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Fluorimetric determination of histamine in wine and cider by using an anion-exchange column-FIA system and factorial design study

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This article is dedicated to Prof. Cecilia Sarasola.

Abstract

A study has been performed of the conditions for the reaction of histamine with *o*-phthaldehyde in a flow injection analysis system employing three channels, using an anion-exchange column to eliminate sample matrix interferences. Factorial design was used to determine which operational parameters should be included in the optimization and their optimal values were found. The method developed shows good selectivity for histamine determination in alcoholic beverages. A linear response of up to 2.0 mg l^{-1} was observed and the detection and quantification limits were 30 and 101 μg l^{-1}, respectively. The repeatability, measured by the R.S.D. for 10 replicate injections, was 0.84 and 0.52% for histamine solutions of 0.20 and 2.0 mg l^{-1}, respectively. The recoveries obtained in wine and cider samples were close to 100% and a sample frequency of 24 samples per hour was achieved.

Keywords: FIA; Histamine; Factorial design; Wine; Cider

1. Introduction

The literature on the occurrence of histamine in certain foods and the resulting public health implications has been extensively reviewed [1–4]. Usual methods for histamine determination frequently involve preliminary steps of extraction and chromatography separation to overcome the problem of interfering species, particularly histidine [5,6]. Detection step includes the condensation reaction between histamine and *o*-phthaldialdehide (OPA) and fluorimetric quantitation. Since the discovery of this reaction by Shore et al. [7] many studies have been performed to identify its characteristics. Taylor and Lieber compared the sensitivity and specificity (22 amino acids, 6 peptides and 27 amines tested) of histamine measurement by six fluorimetric assays [8] determining that the one based on Shore method reached the lowest detection limit with the highest selectivity. At equimolecular concentrations of histamine and compounds tested, the fluorescence intensity increased 9% in presence of histidine; from 6 to 0.5% for different histidyl peptides and, considering only the amines occurring in the wine, cadaverine caused the highest increase, of 0.2%. The Shore method, as modified by Michaelson and Coffman [9] was altered slightly by Ough to measure histamine content in California wines [10]. Histidine was separated from histamine by passing the sample, at pH 6.0, through a Dowex 1 × 8 anionic resin, in these conditions histamine passed through the column while the histidine was successfully retained. AOAC method to determine histamine in seafood [11] also uses an anion-exchange column, to the separation of interfering compounds, and subsequent reaction of the eluate with OPA at alkaline pH. This reaction was also satisfactorily applied to determination of histamine in wine and canned tuna samples without a separation step, using derivative synchronous fluorescence spectrometry [12]. The histamine–OPA reaction develops optimally in a basic medium where histamine occurs as a free base, but the determination is performed in acidic medium, where the flu-

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orescence intensity is more stable [13]. The OPA derivative is unstable and the fluorescence intensity diminishes quickly, mainly in alkaline medium, requiring a strict control of reaction time.

A variety of alternative methods exist for the determination of histamine and other biogenic amines in foods and most of them involve chromatography of amine derivatives using HPLC, GC and more recently, capillary electrophoresis [14]. Many of these methods are based on previous derivatization of the amines with OPA and SH-containing reagents in basic medium, in these conditions, amino acids, peptides and amines produce fluorescent compounds [15]. Flow injection analysis (FIA) technique has also been applied to histamine determination. Hungerford et al. reported a method [16,17], which precludes the need for separation steps, the selectivity for histamine versus interfering compounds, appears to be based on differences in the reaction rates with OPA because histamine reacted more quickly than the remainder compounds. The method was applied to fish samples, being adequate for the purpose of screening fish tissues at levels of regulatory interest. Takagi and Shikata developed a new FIA method [18] using a histamine dehydrogenase-based electrode, the FIA system was applied to the determination of histamine in fish samples.

Research on the biogenic amines content in wines has recently gained interest, since the toxicity of these compounds is now better documented and food controls are more frequently required. Among the biogenic amines, histamine plays a special role as an indicator amine and its determination is used, together with bacteriological monitoring, to assess the freshness and quality of several foods. Although no legal limit has been defined for histamine content in wines, a value of 8 mg l^{-1} has been quoted which may induce headaches when large amounts of wine are ingested, and the recommended upper limit for histamine varies from 2 mg l^{-1} in Germany to 10 mg l^{-1} in Switzerland [4].

The total concentration of biogenic amines in wines has been reported to range from a few mg l^{-1} to about 50 mg l^{-1} depending on the quality of wine [4], whilst histamine content varied from 0 to 22 mg l^{-1} in 100 samples of European, North African and Chilean wines [19], and from 0.3 to 15.5 mg l^{-1} in 248 Californian wines with an average value of 2.82 mg l^{-1} [10]. Because of these relatively low quantities, sensitive and selective analytical methods are required and the separation of histamine from matrix interference is frequently a necessary prelude to any analysis based on the reaction between histamine and OPA. The inherent dynamic characteristics of FIA have proven to be well suited to accurate control of timing. Moreover, interference suppression procedures can be implemented on-line improving analytical selectivity and saving time, human intervention and costs.

