Sequential injection-LOV format for peak height and kinetic measurement modes in the spectrophotometric enzymatic determination of ethanol: Application to different alcoholic beverages

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Abstract

The objective of this work was to make a contribution to study the potential of the sequential injection-lab-on-valve (SI-LOV) format for the miniaturization of enzymatic assays, by using different measurement modes (peak height and initialrate-based measurement). A LOV system was developed for the enzymatic assay of ethanol in beverages, based on the conversion of ethanol to acetaldehyde by alcohol dehydrogenase, using spectrophotometric detection. The use of the kinetic-based approach permits the applicability of the enzymatic determination to samples with intrinsic absorption, with a higher determination throughput.

A linear dynamic application range up to 0.040% (v/v) was achieved for both initial rate and for the peak height measurement, with good repeatability (R.S.D. < 5.0% and <1.0%, respectively). Enzyme, NAD^+, buffer and sample consumption per assay were 0.12 U, 0.066 mg, 150 and 15 μL, respectively. The determination rate achieved was 37 and 27 determinations h^{-1} for the initial rate and for the peak height measurement, respectively. The results obtained for several alcoholic beverages, including a certified sample material, were not statistically different from those obtained by the reference procedures.

Introduction

Flow analysis systems have been gaining an increased importance in wet analytical chemistry. This fact can be mainly explained by the possibility of automating analytical chemical procedures with a simultaneous dramatic decrease in reagents consumption. The trend for automation and miniaturization is particularly important for biochemical methods of analysis, due to the elevated costs of the reagents involved in enzymatic and immuno-assays, as well as by the often-limited amount of the samples available. Therefore, the possibility to perform the biochemical assays within small-integrated manifolds through flow-based automation of sample and reagent handling has received increased attention.

In this scenario, flow methods became widely popular among the scientific community, due to the possibility of automatic sample handing resorting to mostly simple and low cost apparatus. These approaches consist in the injection of a well-defined volume of sample solution into a carrier stream in a reproducible way [1]; one or more reagents can be added downstream and the product is measured in a suitable flow through detector.

Although these systems have proven to be an effective way of automating various biochemical methods, further downscaling would be important. In this context of downscaling and miniaturization one of the frequent objections to the micro- or nano-scale analytical circuits is their probable susceptibility to clogging when "real" samples are to be analyzed. In the sequential injection-lab-on-valve (SI-LOV) format the circuits are downsized only to the microlitre scale, being still easy to manipulate, but already in a compact format [2]. Like in sequential injection analysis (SIA), in a SI-LOV system, a well-defined volume of sample and reagents are sequentially aspirated to the holding coil and then propelled by reversed flow to the detector. In this equipment the manifold/detector is integrated on the top of the selection valve in a robust way, so the miniaturization is possible because of the proximity of the injection port to the flow cell [3]. Unlike traditional SIA, there is no need for the plugs to travel a distant path in the LOV format, since the volumes of sample and reagents involved are in a microliter scale. Therefore, an efficient overlapping of reagent zones is achieved in a repeatable way. The physical configurations of the flow channels are also designed to improve the mixing conditions. This way, one of the limitations attributed to SIA, the lack of efficient reagent/sample overlapping as a source of inaccuracy, especially when sample is contaminated by interfering species that also consume reagent in the overlapping zone, can be overcome.

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The objective of this work was to study the potential of the SI-LOV format for the miniaturization of enzymatic assays. As a case study, we selected the enzymatic determination of ethanol in alcoholic beverages, an important parameter to define the quality and the stability of the product. The reference methods proposed by Office International de la Vigne et du Vin (OIV) are complex, laborious and most of them require distillation as sample pre-treatment [4–6]. To overcome these disadvantages enzymatic flow systems were developed for this determination [7–17]. Most of these methods exploit the reaction between the immobilized alcohol dehydrogenase with ethanol in the presence of NAD^+ to produce acetaldehyde and NADH. The detection is generally spectrophotometric of the produced NADH at 340 nm. The methods are fast, simple and do not require any other sample pre-treatment than dilution, however the process of enzyme immobilization frequently involves the manipulation of toxic reagents (glutaraldehyde), and the support material used for the immobilization process (controlled pore glass) can add to the cost of analysis.

