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Flow Injection Analysis with On-Line Solid Phase Extraction for Spectrophotometric Determination of Ponceau 4R and its Subsidiary Unsulfonated Dye in Sweets and Cosmetic Products

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Abstract. An integrated solid-phase spectrophotometry/flow injection analysis (FIA) method is proposed for the determination of the synthetic colorant matter Ponceau 4R (P4R) in the presence of its unsulfonated derivative 2-hydroxy-1-[(naphthalenyl)azo] naphthalene (N2N). The procedure is based on the measurement of P4R at $\lambda = 508$, followed by retention and preconcentration of the low level concentration of N2N on a C_{18} silica gel minicolumn and subsequent measurement of the absorbance of N2N at $\lambda = 508$ nm after its elution. The applicable concentration range, the detection and the relative standard were the following: for P4R, from 0.30 to 20.0 mg/L; 0.052 mg/L 1.1%; and for N2N, between 0.020 to 3.0 mg/L 0.003 mg/L and 1.1%. The method was applied to the determination of small amounts of N2N present in P4R in food and cosmetic products. Percentages of recovery between 95 and 105% were obtained in all instances. The method was applied satisfactorily to the determination of the compounds P4R and N2N in samples of sweets and cosmetic products when compared to results offered by a HPLC reference method.

Key words: Ponceau 4R determination; unsulfonated Ponceau 4R determination; flow injection analysis; on-line solid phase extraction; sweets; cosmetic products.

The analysis and monitoring of food additives that are permitted by government regulatory agencies is a major concern in many countries, largely because of reports on the toxic and/or carcinogenic effects that the use of these chemicals can cause.

Most of the colorants used as additives in the food industry are synthetic products, usually azo-colorants with aromatic rings, containing sulfonic groups to ensure their solubility in aqueous media. The raw dyes used in manufacturing can contain variable amounts of by-products from the synthetic procedures such as other sulfonated derivatives, unsulfonated aromatic amines, the corresponding unsulfonated dyes and chlorinated derivatives of the unsulfonated dyes [1].

Many countries and health organizations have published guidelines to control the use, purity and permitted amounts of colorants in foods and also to specify permitted percentages of soluble and insoluble colorant materials. The amounts of impurities in raw dyes are usually limited, such that a maximum of only 0.2% of the total amount of color additive is allowed as ether soluble matter [2].

One of the synthetic sulfonated colorants widely used in the food industry is the 7-hydroxy-8-[(4-sulfo-1-naphthalenyl)azo] 1,3-naphthalenedisulfonic acid trisodium salt, commonly named Ponceau 4R (C.I. 16255; E-124). It is the water-soluble colorant derived from the unsulfonated colorant 2-hydroxy-1-[(naphthalenyl)azo] naphthalene (N2N) also named Ponceau 4R Spirit Soluble.

The E-124 colorant is found in the colorants which are permitted within the European Community, where their use is authorized in sweets, cakes and cookies, ice creams, syrups, delicatessen drinks and other desserts, with the daily acceptable dose (DDA)

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established temporarily at 0.15 mg/kg of body weight. The European Union recommends a maximum 0.2% presence of subsidiary N2N with the colorant Ponceau 4R (P4R). There is a clear need for a good method to determine residual amounts of N2N initially present in the P4R used in food manufacturing.

Different analytical techniques have been proposed for the determination of P4R. Fogg et al. used electrochemical techniques for the determination of several synthetic colorants, including P4R; these included voltammetry using stationary glassy carbon electrode [3] or mercury-drop electrode [4], and differential pulse polarography [5]. Later, Sánchez Batanero et al. [6] studied the electrochemical behavior of P4R using voltammetric, chronopotentiometric and coulometric techniques with a mercury drop electrode in different media and at different pH values. Yongnian et al. propose an adsorptive voltammetric analysis for a mixture of colorants by a multivariate calibration approach [7].

Chromatographic separations of colorants in food and pharmaceutical preparations have also been proposed. Colorants are retained in a column with resin followed by a spectrophotometric determination [8]. Separation of several colorants by high-performance liquid chromatography [9], high-performance ion chromatography [10], conventional thin layer chromatography [11] or high-pressure thin layer chromatography [12] has also been used, followed by absorbance measurements to determine the amounts of the colorants. More recently, mixtures of two or three food colorants (one of then P4R) were spectrophotometrically resolved using derivative and ratio spectra [13, 14, 15] or solid-phase spectrophotometry and multicalibration procedures [16, 17]. In all instances only the sulphonated products, or the total amount of sulphonated plus subsidiary products, were determined.