In this work, a FIA method for the determination of μg l^{-1} of histamine in alcoholic beverages based on its reaction with OPA, was developed. Factorial design was used to establish the optimal conditions for histamine determination. The experimental designs not only determine the influence of the variables to be optimized on the response, but also enable the response function to be obtained and optimized. An anionic-exchange mini-column filled with Dowex 1 × 8 resin in –OH form was examined for on-line separation of interfering species, mainly histidine. The suitability of the proposed method, for its application to the determination of histamine in wine and cider, in terms of accuracy, repeatability and linearity were studied, and the detection and quantification limits were found.

2. Experimental

2.1. Apparatus and software

Fluorescence measurements were performed on a Shimadzu RF-540 spectrofluorimeter (Kyoto, Japan) equipped with a flow-through compact cell (inner volume, 12 μl; pathlength, 1 cm). The four-channel peristaltic pump was a Gilson Minipuls 2 (Worthington, OH, USA) and the injection valve was a Rheodyne RH-5020 rotary Teflon valve (Cotati, CA, USA). The manifold and reaction coil tubing was 0.7 mm i.d. PTFE and was wound around glass tubes with an outside diameter of 5 mm. A water-bath Selecta Tectron Digiterm (Barcelona, Spain) was used for temperature control.

The FIA manifold, schematically shown in Fig. 1, was designed and constructed so that histamine detection can be directly couple with on-line separation of interfering compounds. A sample solution (S) is introduced into the system via the rotary injection valve using H_2O as a carrier and it passes through an anionic-exchange column (AEC), to eliminate interfering compounds. A stream, containing 100 mg l^{-1} *o*-phthaldialdehyde (OPA), 4% ethanol and 150 mM NaOH joins before L_1 and the reaction between the histamine and OPA takes place in an alkaline medium. A second stream containing 40 mM H_3PO_4 joins before L_2. The resulting compound is measured fluorimetrically (λ_{ex}: 355 nm; λ_{em}: 445 nm).

The STATISTICA [20] and SPSS [21] statistical software packages were used for data treatment.

2.2. Reagents, standards, samples

All solutions were prepared with analytical-grade chemicals, unless otherwise stated, and twice distilled water. A stock solution (1000 mg l^{-1}) of histamine was prepared by dissolving 0.1691 g of histamine dihydrochloride (minimum 99%, Sigma, St. Louis, MO, USA) into 100 ml volumetric flask and diluted to volume with 0.1 M HCl (Panreac, Barcelona, Spain). This solution was prepared weekly and stored in a refrigerator at 4 °C. Stock histamine solution was diluted with water to yield standard solutions of 1.0 mg l^{-1} for optimization work and found the calibration line in the range 0.2–2.0 mg l^{-1}. When standard addition calibration was used, the histamine concentrations added were 20.0, 40.0 and 80.0 mg l^{-1} The OPA

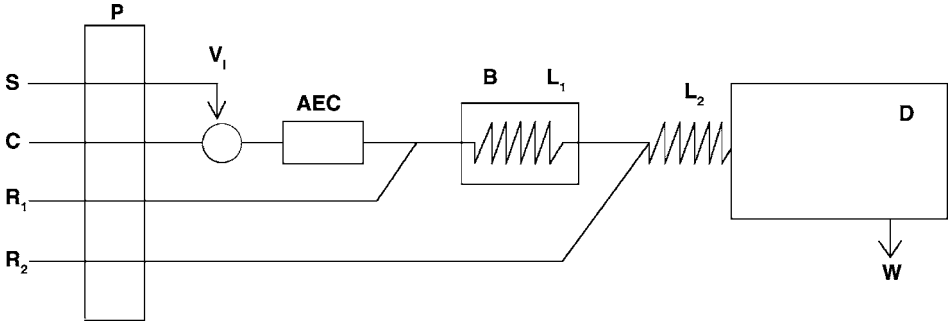

Fig. 1. FIA assembly for determination of histamine. S, sample diluted at pH 6.0; C, H_2O; R_1, 0.01% (w/v) OPA in 4% (v/v) ethanol and 150 mM NaOH; R_2, 40 mM H_3PO_4; P, peristaltic pump (1.5 ml min^{-1}); AEC, anion-exchange column; V_i, injection valve (sample loop 100 μl); B, water bath at 25 °C; L_1 and L_2, reaction coils (500 and 20 cm, respectively); D, fluorimeter equipped with a cuvette of 1.0 cm pathlength (λ_{ex} = 355 nm, λ_{em} = 445 nm); W, waste.