In this scenario, our study comprised two main aspects: (i) the use of the SI-LOV format to further miniaturize the flow system and prevent the need for the enzyme immobilization; this study implied the assessment of the efficiency of the zones overlapping in LOV; (ii) comparison of two quantification methodologies, peak height based versus initial rate measurement; the latter one to cope with the possible interference of the sample intrinsic absorption [3,18].

Experimental

Reagent and solutions

All chemicals were of analytical reagent grade, and boiled Milli-Q water (resistivity 18 MΩ cm^{-1}) was used throughout the work. The buffer solution (pH 9.5) was prepared weekly by dissolving 5.58 g of $Na_4P_2O_7{\cdot}10H_2O$ in 250.0 mL of water. The pH of this solution was adjusted with phosphoric acid 8%, v/v. The buffer for the enzyme suspension (pH 7.5) was prepared by dissolving 0.178 g of $Na_2HPO_4{\cdot}2H_2O$ in 100.0 mL of water.

To daily prepare the working enzyme solution, 1 mg of lyophilized ADH (alcohol dehydrogenase, EC 1.1.1.1, from baker yeast, A7011, Sigma) was re-suspended in 1.00 mL of enzyme buffer pH 7.5, and this suspension was further diluted to 1.00 mL in the same buffer in a way to achieve the concentration of 24 U mL^{-1} in the working reagent solution. The cofactor solution was prepared daily by dissolving 0.0664 g of NAD^+ (NAD^+, free acid grade II, ~98%, 10621650001, Roche) in 5.00 mL of water.

The working standard solutions of ethanol were prepared daily from the stock standard solution (ethanol absolute pro analysis, 1210861212, UN1170, Panreac Quimica, SA) in a range between 0.010% and 0.040% (v/v).

For the zone overlapping study, a 24 mg L^{-1} bromothymol blue solution was prepared as described by Ruzicka and Hansen [1]. The carrier solution used for this study was borax 0.01 M.

Samples

Samples were purchased in a local supermarket. A total of 10 samples were analyzed, using the content of the same bottle for the reference and for the developed flow methods. For the table wine and spirit samples no other treatment than dilution was applied before sample analysis. Table wines were 350 times diluted, and the spirit samples were diluted 2500 times. The beer samples were filtered, degassed and 400 times diluted before analysis.

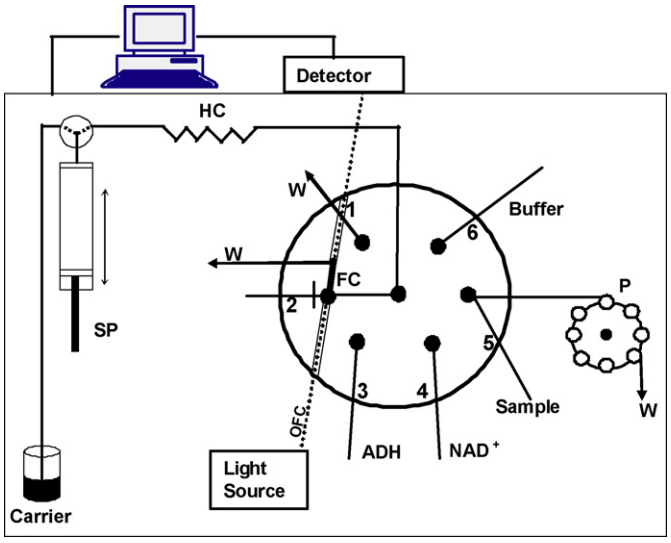

Fig. 1. Configuration of μSI-LOV system for the determination of ethanol; ADH, alcohol dehydrogenase 24 U mL^{-1}; NAD^+, cofactor 20 mM; Buffer, phosphate buffer pH 9.5; W, waste; Carrier, water; SP, syringe pump (2.5 mL); HC, holding coil; FC, flow cell; OFC, optical fibre cable; P, peristaltic pump; Detector, diode-array spectrophotometer.

A certified reference sample of low alcohol level wine was also analyzed (CRM 653, wine, nominal 0.5%, v/v). This sample was 20 times diluted before introduction into the SI-LOV system.

Apparatus

The SI-LOV system (FIAlab-3500, FIAlab Instruments, Medina, WA, USA) presented in Fig. 1 consisting of a bi-directional syringe pump (2500 μL of volume), a holding coil, a bi-directional variable speed peristaltic pump and a lab-on-valve manifold mounted on the top of a six-port multi-position valve, was used.

