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A sensitive flow analysis system for the fluorimetric determination of low levels of formaldehyde in alcoholic beverages

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Abstract

A sensitive FIA method was developed for the selective determination of formaldehyde in alcoholic beverages. This method is based on the reaction of Fluoral-P (4-amine-3-pentene-2-one) with formaldehyde, leading to the formation of 3,5-diacetyl-1,4-dihydrolutidine (DDL), which fluoresces at $\lambda_{ex} = 410$ nm and $\lambda_{em} = 510$ nm. The analytical parameters were optimized by the response surface method using the Box–Behnken design. The proposed flow injection system allowed for the determination of up to 3.33×10^{-5} mol L^{-1} of formaldehyde with R.S.D. < 2.5% and a detection limit of 3.1 ng mL^{-1}. The method was successfully applied to determine formaldehyde in alcoholic beverages, without requiring any sample pretreatment, and the results agreed with the reference at a 95% confidence level by paired *t*-test. In the optimized condition, the FIA system proved able to analyze up to 60 samples/h.

Keywords: Formaldehyde determination; Alcoholic beverages; Fluoral-P; Box–Behnken design

1. Introduction

In recent years, efforts have focused increasingly on quantifying carbonyl compound levels in alcoholic beverages [1–11]. The importance of such analyses is understandable in view of the known toxicity of several aldehydes, including formaldehyde, acetaldehyde, acrolein and benzaldehyde [12–14]. In this context, information regarding aldehyde profiles may be a valuable tool in assessing the authenticity and/or aging conditions of different alcoholic beverages [15] since aldehydes are extracted from wood into alcoholic beverages during the aging process, thereby contributing to their final flavor [5].

The formaldehyde level is important to evaluate the quality of alcoholic beverages, for it presents toxic activity at levels above 16.65×10^{-5} mol L^{-1} [16]. Formaldehyde can be formed during the alcoholic fermentation process, or it can occur due to aldehyde contamination when plastic bottles are employed.

The development of automatic methods for formaldehyde determination in spirits is important in view of the growing interest of Brazilian government agencies to establish chemical profiles and markers that differentiate Brazilian *cachaça* (sugar-cane brandy) from other types of beverages such as rum.

Several methods have been developed for aldehyde determination, including color reaction with chromotropic acid [17,18], pararosaniline-bisulfite (Schiff reagent) [19,20], malachite green-bisulfite [21,22], brilliant green-bisulfite [23], enzymatic methods [8–10], liquid chromatography by derivatization with 2,4-dinitrophenylhydrazine [24–28], reaction with 3-methyl-2-benzothiazolone hydrazone (MBTH) [29–33] and reaction with Fluoral-P [1,11,34–43].

The chromotropic acid method employs concentrated sulfuric acid (>85%), which increases the viscosity of reaction media, making its application in flow systems difficult. Spectrophotometric methods based on the reaction with pararosaniline, malachite green or brilliant green associated with HSO_3^- are subject to interference from low levels of ethanol [21–23], making them unsuitable for determining formaldehyde in alcoholic beverages.

The standard method for determining carbonyl compounds in atmospheric air samples is HPLC, using 2,4-dinitrophenilhydrazine (2,4-DNPH) as the chromogenic reagent and spectrophotometric detection at 365 nm, which offers excellent sensitivity and selectivity [24–28]. Nonetheless, the development of automatic flow systems using 2,4-DNPH without the

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chromatographic separation step is considered unfeasible, since the reagent and reaction products have absorption maxima in the same wavelength range. The MBTH method has been employed for the determination of total aliphatic aldehydes in several samples [29–33]. On the other hand, the MBTH method is not selective for formaldehyde determination and several other aliphatic aldehydes can interfere in this determination.

The Fluoral-P method [34-43] is based on the reaction of this compound (4-amino-3-penten-2-one) with formaldehyde, producing 3,5-diacetyl-1,4-dihydrolutidine (DDL). When excited at 410 nm, DDL fluoresces at 510 nm. Although Fluoral-P presents reactions similar to those of other aliphatic aldehydes, the reaction product with formaldehyde is the only one that produces a high fluorescent emission. Thus, the Fluoral-P method is specific for formaldehyde [1,11,34–43], allowing for the determination of this analyte even in the presence of acetaldehyde concentrations 1000 times higher than formaldehyde [1].

In this work, a flow injection system was developed for the selective determination of formaldehyde in different alcoholic beverages. The proposed flow system was based on the reaction of formaldehyde with Fluoral-P with the selective production of a fluorescent compound (λ_{ex} = 410 nm and λ_{em} = 510 nm).