(*o*-phthaldialdehyde) (99%, Sigma–Aldrich, Steinheim, Germany) stock solution (0.1%, w/v) was prepared daily by dissolving the reagent in ethanol (Panreac) and adding 1.0 M NaOH (Panreac) as required. From this stock solution, working solutions in the range 20–120 mg l^{-1} OPA were recently prepared. Phosphoric acid (Panreac) at concentrations from 20 to 200 mM was prepared by dilution of 85% (w/w) concentrate acid. For interference studies L-histidine, tyramine, 2-phenylethylamine and methylamine (as monohydrochlorides), ethylamine (70% aqueous solution), 1-methylhistamine, cadaverine and putrescine (as dihydrochlorides) were supplied by Sigma–Aldrich. Aqueous solutions were prepared in the range 1.0–20.0 mg l^{-1}, excepting histidine which was tested up to 200 mg l^{-1}.

The exchange column was prepared as described Townshed [22]. The strongly basic anion-exchange resin Dowex 1 × 8 (200–400 mesh) in the chloride form (Aldrich, Milwaukee, WI, USA) was used and converted to the form –OH [18]. The resin was filtered off and packed into a polypropylene tube of 7.0 cm length × 2 mm i.d. A thin layer of glass wool was put at both ends of the column.

To evaluate the validity of the method, samples of wine from La Mancha (Spain) and cider from Gipuzkoa (Spain) were used. The samples of white wine only required the appropriate dilution of the sample and usually a dilution of 20 ml up to 50 ml was adequate. The samples of red wine were diluted ten times and filtered through a 0.22 μm nylon membrane micro-filter to retain micro-particulate mater (mainly tannic compounds), which reduced the column lifetime. The cider samples were diluted two times (25 ml up to 50 ml total volume) and de-gasified, if they showed turbidity, they also were filtered.

3. Results and discussion

To improve the performance of the proposed system, both chemical and flow conditions were sequentially optimized. The factorial design was evaluated using the fluorescence intensity as response. In all factorial designs, each experiment was carried out three times by successive injections of the sample and the average value of the experimental response was considered. Firstly, the full factorial design was used as a screening method to determine the significance of the individual experimental parameters and the centre composite design was applied to the significant parameters as an optimization method. All experiments were carried out in random order to eliminate environmental variance. Secondly, the influence of the two, the column length and the particle size of the anionic exchange resin on the selectivity of the procedure, were studied. Finally, to evaluate if the system found had the desired characteristics, its analytical figures of merit were determined.

3.1. Factorial designs

Chemical conditions studied in batch mode showed that the formation of the fluorescent adduct OPA–histamine proceeded more quickly at alkaline medium (pH about 12.5) [7], although it is also produced in aqueous medium [23]. Depending on experimental conditions different reaction times were applied for this reaction, which varied from 3 min [10] up to 17 h [24]. The stability of the fluorescent product varies according to the method of derivatization used [25] and is frequently increased by acidification [26]. Conditions of the AOAC method [11] involve OPA and methanol concentrations into reaction erlenmeyer, about 50 mg l^{-1} and 5%, respectively, and a reaction time of 4 min, without termostatization. From this background, the chemical conditions were firstly studied to screen the significant factors and to determine the experimental domain.

System conditions were fixed as follows: flow rate, 1.50 ml min^{-1}; injection volume, 100 μl; lengths of the reaction coils, 450 and 200 cm for L_1 and L_2, respectively. A full factorial design at two levels was applied. The factors studied included OPA, ethanol, NaOH and H_3PO_4 concentrations. Moreover, three replicates were randomly measured in the centre point to obtain an estimate of the experimental uncertainty, although these measures corresponded only to the centre point, it is considered to be an estimate for the whole experimental domain [27]. In total, 19 experiments were carried out. The experimental values corresponding to the high

Table 1
Experimental design matrix with the chemical parameters and results obtained for the 2^4 full factorial design

Run	OPA (mg l^{-1})	Ethanol (%, v/v)	NaOH (mM)	H_3PO_4 (mM)	Fluorescence intensity
1	20	0.0	100	20	41.0
2	120	0.0	100	20	38.9
3	20	8.0	100	20	43.0
4	120	8.0	100	20	39.6
5	20	0.0	220	20	13.0
6	120	0.0	220	20	12.4
7	20	8.0	220	20	14.2
8	120	8.0	220	20	13.8
9	20	0.0	100	200	18.1
10	120	0.0	100	200	16.9
11	20	8.0	100	200	19.0
12	120	8.0	100	200	17.9
13	20	0.0	220	200	41.2
14	120	0.0	220	200	40.4
15	20	8.0	220	200	43.7
16	120	8.0	220	200	40.2
17	70	4.0	160	110	32.3
18	70	4.0	160	110	30.4
19	70	4.0	160	110	28.9

The 19 runs were randomly performed.

and low levels and the centre points, as well as the fluorescence intensities obtained for each run of the experimental design, are shown in Table 1. Analysis of variance (ANOVA) is a convenient method of analyzing the significance of effects in the analysis of a two-level factorial design, and was applied to design with the results presented in Table 2. ANOVA demonstrated that the only significant factors ($p < 0.05$) were the H_3PO_4 and NaOH concentrations and their interaction.