Determination of the N2N in the presence of large amounts of P4R can be achieved by means of [18], where an ion exchanger is used for the retention of sulphonated dyes and C-18 silica for the corresponding unsulphonated dye, with subsequent measurement directly on the solid support. This is a simple, sensitive, and selective technique that enables the separation and preconcentration of both colorants in different matrixes, making possible their determination with conventional instrumentation. However, these are batch methods in the double sense that they are both discontinuous and also analyze only one compound, sulphonated or unsulphonated, each time.

The approach used in this paper is a flow system with a minicolumn [19, 20] to retain the unsulphonated derivative without the use of additional reagents, which allows us 1) to retain and hence separate the unsulphonated from the ionic sulphonated dye and 2) to preconcentrate the first compound. In this way, the analytical problem that arises from there being two different magnitudes of concentration can be solved. In this paper we have developed a flow system for a simple and inexpensive methodology for the analysis of P4R and N2N in sweets and cosmetic products.

Experimental

Chemicals

All chemicals used were of analytical-reagent grade. Reserve-osmosis type quality water (Milli-RO 12 plus Milli-Q station from Millipore) was used throughout.

Ponceau 4R Spirit Soluble (N2N) stock solution (7.0 mg/L) was synthesized and purified by us [21, 22] and prepared by exact weighing and dissolution in methanol. This solution was stable for at least two months. The working solution was prepared by appropriate dilutions with water while maintaining 75% (v/v) methanol in all instances.

Ponceau 4R water soluble (P4R) stock solution (20.0 mg/L) was used and prepared by exact weighing of the compound (Utter Laboratory Spain S.L) and dissolution in water. This solution was stable for at least two months. Solutions of lower concentration were obtained by dilution with water. As the manufacturer indicates a 82% purity level of P4R, we purified it further by repeated treatment of the solid with trichloromethane. The solutions of both colorants were stored under refrigeration at 4 °C in dark bottles.

C_{18} Sep-Pak® cartridge (Water, Millipore Corporation) packed with 300 mg of C_{18} silica with average particle sizes of 55–105 μm was tested as a solid support. Ion exchange gels Sephadex SP C-25, Sephadex DEAE A-25, and QAE A-25 (Sigma) and the adsorbent Sephadex G-15 were also tested. As the carrier solution, a 75:25 (v/v) methanol-water (methanol HPLC grade Panreac) was used. Pure methanol was used as the desorbing solution.

Apparatus, Software and Flow Diagram

Absorption measurements were made with a Perkin Elmer Lambda-2 and a Bausch Lomb Spectronic 2000 spectrophotometers equipped both with Hellma 176.052 QS flow glass cells with 10 mm light path and 25 μL volume. A Crison Digit pH-meter equipped with a combined glass-calomel electrode was used. Hewlett-Packard 1050 series liquid chromatograph with DAD detector and a C_{18} Sugelabor column were used.

The flow analysis set-up consisted of a Gilson Minipuls-2 four channel peristaltic pump working at a constant flow-rate, three variable volume Rheodyne 5041 Teflon rotary valves controlled electromechanically by a method developed in our laboratory; and the spectrophotometers cited above. All three were connected to a conventional microprocessor that controlled the pump, valves and spectrophotometer using a software designed by us (written in Basic

language and compiled). This set-up was interfaced using RS-232C interface to a microprocessor. PTFE tubing (Omnifit, Cambridge, England) (0.8 mm i.d. and 1.6 mm o.d.) and various end-fittings and connectors of different diameters (Omnifit also) were used. The minicolumn used was a Sep-Pak cartridge packed with C_{18} silica.

Software programs used for the treatment of the data were: Statgraphics software package, ver.6.0 STSC, Inc. Statistical Graphics Corporations, USA, 1993, Grams/386 software package ver.1.0 and Add. Galactic Industries, Salem, USA and Data Leader software package, Beckman, Fullerton, CA, 1987.