The USB 2000 Ocean Optics, a diode array spectrophotometer equipped with fiber optics (i.d.: 200 μm), and a DH-2000-BAL Mikropack, UV/Vis/NIR light source, was used. FIAlab for windows 5.0 software on an Intel Pentium III Computer (995 mHz, 128 MB) controlled the system.

Flow procedure

Peak height measurement

The initial steps (A–F) in Table 1 of the flow protocol were similar for the peak height and for the initial rate measurement. Those steps consisted in aspiration of carrier, buffer, sample and reagents to the holding coil: 1000 μL of carrier, then 50 μL of buffer, 15 μL of sample solution, 5 μL of ADH, 5 μL of NAD^+ and finally 100 μL of buffer. In the case of the peak height measurement, the following steps (G–J) consisted in reversing the flow and propelling the mixture, after 30 s of stop time in the holding coil, to the flow cell where the absorbance was measured.

Initial rate measurement

In this case, the stacked zones (steps K–M) were directed to the flow cell and the flow was stopped and the change in absorbance was monitored during 15 s. Afterwards, the reaction zone was dispensed.

Reference procedures

The reference methods used for beer and wine samples consisted in the distillation of the sample [4,5], and Anton Paar DMA

Table 1
Flow protocol

Step	Description	Volume (μL)	Flow rate (μL/s)	Selection valve position
A	Aspirate carrier to HC	1000	100	–
B	Aspirate buffer to HC	50	80	6
C	Aspirate sample to HC	15	25	5
D	Aspirate enzyme to HC	5	25	3
E	Aspirate cofactor to HC	5	25	4
F	Aspirate buffer to HC	100	25	6
Peak height measurement				
G	Reverse flow, reference scan	10	15	2
H	Stop period (30 s)	–	–	–
I	Dispense HC content, data acquisition	450	15	2
J	System washing, SP empty	–	100	2
Initial rate measurement				
K	Dispense selection of stacked zones	100	15	2
L	Stop period (15 s), data acquisition	–	–	–
M	System washing, SP empty	–	100	2

5000 Density meter was used for the measurement of the volumetric alcohol content of the distillates [4]. The spirit samples were diluted [6] before Density meter reading [4].

Results and discussion

Study of the overlapping of reagent zones

Enzymatic assays usually comprise the mixing of various solutions like sample, enzyme, buffer and frequently cofactor solutions. Therefore, when these assays are carried out in SIA, the number of plugs and the aspiration order are determined by the involved enzymatic reaction. In this work, the enzymatic assay is based on the reaction between ethanol and alcohol dehydrogenase in the presence of NAD^+ (cofactor), producing acetaldehyde and NADH. The formation of the reduced cofactor is measured spectrophotometrically at 340 nm. Additionally, the reaction occurs under controlled pH. Based on these conditions, the overlapping and mixing of the reagent zones is of great importance. Firstly the aspiration sequence was defined as buffer–sample–enzyme–cofactor–buffer; buffer solutions in the front and in the rear part of the sequence were used to sandwich the other reagents and assure the adjustment of the reaction pH. Within the sandwiched zone the sequence of the other reagents was selected to promote the penetration of the sample zone through the enzyme and cofactor sections [19,20]. In preliminary experiments, the aspiration flow rates were studied with the objective of using low sampling volumes with good repeatability. These preliminary studies were carried out using a bromothymol blue model solution (24 mg L^{-1}): when a combination of aspiration flow rate and volume uptaken fulfilled the criteria of R.S.D. < 5% (n = 10) for the absorbance values, it was applied in the enzymatic method. To assess the effect of the dimensions of the introduced reagent zones on the efficiency of the mixing, a study similar to Gübeli et al. [20] was carried out using also the model solution of bromothymol blue (24 mg L^{-1}). Using a borax solution (0.01 M) as carrier, the bromothymol blue solution was introduced sequentially in each port involved and the resulted peak profiles were recorded. The distance across the dispersed zone (W_t), the zone of total overlapping between the reagents (W_o) and the peak height absorbance (H) were measured. These parameters and the volumes studied are resumed in Table 2.