All concentrations of NaOH (100–220 mM) adjusted the pH of the OPA solutions at values higher than 12.5, even after its dilution when mixed with the carrier stream, this being considered as the optimum pH for histamine determination [7]. Under the experimental conditions fixed, the reaction time was about 1.2 min in L_1 (alkaline medium). After mixing with H_3PO_4, the reaction continued for about 0.5 min in L_2 reaction coil at a lower pH value than the initial one, which varied depending on NaOH and H_3PO_4 concentrations. From the results in Table 1, it can be observed that there are two different series of conditions with high fluorescence intensities: runs 1–4 (low levels for NaOH and H_3PO_4 factors) and 13–16 (high levels for NaOH and H_3PO_4 factors). Evidently, the pH of the reaction is very important, but in the flux conditions of the system is not possible to know exactly the impact of its variation on the kinetics of the reactions involved. Observations in runs 1–4 could be explained by a short advance of the histamine–OPA reaction in L_1 (the low level of NaOH factor should decrease the reaction rate), in L_2 this reaction should continue since the low concentration of H_3PO_4 is not sufficient to significantly decrease the pH value, producing higher fluorophore amount and increasing the fluorescence intensity. The high fluorescence intensity observed throughout runs 12–16 could be the result of a quick reaction in L_1 with a high advance of the reaction (high level of NaOH factor) and a stabilization of the reaction product caused by the decrease of pH up to an acid value (high level of H_3PO_4 factor). Considering that the high concentrations of both, alkali and acid, should attack easier the tubing used in FIA system, more reduced concentrations of H_3PO_4 were fixed in the subsequent assays.

The OPA factor does not affect the fluorescence intensity, indicating that all OPA concentrations tested were in sufficient excess to successfully develop the condensation

Table 2
Analysis of variance for intensity fluorescence with the data in Table 1

Factor	Sum of squares	Degrees of freedom	Mean squares	F ratio	p Level
(1) OPA	10.726	1	10.726	3.8799	0.0844
(2) Ethanol	5.641	1	5.641	2.0404	0.1910
(3) NaOH	15.016	1	15.016	5.4318	0.0481[a]
(4) H_3PO_4	28.891	1	28.891	10.4509	0.0120[a]
(1) × (2)	0.856	1	0.856	0.3095	0.5932
(1) × (3)	0.391	1	0.391	0.1413	0.7168
(1) × (4)	0.001	1	0.001	0.0002	0.9884
(2) × (3)	0.006	1	0.006	0.0020	0.9651
(2) × (4)	0.076	1	0.076	0.0274	0.8727
(3) × (4)	2567.956	1	2567.956	928.9324	0.0000[a]

[a] Significant factor at $p < 0.05$.

reaction with histamine. Within the experimental range studied, OPA and ethanol concentrations were not significant factors, and their values were maintained at 100 mg l^{-1} and 4% (v/v), respectively in the subsequent assays. This concentration of 100 mg l^{-1} OPA was chosen by considering that, after its dilution when mixed with the carrier stream, the resulting concentration should be about the half, 50 mg l^{-1}, this being the value fixed in the AOAC method [11]. Although at tested concentrations, ethanol scarcely affected the fluorescent intensity, it showed a favourable effect on the OPA solubilization and on the solution stabilization, as well as on the repeatability of the FIA signals, and a 4% concentration was considered to be a convenient value.

To optimize NaOH and H_3PO_4 concentrations a central composite design (CCD) was applied. This design is especially useful because it provides sufficient factor combinations to fit the full second order polynomial model and this model can be used to approximate almost any smooth surface over a limited domain. CCD consisted of a two-level factorial design (2^2), added with four star points situated at a distance ± 1.414 from the centre of the design, and three replicates at the centre. The design was rotatable, this means that the variance of the prediction does not depend on the direction in which one looks starting from the centre point, but only on the distance from the centre point [27]. The experimental design matrix and corresponding responses for fluorescence intensity are shown in Table 3. The results of ANOVA, included in Table 4, confirmed that the most significant factor influencing on the response was [NaOH] × [H_3PO_4] and the term [NaOH]^2 was also significant ($p < 0.05$) in the model. The R^2 value was 0.902 indicating that the model explained 90.2% of the variability in the response. The 3D plot of the response surface in Fig. 2 clearly demonstrates the effect (both magnitude and direction) of the interaction between the two parameters considered. Fluorescence intensity increased at NaOH concentration about 150 mM and low H_3PO_4 concentrations, and also at high concentrations of both reagents, underlying the importance of the reaction pH on its rate and thus on the fluorescence. Table 3 shows that the highest fluorescence value corresponded at run 1, if we consider the concentrations of NaOH and H_3PO_4 in this assay, the pH in L_1 should be about 12.9 and in L_2 the pH should correspond to buffer $H_2PO_4^-/HPO_4^{2-}$, being about 8.0. Fig. 2 indicates a second direction to found a high fluorescence, according to the results obtained in the screening step, this corresponds to more concentred alkaline and acid solutions. Because less concentrated solutions were preferred, the con-

