97% and from 1.83 to 2.99%, respectively. These findings show that the proposed method and extraction cartridge are applicable for the measurement of RhB and EsY in real samples.

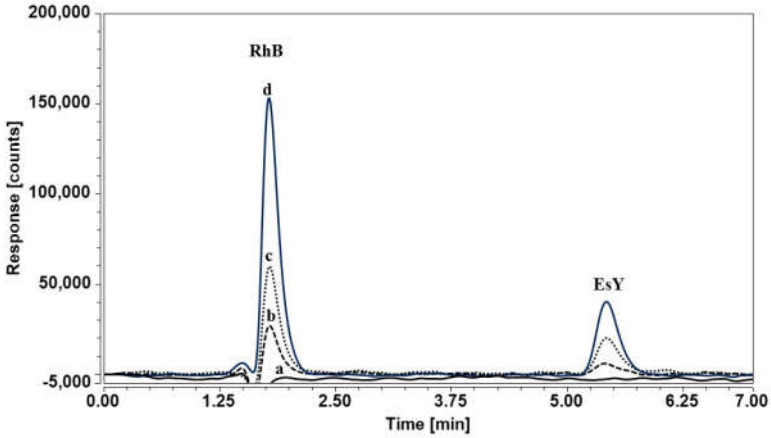

Figure 4. HPLC-FLD chromatogram of a negative chili sauce sample before (a) and after spike with different concentration levels of RhB and EsY (b, c and d).

Table 2. Comparison of the proposed method with some reported methods.

Analyte	Method	Linear range	LOD	Ref.
Rhodamine B	On-chip SPE-Spectrophotometry	10-800 ng/mL	7 ng/mL	[16]
	MD- µ-SPE-Spectrophotometry	5-200 ng/mL	1.6 ng/mL	[17]
	SPE-Spectrophotometry	0.25-3.0 µg/mL	3.14 ng/mL	[36]
	MSPE-Fluorescence	0.40-140.00 ng/mL	0.06 ng/mL	[21]
	polyamide-SPE-HPLC-UV	0.2–50 µg/mL	0.107 µg/mL	[24]
	C18-SPE-LC-UV	0.05 – 10 mg/ mL	0.14 µg/g	[25]
	Fe_3O_4 @COF-MSPE-HPLC-UV	0.05–5 µg/mL	3.8 ng/mL	[23]
	HPLC-UV	0.025−20 µg/mL	0.14 µg/g	[34]
	HPLC-FLD	0.5–10 ng/mL	3.7 ng/g	[2]
	UHPLC-HRMS	0.5−100 ng/mL	1.67 ng/g	[31]
	HPLC-ESI-MS/MS	0.2–10 ng/mL	0.05 ng/g	[27]
	UHPLC-MS/MS	0.5 to 100 ng/mL	0.1 ng/mL	[28]
	HPLC-MS/MS	0.05–100 µg/g	0.05 µg/g	[30]
	SPE-HPLC-FLD-zero order emission mode	0.5- 300 ng/ mL	0.344 ng/mL (0.355 ng/g)	This work
Eosin Y	Spectrophotometry	0.115-2.53 µg/mL	0.03 µg/mL	[15]
	HPLC-UV	0.025−20 µg/mL	0.14 µg/g	[34]
	SPE-HPLC-FLD-zero order emission mode	0.5- 300 ng/ mL	0.215 ng/mL (0.222 ng/g)	This work

Comparison with other methods

The values of the linear range and LOD for the proposed approach for the simultaneous quantification of RhB and EsY were compared with those of previously reported approaches, as shown in Table 2. The LOD values of RhB obtained in this study were far lower than those of on-chip SPE spectrophotometry [16], MD µ-SPE spectrophotometry [17], SPE spectrophotometry [36], polyamide-SPE-HPLC-UV [24], C18-SPE-LC-UV [25], Fe_3O_4@COF-MSPE-HPLC-UV [23], HPLC-UV [34], HPLC-FLD [2] and UHPLC-HRMS [31]. However, it was higher than that of MSPE-fluorescence [21], HPLC-ESI-MS/MS [27], UHPLC-MS/MS [28] and HPLC-MS/MS

[30]. In general, the presented method has satisfactory sensitivity for the estimation of RhB in real samples compared with the reported method. The LOD of EsY obtained using this approach is far lower than that of all reported methods. The results obtained for the simultaneous determination of RhB and EsY showed that the developed Sep-Pak® C18 SPE-HPLC-FLD-zero-order emission mode method was satisfactory.

CONCLUSION

In this study, the first HPLC-FLD method was successfully applied for the concurrent estimation of RhB and EsY in bottled chili sauce. The proposed approach has several benefits including time savings, good linearity, high sensitivity, and good recovery. The high efficiency in avoiding matrix interference was achieved by the Sep-Pak® C18 SPE cartridge. Based on the aforementioned advantages and results, the Sep-Pak® C18 SPE-HPLC-FLD-zero order emission mode method can be used for the extraction, preconcentration, and detection of RhB and EsY in real samples and can be applied in routine food safety examinations. RhB was detected only in one sample at a concentration of 0.388 ng/mL while EsY was not found in any sample.

ACKNOWLEDGEMENTS

This work was funded by the Deanship of Scientific Research at Jouf University under grant no. (DSR-2021-03-0345).

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