Procedure

The sample solution (700 µL) containing between 0.30 and 20.0 mg/L of P4R and/or between 0.020 to 3.0 mg/L of N2N and the same hydroalcoholic medium as carrier, was inserted into the carrier stream (methanol-water 75:25 v/v) at a flow-rate of 1.10 mL/min. When this reached the cartridge containing C_{18} silica, the N2N was retained whereas the P4R was transported to the flow cell, which measured its absorbance. When the absorbance reached the background signal, the selecting valve was switched to the eluent stream (methanol) at a flow-rate of 1.1 mL/min for 60 seconds, which removed the N2N from the support and made it possible to measure the N2N in the flow cell. After the maximum absorbance was reached, the system was conditioned by passing carrier for 1 minute. In both cases, an absorbance increase at 508 nm was recorded. Also, in both cases, the relation between concentrations and height or area was established by the calibration graphs.

As a previous step in the analysis of sweets samples (after dissolving by heating, if necessary), the only preparation needed was to filter the sample through a 0.45 µm membrane filter. Then we operated as indicated before. In the case of soft drinks, it was only necessary to degasify the liquid. For cosmetic products, the sample was treated in an ultrasonic bath and filtered through a 0.45 µm membrane filter. In all cases, it was necessary to adjust the proportion of methanol to 75%. As a reference method, the HPLC procedure proposed by Lawrence et al. [23] was used.

Results and Discussion

In the VIS region both P4R and N2N dyes are highly absorbing species with identical spectra (maximum 508 nm for the two compounds). This and the two orders of difference in concentrations enable its direct determination.

The variables influencing the system can be divided into three groups: those related to the retention unit, chemical variables and system variables. Optimization studies were carried out for each individual constituent and compromise values of the experimental variables were selected.

Variables of the Retention Unit

To achieve the separation and preconcentration of both dyes, several alternatives for the adsorbent position and type of column were tested.

From the different configurations tested to separate the colorants, the use of a single column filled with an absorbent in order to achieve the selective retention and preconcentration of the unsulfonated dye N2N offers better results. Of the different adsorbents that we studied (cation and anion exchanger and sorbents of Sephadex type and silica C-18) we selected, taking into account the results obtained, the Sep-Pak cartridge filled with 300 mg C-18 silica placed 15 cm before the detection cell.

Chemical Variables

The effect of pH on the sorption of N2N and in the absorbance measurement of both analytes was determined to be unimportant, because the signal was constant in the 1–10 pH range.

For the carrier we tested several acids (sulfuric acid, hydrochloric acid), bases (sodium hydroxide, ammonia) and buffers (acetic acid/sodium acetate, ammonium chloride/ammonia) with different hydroalcoholic proportions of ethanol or methanol. Considering that P4R was not retained independently of the percentage of methanol, but that N2N is completely retained if the methanol percentage was lower than 75%, we selected as a carrier a 75:25 methanol-water (v/v) mixture. The breakthrough concentration for the Sep-Pack cartridge used was 10 mg/L of N2N for a volume of 700 µL.

In order to elute the N2N retained in the cartridge, ethanol, methanol, and acetone were tested in water-organic solvent mixtures ranging between 10 and 100%. The use of pure methanol (1.1 mL) resulted in the fastest elution.

Aqueous methanol provided a good FIA signal minimizing a baseline shift. So, the samples were conditioned with the same hydroalcoholic percentage as the carrier.

FIA Variables

The width and height of the peaks in the FIA-curve depended on the flow rate, elution time and sample volume, that is, the length of the loop. Decreased flow-rates studied between 1.0 and 4.5 mL/min resulted, as expected, in increased residence times and decreased peak heights for P4R; but with a flow-rate of 1.1 mL/min, N2N is fixed in optimal conditions and decreases at high speeds. A flow-rate of 1.1 mL/min was selected. Increasing the sample volume

between 300 and 2.000 µL yielded increasing analytical signals and residence times, as a result of the larger amount of analyte in the flow system, but several problems were observed: a decrease in sample frequency, adsorption of the sulfonated dyes in the C_{18} minicolumn, and saturation of the C_{18} silica with the N2N. A sample volume of 700 µl was chosen. The complete elution of N2N from C_{18} silica with the minimal consumption of methanol was achieved by passing methanol for 60 s at the same rate as the previous flow (i.e. a volume of 1.1 mL).