Table 3
Experimental design matrix with the chemical parameters and results obtained using central composite design

Run	NaOH (mM)	H_3PO_4 (mM)	Fluorescence intensity
1	150	40	73.9
2	150	120	35.0
3	250	40	29.1
4	250	120	57.3
5	130	80	37.6
6	270	80	46.5
7	200	20	58.9
8	200	140	57.7
9	200	80	58.8
10	200	80	56.5
11	200	80	57.6

The 11 runs were randomly performed.

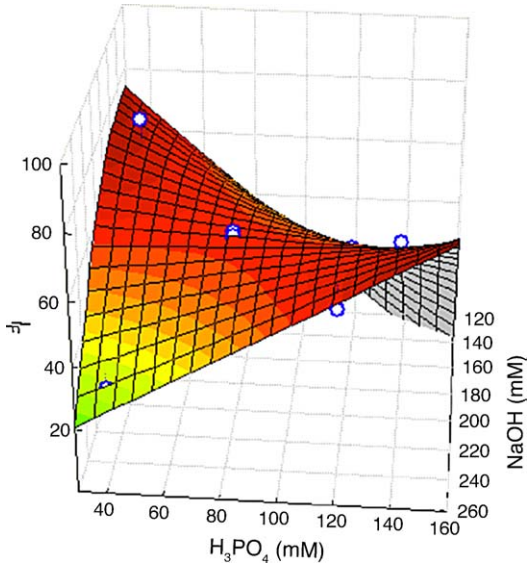

Fig. 2. 3D plot of the response surface for significant chemical parameters estimated using the central composite design.

Table 4
Analysis of variance for intensity fluorescence with the data in Table 3

Factor	Sum of squares	Degrees of freedom	Mean squares	F ratio	p Level
(1) NaOH (L)	12.727	1	12.727	0.3747	0.5672
NaOH (Q)	366.169	1	366.169	10.7809	0.0219[a]
(2) H_3PO_4 (L)	18.382	1	18.382	0.5412	0.4950
H_3PO_4 (Q)	0.038	1	0.038	0.0011	0.9746
(1) × (2)	1125.603	1	1125.603	33.1404	0.0022[a]
Error	169.823	5	33.965		
Total sum of squares	1735.796	10			

[a] Significant factor at $p < 0.05$. (L) Linear term; (Q) quadratic term.

Table 5
Experimental design matrix with the FIA parameters and results obtained for the 2^4 full factorial design

Run	Q (ml min^{-1})	L_1 (cm)	L_2 (cm)	V_i (μl)	Fluorescence intensity
1	1.0	250	20	30	33.0
2	2.0	250	20	30	27.6
3	1.0	450	20	30	48.8
4	2.0	450	20	30	39.2
5	1.0	250	400	30	18.4
6	2.0	250	400	30	16.7
7	1.0	450	400	30	23.7
8	2.0	450	400	30	20.8
9	1.0	250	20	130	58.2
10	2.0	250	20	130	49.0
11	1.0	450	20	130	86.5
12	2.0	450	20	130	72.6
13	1.0	250	400	130	41.0
14	2.0	250	400	130	36.8
15	1.0	450	400	130	53.2
16	2.0	450	400	130	46.3
17	1.5	350	210	80	52.8
18	1.5	350	210	80	53.5
19	1.5	350	210	80	50.1

The 19 runs were randomly performed.

centrations fixed were 150 mM for NaOH and 40 mM for H_3PO_4.

To find the significant FIA parameters and provide a measurement of their effect, the flow rate (Q), the lengths of the reaction coils (L_1 and L_2) and the sample volume injected (V_i) were considered. The i.d. of the tubes for all the solutions was constant in the experiments. A two-level full factorial 2^4 design with three centre point replicates was carried out. The experimental design matrix and the response for each run are shown in Table 5. The data obtained was evaluated by ANOVA and the statistical results are included in Table 6. The main effect of each factor was determined using the relationship $E_x = (\Sigma y+ - \Sigma y-)/2^{k-1}$, where $y+$ and $y-$ are the responses at higher (+) and lower (−) levels, respectively, and k the number of factors [27]. Injection volume was the most important parameter, with a positive effect of 26.9. The length of L_2 had a negative effect (−19.8). This can be explained considering that the reaction of the fluorophore (formed in L_1) with H_3PO_4 (joined just after L_1) is very fast and the increase of L_2 length increased the dispersion of the injected sample zone, decreasing the height peak. Within the examined range, the L_1 length had a positive effect (13.8), in L_1 proceeds the condensation reaction histamine–OPA, and its length determines the reaction time, thus the response increased as reaction time increased. The flow rate had a negative effect (−6.7), which can also be explained by its effect on the reaction time, but this factor was not significant ($p < 0.05$) within the experimental range examined, and a value of 1.5 ml min^{-1} was set in further measurements. As can also be seen in Table 6, the effects of the interactions were negligible.