Because many parameters related to the flow system can affect the fluorescent response, a multivariate optimization approach was adopted. This optimization strategy presents several advantages such as: (a) valuable information is obtained with fewer experiments than when using traditional one-factor-at-a-time optimization; (b) a mathematical model that describes the dependency of the experimental response and evaluated parameters is obtained; (c) it provides information regarding the interactions between the factors under study; (d) even if optimal conditions are not located in the evaluated domain, the mathematical model can indicate the optimal direction and steepest ascent method that can be used.

2. Experimental

2.1. Reagents and solutions

All the solutions were prepared from analytical grade reagents using distilled and deionised water with a resistivity greater than 18 MΩ cm^{-1}.

The Fluoral-P solution was prepared by reacting 0.2 mL of acetylacetone with 15.4 g of ammonium acetate in the presence of 0.3 mL of acetic acid. The volume was adjusted to 100 mL with distilled water.

A 0.033 mol L^{-1} formaldehyde stock solution was prepared by diluting 2.5 mL of 37% formaldehyde solution with distilled water and standardized by 2,4-DNPH/HPLC method [25].

Samples of alcoholic beverages were purchased in the local market and analyzed by the proposed flow system without any further pretreatment.

2.2. Apparatus

A Rheodyne, USA, model 5020 six-port rotary injection valve was employed for sampling aliquots in the flow path and a Minipuls 3 (Gilson, France) peristaltic pump was utilized for fluid propulsion. Polyethylene and PTFE tubes (0.8 mm i.d.) were used in the flow systems as peristaltic and connection tubes, respectively. A Spectra-Physics FS-970D-A1 fluorimeter whose excitation wavelength was adjusted at 410 nm was used with a high-pass filter (which transmits at wavelengths above 440 nm, transmittance at 440 nm being 0.8) to collect maximum DDL fluorescent emission at 510 nm. Bathwise fluorescence determinations were carried out with a spectrofluorimeter (Jasco, model FP-777) equipped with a 1.0 cm quartz cell. An Intralab 4290 integrator was used to record transient signals produced by the FIA system.

Statistica 6.0 (Statsoft, USA) was employed for calculations related to multivariate response surface optimization.

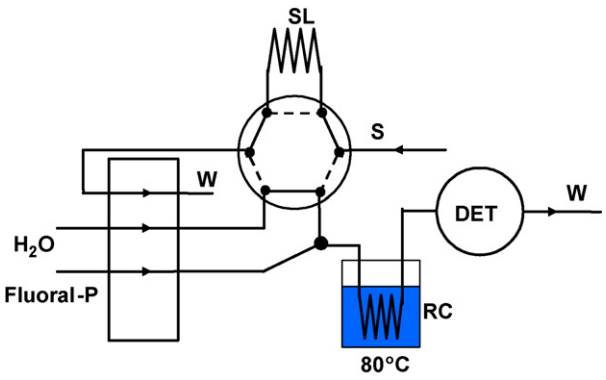

Fig. 1. Flow manifold for the fluorimetric determination of formaldehyde in alcoholic beverage samples. S = sample, SL = sampling loop, RC = reaction coil, DET = fluorimetric detector (λ_{ex} = 410 nm and λ_{em} = 510 nm), W = waste. Flow rate = 0.95 mL min^{-1} and sample volume = 414 μL.

2.3. Flow system

Fig. 1 shows a schematic diagram of the flow manifold. In the FIA manifold an aliquot of 414 μL of alcoholic beverage sample solution was inserted into the flow system and the Fluoral-P reagent was continuously added by confluence. The mixture was then directed to a reaction coil heated in a thermostatic bath at 80 °C in order to improve the sample/reagent mixture and reaction rate, then to the fluorimetric detector (λ_{ex} = 410 nm and λ_{em} = 510 nm) where the signal was acquired and recorded by an integrator.

3. Results and discussion

3.1. Effect of reaction coil temperature

Previous work showed that, in the batch condition, the highest and most stable fluorescent responses were obtained several hours after the addition of reagent at room temperature (25 °C) or after 20 min of sonication [1]. To reduce the long time interval required to complete the reaction of formaldehyde with Fluoral-P, a reaction coil was inserted into a thermostatic water bath to heat the Fluoral-P—sample segment and thus accelerate DDL formation.

The effect of heating time on fluorescent emission was evaluated and the heating temperature of 80 °C was selected, since higher temperatures did not significantly increase the analytical signals and led to the formation of air bubbles.