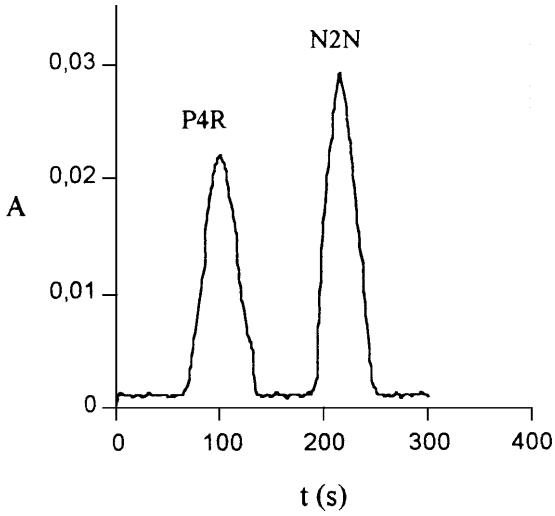

Fig. 1. Typical FIA recording. Conditions: Flow rate 1.1 mL/min; Sample volume 700 µL; Carrier methanol:water 75:25 v/v; Desorbing agent: methanol; Elution time: 60 s. [P4R]: 6.0 mg/L; [N2N]: 3.0 mg/L

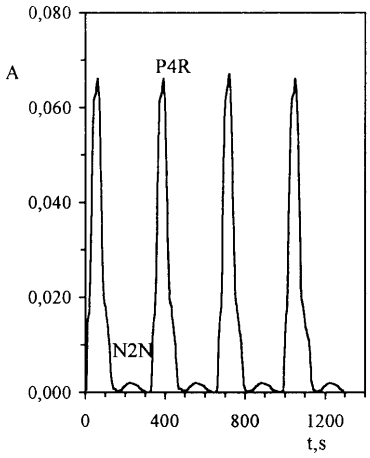

Fig. 2. Typical FIA recording. Conditions: Flow rate 1.1 mL/min; Sample volume 700 µL; Carrier methanol:water 75:25 v/v; Desorbing agent: methanol; Elution time: 60 s. [P4R]: 20.0 mg/L; [N2N]: 0.2 mg/L

Analytical Features

For analytical signals, the height or areas of the FIA peaks (Figs. 1, 2) can be used. The calibration graphs are linear for both compounds in the range 0.30–20.0 mg/L for P4R and 0.020–3.0 mg/L for N2N. The adjustment of those analytical data was carried out by linear regression, with the lack-of-fit test applied to test its linearity (three replicates of each standard and seven standards for each calibration graph), as suggested by Analytical Methods Committee [24]. Table 1 shows the analytical parameters calculated. This table shows that the use of areas increases the sensitivity and thus this analytical parameter was selected. The detection limit

Table 1. Analytical parameters

Parameters	P4R*		N2N*	
	Area	Height	Area	Height
Slope(b)	0.4451	$3.3\ 10^{-3}$	0.9671	$9.45\ 10^{-3}$
SD(b)	0.0123	$1.0\ 10^{-4}$	0.0522	$5.60\ 10^{-4}$
Intercept(a)	0.1169	$2.64\ 10^{-4}$	0.0253	$2.0\ 10^{-4}$
SD(a)	0.0822	$6.42\ 10^{-4}$	0.0232	$2.5\ 10^{-3}$
R	0.998	0.999	0.996	0.995
PL(%)	11.37	63.91	11.29	21.68
LDR	0.30–20.0	2.0–20.0	0.020–3.0	0.20–3.0
DL	0.052	0.439	0.003	0.050
QL	0.176	1.460	0.010	0.166
RSD(%)	1.1	1.9	1.1	2.5

The units for P4R and N2N were mg/L.
PL Probability level of lack-of-fit test.