Table 7
Experimental design matrix with the FIA parameters and results obtained using the design central composite

Run	L_1 (cm)	L_2 (cm)	V_i (μl)	Fluorescence intensity
1	250	50	50	45.6
2	450	50	50	59.2
3	250	150	50	35.5
4	450	150	50	50.7
5	250	50	130	60.6
6	450	50	130	68.4
7	250	150	130	61.0
8	450	150	130	57.2
9	182	100	90	54.6
10	518	100	90	49.5
11	350	16	90	75.8
12	350	184	90	64.5
13	350	100	23	21.2
14	350	100	150	59.3
15	350	100	90	69.0
16	350	100	90	68.8
17	350	100	90	69.1

The 17 runs were randomly performed.

The central composite design, in Table 7, was used to optimize V_i and the lengths of L_1 and L_2. Star points spread the experimental range up to ±1.64 from centre levels. The lower and upper levels for L_1 were maintained in 250 and 450 cm, respectively, whilst the experimental domain for L_2 was now reduced owing to the negative effect of this factor. Also the experimental domain for V_i was changed to can include a minimum positive value corresponding to star point at −1.64. ANOVA results (Table 8) showed as significant

Table 6
Analysis of variance for intensity fluorescence with the data in Table 5

Factor	Sum of squares	Degrees of freedom	Mean squares	F ratio	p Level
(1) Q	180.903	1	180.903	5.2089	0.0519
(2) L_1	761.760	1	761.760	21.9339	0.0016[a]
(3) L_2	1560.250	1	1560.250	44.9255	0.0002[a]
(4) V_i	2899.822	1	2899.822	83.4968	0.0000[a]
(1) × (2)	10.240	1	10.240	0.2949	0.6019
(1) × (3)	31.360	1	31.360	0.9030	0.3698
(1) × (4)	13.323	1	13.323	0.3836	0.5529
(2) × (3)	145.202	1	145.202	4.1809	0.0751
(2) × (4)	84.640	1	84.640	2.4371	0.1571
(3) × (4)	25.000	1	25.000	0.7198	0.4209

[a] Significant factor at $p < 0.05$.

Table 8
Analysis of variance for intensity fluorescence with the data in Table 7

Factor	Sum of squares	Degrees of freedom	Mean squares	*F* ratio	*p* Level
(1) L_1 (L)	43.034	1	43.134	1.7187	0.2312
L_1 (Q)	343.202	1	343.202	13.7072	0.0076[a]
(2) L_2 (L)	171.568	1	171.568	6.8523	0.0345[a]
L_2 (Q)	9.894	1	9.894	0.3952	0.5496
(3) V_i (L)	879.238	1	879.238	35.1160	0.0006[a]
V_i (Q)	1071.099	1	1071.099	42.7788	0.0003[a]
(1) × (2)	12.500	1	12.500	0.4992	0.5027
(1) × (3)	76.880	1	76.880	3.0705	0.1232
(2) × (3)	7.605	1	7.605	0.3037	0.5987
Error	175.267	7	25.038		
Total sum of squares	2983.921	16			

[a] Significant factor at $p < 0.05$. (L) Linear term; (Q) quadratic term.

effects ($p < 0.05$): V_i^2, V_i, L_1^2 and L_2, and the R^2 value indicates that the model explained 94.1% of the variability in the response. The response surfaces keeping one of the variables fixed (Fig. 3), provided a maximum of fluorescence intensity at injection volume: 100 μl, L_1: 500 cm and L_2: 20 cm.

Histamine determination is usually carried out at room temperature in order to simplify the procedure. This is possible because the reaction for fluorophore formation occurs at a convenient rate on interval 15–27 °C [12]. Therefore, this variable was not included in the experimental designs. To determine its effect upon fluorescence intensity a study was performed using temperatures of 25, 30, 35, 40, 50 and 60 °C with the remaining parameters fixed in the optimal values previously found. Temperature proved to be a relevant factor, increasing the water bath temperature within 25 and 35 °C, fluorescence intensity decreased at a rate of 0.2 units $°C^{-1}$, but at temperatures higher than 35 °C, the decrease rate was higher (2.3 units $°C^{-1}$), decreasing analytical sensitivity and increasing the bubble formation along the system, consequently the selected temperature was 25 °C.

The resulting conditions for histamine determination are shown in Fig. 1.