Because temperature affects the fluorescence emission of several compounds, the addition of a cooling coil before the fluorescence detector was evaluated. The use of the cooling coil led to a 6% increase in the analytical signal, and this cooling step was not employed in further experiments due to the low gain in sensitivity and the longer analysis time interval.

3.2. Optimization by response surface method

In this work, we employed the response surface method and Box–Behnken [44,45] design to optimize the sensitivity of the proposed flow system. The Box–Behnken [44,45] design can be considered a highly fractionalized three-level factorial design where the treatment combinations are the midpoints of edges of factor levels and the center point. These designs are rotatable (or nearly rotatable) and require three levels of each factor under study. Like other designs such as central composite [45] and Doehlert [46], Box–Behnken designs can fit full quadratic response surface models and offer advantages over other designs.

The advantages of the Box–Behnken design over other response surface designs are: (a) it needs fewer experiments than central composite design and similar ones used for Doehlert designs; (b) in contrast to central composite and Doehlert designs, it has only three levels; (c) it is easier to arrange and interpret than other designs; (d) it can be expanded, contracted or even translated; and (e) it avoids combined factor extremes since midpoints of edges of factors are always used.

To optimize the sensitivity of the proposed flow system, a multivariate optimization design was adopted to evaluate the influence of the flow rate ($flow_R$), reaction coil length (l_{RC}), and sampling loop length (l_{SL}) on the analytical signal. The univariate evaluation of these flow parameters can be difficult for, although the sampling loop and reaction coil length ratios are closely related to the mixture condition and sample zone dispersion, the flow rate determines the residence time of the sample zone in the heating bath, so interactions among these parameters can occur.

A Box–Behnken design was employed for multivariate optimization, and the levels of the evaluated variables are presented in Table 1. The response surface obtained by Box–Behnken design (Fig. 2) was described by the equation $S = 16.5 + 2.5l_{RC} - 1.7l_{RC}^2 + 1.6l_{SL} - 1.8l_{SL}^2 - 2.7\,flow_R - 1.4\,flow_R^2$, with the variables at coded levels, revealing a parabolic response surface whose maximum was located within the evaluated domain. The equation obtained by the response surface method revealed that linear and quadratic terms were significant while interaction terms were not.

Table 1
Experimental levels employed for Box–Behnken optimization design

Variable	Coded variable		
	(−1)	(0)	(+1)
Sampling loop (cm)	20	55	90
Reaction coil (cm)	55	125.5	200
Flow rate (mL min^{-1})	0.90	1.75	2.60

The optimized values of flow variables obtained by the Box–Behnken method were calculated by Statistica 6.0 using derivative techniques [44,46]. Flow system optimal conditions were attained using a sampling loop with 82.4 cm, reaction coil with 158.0 cm and flow rate at 0.95 mL min^{-1}. The optimization methodology allowed for maximization of the analytical sensitivity with only 15 experiments.

3.3. Interference study

3.3.1. Evaluation of ethanol level

The ethanol level in alcoholic beverages can be related to the type of beverage (cachaça, rum, wine, vodka and others), to the producer, and to variations in production processes. Thus, the proposed method must be insensitive to ethanol variations within the range expected for this kind of sample.

The effect of the ethanol level in samples was evaluated, since variations in solvent composition could lead to fluctuating fluorescence signals. Additionally, flow systems with poor mixing conditions can lead to strong solvent concentration gradients when an alcoholic sample is inserted into an aqueous carrier stream, resulting in the occurrence of a liquid lens in the ethanolic/aqueous medium interface [47] due to differences in the refraction indexes. This phenomenon, known as the Schlieren effect [47], is well described in light absorption measurements, but there is little information about its extension to fluorescence and how it affects signal repeatability.

The results obtained by these experiments showed that the ethanol level had a non-significant effect on the fluorescent signal magnitude and repeatability up to 50% (v/v) of ethanol. The observation of non-significant Schlieren effect may be ascribed to the fact that the excitation beam diffracted by a liquid lens was blocked by a high-pass filter positioned in front of the photomultiplier. Additionally, the geometry of the fluorimeter and flow cell produces an incident/excitation beam angle of 45° in relation to the collected fluorescent emission beam, and diffraction of the excitation beam by Schlieren's effect at such a high angle is not expected to occur.

Hence, the results indicated that the proposed system is insensitive to ethanol levels within the evaluated range, and is suitable for determining formaldehyde in the alcoholic media of beverages without requiring analyte/ethanol separations.