Table 2. Calibration graphs of P4R and N2N in mixtures*

Compound determined	Units	Co-existing compound	Amounts	Slope	Intercept
P4R	mg/L	N2N	–	0.4451	0.1169
		N2N	2.0 mg/L	0.4397	0.0983
N2N	mg/L	P4R	–	0.9671	0.0253
		P4R	5.0 mg/L	0.9573	0.0144

*The values are the mean of five standards for each concentration and five different standards. The calibration graphs were carried out between 0.30–20.0 mg/L for P4R and 0.020–3.0 mg/L for N2N. There is no difference significant in slopes (t_{cal} 0.214 and t_{cal} 0.088 for N2N and P4R, respectively).

Table 3. Effect of foreign species on the determination of 10 µg mL^{-1} of P4R or 0.2 µg mL^{-1} of N2N

Interferent	Tolerance level µg mL^{-1}			
	P4R		N2N	
	H	Area	H	Area
Glucose	600	400	1000	1000
Fructose	500	300	1000	1000
Sacarose	400	200	800	500

[25, 26] and the quantification limit [27] were calculated according to IUPAC. The repeatability of the method (RSD) was determined by a series of ten samples, one each for each colorant.

In order to test the mutual independence of the analytical signals for P4R and N2N and to show that the signal produced by each analyte was independent of the concentration of the other analyte, several

Table 4. Statistics for validation the P4R and N2N procedures in real samples

Sample	Parameter	P4R (mg/L)			N2N (mg/L)		
		SC	AC	YC	SC	AC	YC
Mouthwash	b	4.45×10^{-1}	4.48×10^{-1}	1.213	9.67×10^{-1}	9.43×10^{-1}	3.70×10^{-1}
	n	10	18	5	15	10	5
	s_b	1.23×10^{-2}	4.35×10^{-2}	1.22×10^{-1}	5.21×10^{-2}	4.82×10^{-2}	2.17×10^{-2}
	s_a	8.22×10^{-2}	3.10×10^{-1}	1.36×10^{-1}	2.32×10^{-2}	2.24×10^{-2}	1.65×10^{-2}
	a	1.17×10^{-2}	1.205	1.98×10^{-1}	2.53×10^{-2}	2.44×10^{-2}	2.00×10^{-2}
	a′	9.54×10^{-2}	1.154	–	2.53×10^{-2}	2.60×10^{-2}	–
	t(b)	0.397		–	1.400		–
Soft drink	b	4.45×10^{-1}	4.53×10^{-1}	2.255	9.67×10^{-1}	9.96×10^{-1}	2.55×10^{-1}
	n	10	18	5	15	10	5
	s_b	1.23×10^{-2}	2.60×10^{-2}	2.69×10^{-2}	5.21×10^{-2}	5.73×10^{-2}	2.44×10^{-2}
	s_a	8.22×10^{-2}	1.76×10^{-1}	2.00×10^{-2}	2.32×10^{-2}	2.66×10^{-2}	1.40×10^{-2}
	a	1.17×10^{-1}	7.12×10^{-1}	2.35×10^{-1}	2.53×10^{-2}	3.59×10^{-2}	2.50×10^{-2}
	a′	9.54×10^{-2}	7.69×10^{-1}	–	2.53×10^{-2}	3.04×10^{-2}	–
	t(b)	1.650		–	1.590		–
Pink panther Candy	b	4.45×10^{-1}	4.45×10^{-1}	4.36×10^{-1}	9.67×10^{-1}	9.42×10^{-1}	1.02×10^{-1}
	n	10	18	5	15	10	5
	s_b	1.23×10^{-2}	2.32×10^{-2}	9.60×10^{-3}	5.21×10^{-2}	5.72×10^{-2}	1.26×10^{-2}
	s_a	8.22×10^{-2}	2.36×10^{-1}	1.12×10^{-1}	2.32×10^{-2}	5.33×10^{-2}	2.20×10^{-3}
	a	1.17×10^{-1}	8.757	2.33×10^{-1}	2.53×10^{-2}	2.48×10^{-2}	1.62×10^{-2}
	a′	9.54×10^{-2}	8.640	–	2.53×10^{-2}	1.83×10^{-2}	–
	t(b)	0.055		–	0.409		–
Strawberry Jam	b	4.45×10^{-1}	4.39×10^{-1}	1.05×10^{-1}	9.67×10^{-1}	9.46×10^{-1}	1.24×10^{-1}
	n	10	18	5	15	10	5
	s_b	1.23×10^{-2}	4.19×10^{-2}	9.70×10^{-3}	5.21×10^{-2}	7.32×10^{-2}	2.56×10^{-2}
	s_a	8.22×10^{-2}	4.62×10^{-1}	5.96×10^{-2}	2.32×10^{-2}	6.82×10^{-2}	2.60×10^{-3}
	a	1.17×10^{-1}	4.603	6.07×10^{-2}	2.53×10^{-2}	3.26×10^{-2}	1.68×10^{-2}
	a′	9.54×10^{-2}	4.532	–	2.53×10^{-2}	2.52×10^{-2}	–
	t(b)	1.071		–	0.297		–
Strawberry Tarts	b	4.45×10^{-1}	4.52×10^{-1}	6.90×10^{-2}	9.67×10^{-1}	1.056	6.52×10^{-1}
	n	10	18	5	15	10	5
	s_b	1.23×10^{-2}	3.50×10^{-2}	8.20×10^{-3}	5.21×10^{-2}	5.56×10^{-2}	3.23×10^{-2}
	s_a	8.22×10^{-2}	3.50×10^{-1}	3.15×10^{-2}	2.32×10^{-2}	5.18×10^{-2}	2.53×10^{-2}
	a	1.17×10^{-1}	3.718	1.58×10^{-1}	2.53×10^{-2}	2.86×10^{-2}	1.51×10^{-2}
	a′	9.54×10^{-2}	3.708	–	2.53×10^{-2}	3.04×10^{-2}	–
	t(b)	1.470		–	1.457		–
Strawberry Gummy Bears	b	4.45×10^{-1}	4.40×10^{-1}	4.45×10^{-1}	9.67×10^{-1}	1.031	3.65×10^{-2}
	n	10	18	5	15	10	5
	s_b	1.23×10^{-2}	1.18×10^{-2}	7.90×10^{-3}	5.21×10^{-2}	4.53×10^{-2}	2.47×10^{-2}
	s_a	8.22×10^{-2}	1.35×10^{-1}	8.00×10^{-3}	2.32×10^{-2}	4.22×10^{-2}	1.45×10^{-2}
	a	1.17×10^{-1}	10.466	2.257	2.53×10^{-2}	2.95×10^{-2}	3.39×10^{-2}
	a′	9.54×10^{-2}	10.425	–	2.53×10^{-2}	3.49×10^{-2}	–
	t(b)	0.975		–	1.128		–
	YB	–	–	2.162	–	–	–