3.2. Incorporation of the anionic-exchange column

In the histamine determination in foods, specificity rather than sensitivity has been the real problem. Because histidine is known to be the major interference in the OPA–histamine reaction, this compound was chosen to study the effect of column parameters on the separation of interfering species. Two lots of five columns were prepared having lengths of 3.0, 5.0, 7.0, 9.0 and 11.0 cm, all with an internal diameter of 2 mm. A lot was filled with a Dowex 1 × 8, 200–400 mesh resin, whilst another lot was filled with identical resin, but 50–100 mesh. A solution of 1 mg l^{-1} histamine was spiked with different concentrations (20, 40, 80 and 200 mg l^{-1}) of histidine and reanalyzed. The best results were obtained using a column length of 7.0 cm and 200–400 mesh resin, this column retained up to 200 mg l^{-1} of histidine, exceeding the expected histidine content in wine samples. Other species tested were: tyramine, 2-phenylethylamine, methylamine, ethylamine, 1-methylhistamine, cadaverine, and putrescine in presence of 1 mg l^{-1} histamine. None of them interfered, at least at a level of 20 times the histamine concentration. The dispersion coefficient (D) of the flow system was determined by the injection of 1 mg l^{-1} histamine, with and without the column present. The D value increased only 2.0% when the column was incorporated in the system. The column was reactivated daily by passing a solution through it that contained 0.1 M NaOH at a flow rate of 2 ml min^{-1}, for 5 min and washing with water at an equal flow rate until a stable baseline was obtained. These operations were analogous to those practiced with the classical columns [11] and mini-columns [22,28] and the recuperation of the resin was effective, each column permitting about 600 injections.

3.3. Analytical features

The standard calibration (SC), standard addition calibration (AC) and Youden calibration (YC) were used to check the accuracy of the analytical results as no reference material was available [29,30]. From the data set obtained for standard calibration, linearity, analytical sensitivity, precision and detection and determination limits for analytical method could be determined. Standard addition calibration was obtained by addition of continuous variations of standard at constant sample volume, including the value of zero addition. The slopes of the lines obtained by the SC and AC calibrations are compared using the t-test. If the difference between the two slopes is significant, then a component of proportional bias is involved, but if this difference is not significant, the recovery studies from histamine standard additions to the samples can be used to evaluate the applicability of the method. Youden calibration was obtained by addition of continuous sample variations without inclusion of the volume zero sample. The blank measured through the Youden regression gives the constant component of bias. If the value for YC intercept is included in the confidence interval value

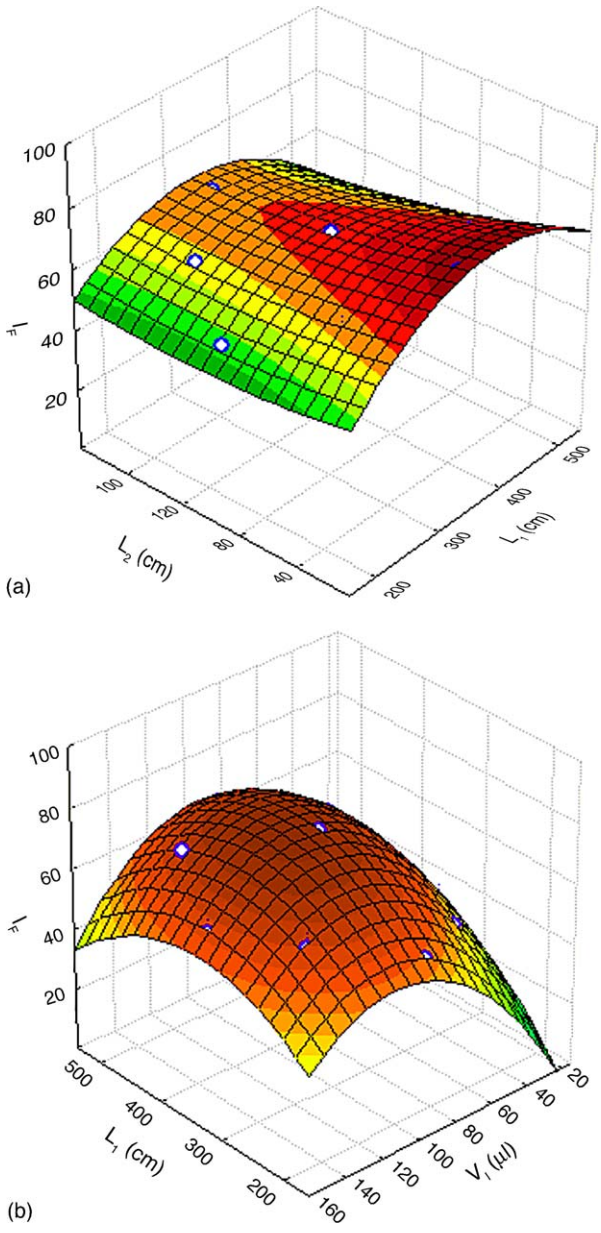

Fig. 3. 3D plot of the response surface for significant FIA parameters estimated using the central composite design, keeping one of the variables fixed: (a) V_i, 100 μl and (b) L_2, 20 cm.

of SC intercept, no difference between both values is found [30] that would indicate absence of systematic error due to the matrix components. By application of SC, AC and YC methods both the constant and proportional errors can be detected and can be corrected for the sample under analysis.