3.3.2. Effect of acetaldehyde level

Aliphatic aldehydes are expected to react with Fluoral-P, producing colored species [38]. On other hand, the literature [1,11,34–43] has shown that the fluorimetric determination of formaldehyde using Fluoral-P as reagent is almost specific, since the product of the reaction of Fluoral-P with other aldehydes does not present a significant fluorescent signal. Despite the selectivity of the Fluoral-P fluorimetric method for formalde-

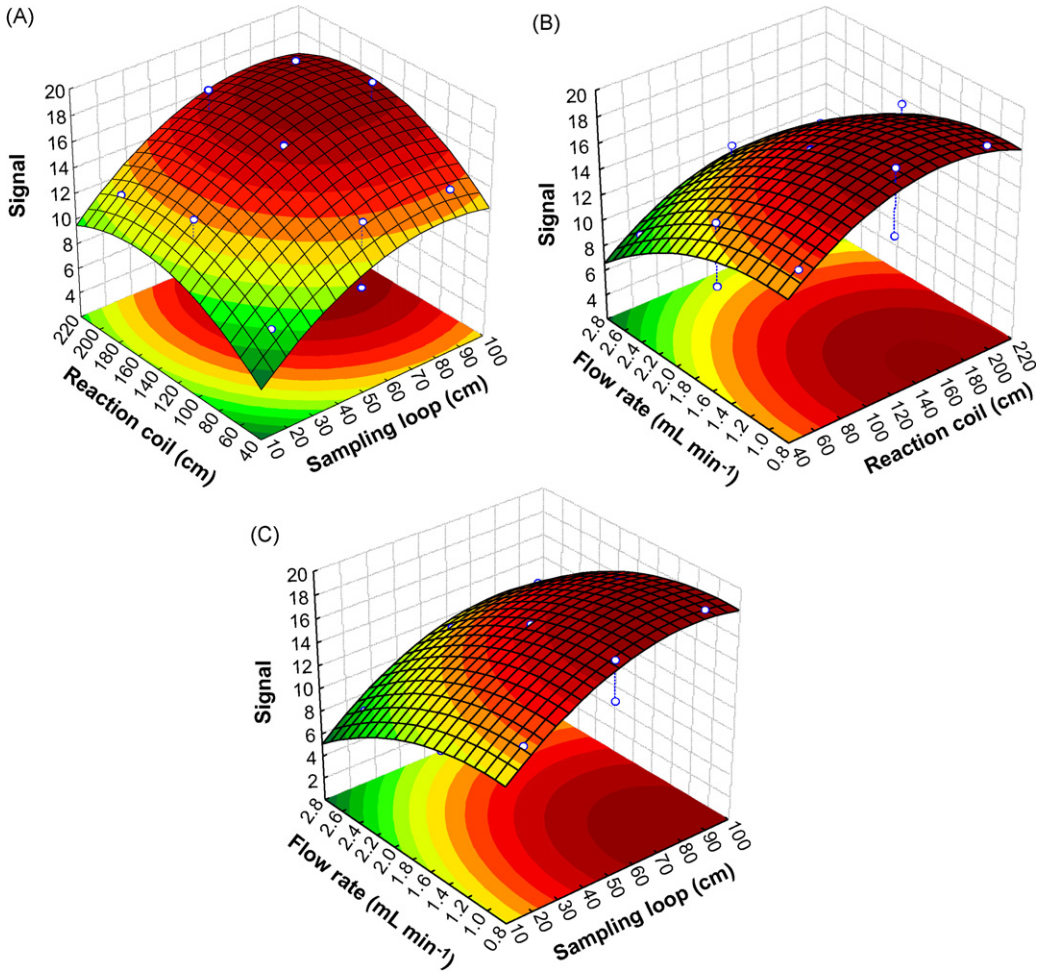

Fig. 2. Response surfaces obtained by the Box–Behnken design for optimization of the flow parameters: (A) sampling loop and reaction coil, (B) reaction coil and flow-rate, and (C) flow-rate and sampling loop.

hyde determination, high levels of other aliphatic aldehydes could lead to interferences due to Fluoral-P consumption and absorption of analyte fluorescent radiation by reaction products of these interferents.

Acetaldehyde is the prevalent carbonyl compound in alcoholic beverages, so interference studies were conducted to evaluate the maximum tolerable level of this compound that would still allow for formaldehyde to be determined accurately.

The results of these experiments indicated that the proposed method is selective for formaldehyde in the presence up to 0.0033 mol L^{-1} of acetaldehyde.