t_{tab} 1.711 ($\alpha = 0.05$; df 24); t_{tab} 1.721 ($\alpha = 0.05$; df 21); *b* slope; *n* replicate number; s_b, standard deviation of slope; s_a, standard deviation of intercept; *a* intercept; *a′* corrected intercept; *t(b)* Student t value calculated comparing slopes SC and AC; YB, Youden blank.

calibration graphs of each colorant in the presence of variable amounts of the other dye were constructed. The slopes of these calibration graphs essentially do not change and the intercepts have no influence on the measured analytical signals. The null hypothesis test was applied to the experimental data to prove these facts (Table 2).

In order to probe the effects that can potentially produce species present in the real samples studied, a systematic study of the effect produced by sugars on the determination of samples containing P4R and N2N was carried out. The potentially interfering species were tested at different concentration levels and if interference occurred, the concentration of the interfering specie was reduced until it produced an error of 5% on the determination of P4R or N2N. The maximum concentration of interfering specie producing an error $\leq 5\%$ was taken as the tolerance level (Table 3).

Analytical Applications

In order to assess the usefulness of the proposed method for the determination of P4R and its unsulfonated homologue, it was applied to soft drinks, sweets and cosmetic products. For the application of the method, we selected different real samples of several commercial products, all of which contained P4R as a single colorant agent and, potentially, N2N as an impurity. Previous to the analysis, as described under Procedure, identification of the coloring present by TLC was carried out to assure that the coloring we found was indeed the same coloring indicated by the manufacturers.