Parameters for SC, AC and YC are shown in Table 9. A set of eleven standard solutions with histamine concentration between 0.20 and 2.00 mg l^{-1} was used to build the calibration line which showed good linearity in the range studied ($R^2 = 0.999$).

Four samples (white and red wines and ciders) were taken as representative matrices containing different levels of histamine [31–33] and known quantities of histamine standard solution were added in triplicate at two levels (Tables 9 and 10). The slopes of the lines obtained were compared with the slope of the regression line obtained in the calibration with standards (t criterion). No significant differences were found between them ($p < 0.05$). The samples used to find the YC lines were prepared by dilution of 1, 2, 4 and 6 ml of a white wine, or 10, 15, 20 and 25 ml of a cider, up to 50 ml in volumetric flasks. Table 9 shows the intercepts of the YC lines for wine and cider and they are included in the confidence interval (CI) of SC intercept (95% CI: −8.999 to +0.323), proving the absence of a constant component of the error.

The accuracy of the FIA method was tested by the standard addition technique. Analytical recoveries were calculated by comparing the results obtained from SC, before and after standard histamine additions. Table 10 shows the data for the recovery of histamine in wine and cider samples measured in triplicate. Recoveries near 100% were obtained for histamine, indicating that the proposed method is suitable to analyse these samples.

The detection limit was calculated according IUPAC [34] as the histamine concentration that provided a signal equal to the blank plus three times its S.D. The blank signal was measured from 12 injections of water which were distributed in four blocks of three injections performed in a day. The blank signal was not equal to zero; however, blanks measured in absence of the ionic exchange column presented not signals, therefore, the column was the main cause of the blank. This is according to previously reported observations [10,11]. The detection limit was 30 μg l^{-1}. Quantification

Table 9
Parameters for SC, standard calibration; AC, standard addition calibration and YC, Youden calibration for the histamine determination in wines and ciders

Sample	Method	Regression line parameters				
		N	Intercept	Slope	s_r	R^2
Standards	SC	12	−0.288 ± 0.301	79.05 ± 0.27	1.042	0.999
White wine A	AC	3	33.64 ± 0.40	78.67 ± 0.78	0.760	0.999
Red wine B	AC	3	46.14 ± 0.66	79.21 ± 1.28	1.251	0.998
Cider C	AC	3	25.39 ± 0.56	78.33 ± 2.32	1.137	0.994
Cider D	AC	3	21.65 ± 0.41	79.42 ± 1.57	0.769	0.997
White wine E	YC	4	0.173 ± 0.478	8.99 ± 0.13	0.843	0.998
Cider F	YC	4	−0.147 ± 0.896	1.96 ± 0.05	0.944	0.994

N, number of samples measured in triplicate.

Table 10
Histamine recoveries (%±S.D.) in wines and ciders measured in triplicate

Sample	Dilution factor	Histamine ($mg\,l^{-1}$)			
		Added	Found	%Recovery	Found in sample
White wine A	2.5	0.00	0.429		1.07 ± 0.03
		0.40	0.818	97.3 ± 2.1	
		0.80	1.227	99.7 ± 1.2	
Red wine B	10	0.00	0.586		5.80 ± 0.11
		0.40	0.992	101.5 ± 5.8	
		0.80	1.387	100.2 ± 1.7	
Cider C	2.0	0.00	0.322		0.64 ± 0.04
		0.20	0.529	103.3 ± 5.5	
		0.40	0.718	99.1 ± 2.7	
Cider D	2.0	0.00	0.274		0.55 ± 0.02
		0.20	0.482	104.4 ± 4.8	
		0.40	0.678	101.0 ± 2.2	

limit was $101\,\mu g\,l^{-1}$, it was calculated using a signal equal to the blank plus 10 times its S.D. The repeatability, measured by the relative standard deviation of replicate injections ($n = 10$) presented values of 0.84 and 0.52%, for histamine solutions of 0.20 and $2.00\,mg\,l^{-1}$, respectively. The sampling rate obtained was $24\,h^{-1}$.

4. Conclusion

A new, fast and simple FIA method with low operating costs is proposed for the determination of histamine in wine and cider. The FIA system includes an anion-exchange mini-column to eliminate interfering compounds. Application of factorial design allowed the optimization of chemical and flow conditions. The proposed method shows high selectivity and a low limit of detection and was successfully applied to the determination of histamine in wine and cider samples.

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