3.3.3. *Effect of bisulfite level*

Bisulfite and S(IV) oxides form a strong oxidation-resistant adduct with formaldehyde, hydroxymethanesulfonate (HMSA) [36]. The kinetics of the decomposition of HMSA is slow at the optimum pH for the formation of DDL, and problems in determining bisulfite-bound formaldehyde are expected to occur. Moreover, the presence of bisulfite is expected in some alcoholic beverages such as wines and beer, since it is employed as antioxidant. Thus, the interference of S(IV) in the determination of formaldehyde by the Fluoral-P method was evaluated.

The results obtained by the proposed flow system revealed that bisulfite levels above 1.0 mg L^{-1} resulted in recoveries of less than 95%. Therefore, the elimination of bisulfite interference through the addition of H_2O_2, followed by alkalinization with NaOH solution [35], must be carefully evaluated to determine total formaldehyde in alcoholic beverages preserved with bisulfite.

3.4. *Figures of merit*

The proposed procedure presented good precision with a relative standard deviation of less than 2.5% for all determinations and an analytical throughput of 60 samples/h. The formaldehyde detection limit was 3.1 ng mL^{-1} and was calculated as three times the standard deviation of the blank signal from 10 replicates divided by the slope of the analytical curve [48]. The linear range for formaldehyde determination was obtained up to 3.33×10^{-5} mol L^{-1} ($S = (40.6 \pm 0.3)$ C (mg L^{-1}) $+ 0.7 \pm 0.1$, $R = 0.9998$).

Table 2 compares the proposed method against other Fluoral-P based fluorimetric methods for determining formaldehyde in alcoholic and other liquid phase samples. The proposed method presents a higher analytical throughput, lower sample/reagent

Table 2
Comparison of the performance of the proposed flow system and other Fluoral-P based methods for determining formaldehyde

	Bathwise method [1]	Previous flow based method [42]	Present work
Reagent volume per determination (mL)	9	2–3	1–2
Sample volume per determination (μL)	1000	25	414
Analytical throughput (sample h^{-1})	2	12	60
Average R.S.D.%	1.7	0.2	2.02
Limit of detection (ng mL^{-1})	2.0	15	3.1

Table 3
Results obtained by the FIA procedure and the batchwise method to determine formaldehyde in samples of alcoholic beverages ($n = 3$)

Sample	Formaldehyde (μg mL^{-1})	
	FIA	Batch
Cachaça 1	0.150 ± 0.001	0.152 ± 0.006
Cachaça 2	0.073 ± 0.002	0.076 ± 0.001
Cachaça 3	0.095 ± 0.002	0.093 ± 0.003
Cachaça 4	0.073 ± 0.002	0.076 ± 0.003
Cachaça 5	0.095 ± 0.001	0.097 ± 0.001
Cachaça 6	0.093 ± 0.003	0.088 ± 0.003
Cachaça 7	0.256 ± 0.004	0.255 ± 0.002
Cachaça 8	0.159 ± 0.003	0.163 ± 0.007
Rum 1	0.125 ± 0.001	0.122 ± 0.001
Rum 2	0.042 ± 0.001	0.044 ± 0.001
Rum 3	0.47 ± 0.01	0.462 ± 0.002
Vodka 1	ND[a]	ND[a]
Vodka 2	0.034 ± 0.001	0.032 ± 0.001

[a] ND = not detected.

consumption and similar detection limits compared with the bathwise method [1]. In comparison with other flow injection systems described in the literature [42], the proposed method was able to determine formaldehyde without any interference removal step, allowing for higher analytical throughput and lower reagent consumption. Moreover, when compared with results described in the literature [42], the proposed flow system allowed for the determination of lower levels of formaldehyde with a low average relative standard deviation (R.S.D.).

3.5. Formaldehyde determination in alcoholic beverages

The proposed method was applied to determine formaldehyde in alcoholic beverage samples with no further pretreatment, which were injected directly into the proposed flow system. All the alcoholic beverages evaluated here except the vodka sample contained formaldehyde in the range of 1.4×10^{-6} to 8.6×10^{-6} mol L^{-1}, reinforcing the need for evaluating the levels of this carbonyl compound in this type of sample. The results obtained by the proposed method were compared with the Fluoral-P bath procedure (Table 3) [1] as reference, and a paired *t*-test [48] confirmed their congruence at a 95% confidence level.

4. Conclusions

The proposed system was successfully applied to determine formaldehyde in alcoholic beverage samples, providing good repeatability and accuracy. The flow analysis system displayed high sensitivity, a low detection limit and a wide linear range. This system proved suitable for routine analyses, as indicated by its high sample throughput, low reagent consumption, minor waste generation, and the fact that it requires no sample pretreatment.

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