As no reference method is available for comparing results of N2N, we tested the quality and accuracy of the proposed methods for P4R and N2N by using the statistical protocol based on standard addition methodology [28, 29]. Standard calibration (SC), standard addition calibration (AC) and Youden calibration (YC) curves were established. The slope, intercept and regression standard deviation for each curve was calculated by applying the linear regression analysis. To find the proposed method valid, we sought the following: a) homogeneity of variances for all calibration curves, b) similarity of slopes and c) that the value of the intercept obtained from the YC curve is included in the confidence interval value of the SC curve. If these conditions are observed, the accuracy of the method is confirmed by comparison of the analyte content in the different calibrations. Both results are similar and the method is accurate, with a significance level greater than 5%, if the null hypothesis test is accepted. Table 4 shows the results of the validation study of P4R and N2N determination in soft drinks, sweets and cosmetic products. We found that there is no constant error bias (YB) and there is no significant difference between AC and SC

Table 5. Determination of P4R in food and cosmetic products (mg/L)

Sample	Proposed method	Reference method (HPLC)	P-value(%)
Soft drink	20.7±0.4	20.4±0.7	63.3
Mouthwash	52.8±1.5	55.3±2.0	11.5
Pink Panther Candy	18.8±0.6	18.5±1.1	57.9
Strawberry Jam	10.1±0.1	10.1±0.2	58.1
Strawberry Tarts	8.0±0.1	8.2±0.4	61.9
Strawberry Gummy Bears	18.5±0.2	18.6±0.2	71.1

Table 6. Weight percentages found in several commercial samples

Sample	P4R (mg/kg)		N2N (mg/kg)	
	SC	AC	SC	AC
Pink Panther Candy	869.0	869.2	0.33	0.40
	t (c) = 0.557		t (c) = 0.018	
Mouthwash	37.70	37.12	0.16	0.18
	t (c) = 0.934		t (c) = 0.036	
Strawberry Tarts	245.9	244.6	2.01	1.93
	t (c) = 1.533		t (c) = 0.032	
Strawberry Jam	91.35	91.25	0.35	0.32
	t (c) = 0.323		t (c) = 0.031	
Strawberry Gummy Bears	140.69	140.70	0.25	0.29
	t (c) = 0.963		t (c) = 0.010	
Soft drink	18.70	18.5	0.33	0.21
	t (c) = 0.804		t (c) = 0.144	

Table 7. Recovery study of P4R and N2N

Sample	P4R (mg/L)			N2N (μg/L)		
	Found	Added	Recovery	Found	Added	Recovery
Soft drink	2.8	2.9	98.9	39.4	39.2	100.5
	0.7	0.7	101.4	102.0	98.2	103.8
Mouthwash	1.5	1.4	104.9	195.0	196.0	99.5
	2.8	2.9	98.3	99.0	98.0	101.0
Strawberry	10.1	10.0	101.0	71.2	70.0	101.7
Gummy Bears	4.9	5.0	98.0	33.2	35.0	94.8
Strawberry Jam	10.2	10.0	102.0	70.2	70.0	100.3
	4.8	5.0	96.0	34.8	35.0	99.4
Strawberry Tarts	9.7	10.0	97.0	69.9	70.0	99.8
	4.7	5.0	94.0	34.9	35.0	99.7
Pink Panther	10.6	10.0	106.0	70.1	70.0	100.1
Candy	5.2	5.0	104.0	34.9	35.0	99.7

slopes. In all instances t_{cal} is lower than the t_{tab} (α 0.05 and 16 freedom degree).

The proposed procedure for Ponceau 4R was found valid also when comparing results with those provided by the HPLC reference method [23], and it yielded statistically matching results (P-values are greater than 5%, the minimum value accepted for the test). Table 5 shows the content of P4R applying the proposed method and the reference method for the samples studied, and Table 6 shows the weight percentages found in those commercial samples.

The accuracy of the proposed method was also proved by means of a recovery study of both colorants in the samples studied. Different amounts of P4R and N2N were added to the samples and the percentage of recovery was determined in the same way as that described in the section Procedure. Table 6 summarizes the results obtained.

Conclusion

This paper describes a practical, simple, rapid and inexpensive method for the simultaneous determination of Ponceau 4R and its unsulfonated derivative at μg/L level in soft drinks, sweets and cosmetic products. Using this method, it is possible to analyze compounds that show the same spectral features at very different concentration levels based on the selective preconcentration of one of them. Results are comparable to the HPLC method with the advantage that no previous treatment is necessary.

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