Table 2
Study of the overlapping of reagent zones using a model solution of bromothymol blue (24 mg L^{-1})

Aspiration sequence	Volume (μL)					
	A	B	C	D	E	F
Plug1: buffer	50	100	50	50	50	50
Plug2: sample	50	50	50	25	15	15
Plug3: enzyme	25	25	25	10	5	5
Plug4: cofactor	25	25	25	10	5	5
Plug5: buffer	50	50	100	100	100	50
W_t[a] (s)	25	32	34	28	20	15
W_o[b] (s)	13	9	16	17	16	8
H[c]	0.331	0.337	0.270	0.138	0.096	0.127

A–F correspond to different configuration and volumes tested.
[a] Total baseline width of the dispersed sample–enzyme–cofactor reagent zones.
[b] Baseline width of the sample–enzyme–cofactor reagent zone with total overlapping.
[c] Peak height absorbance of the enzyme zone.

In configurations A–C, the relative volumes of the buffer zones were studied maintaining the total volume of the other zones at 100 μL. In configuration C; although W_t was the largest, the zone of total overlapping between the reagents had also the largest extension, therefore this combination of the buffer solutions was maintained. To further improve the mixing conditions, the total volume of the sample–enzyme–cofactor sequence was reduced (configurations C–E); as expected, the dispersion of the enzyme zone increased (H decreased) with decreasing the total volume.

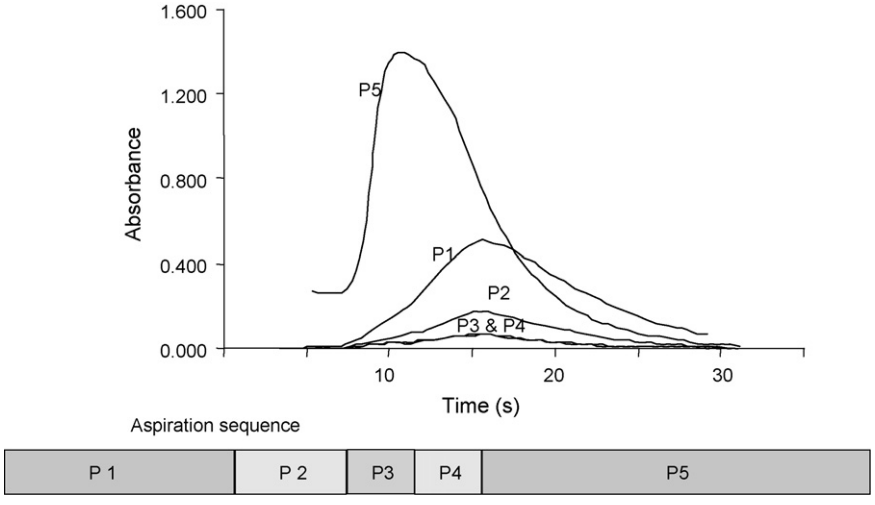

Fig. 2. Recorded peak profiles (P_i) obtained by the injection of 24 mg L^{-1} of bromothymol blue and corresponding aspiration sequence. P1 corresponds to 50 μL of buffer, P2 15 μL of sample, P3 5 μL of enzyme, P4 5 μL of cofactor and P5 100 μL of buffer.

Total overlapping between the sample, the enzyme and the cofactor zone was achieved in configuration E. With the objective of reducing the dispersion of the critical enzyme zone, while maintaining the low injection volumes, the last buffer zone was reduced to half (configuration F), the degree of overlapping of the zones deteriorated. Moreover, there is a need for a larger zone of the buffer solution to reach the flow cell before the reaction zone to perform a reference scan before the measurement. Therefore the configuration of E (Fig. 2) was maintained during the further studies. The repeatability of this aspiration sequence was also evaluated using the bromothymol blue solution. Good repeatability was found ($n = 10$) for all the volumes used in the sequence; 50 μL of buffer (R.S.D. < 0.7%), 15 μL sample (R.S.D. < 1.0%); 5 μL enzyme solution (R.S.D. < 2.9%); 5 μL cofactor solution (R.S.D. < 3.0%) and 100 μL of buffer (R.S.D. < 0.4%).

It can be also concluded from Fig. 2 that in this aspiration sequence the dispersion of the cofactor and the enzyme zone is equal; therefore the order of aspiration of these two zones would not affect the overlapping of the reagent zones.

Study of the enzymatic reaction for peak height measurement mode

After establishing the volume of the reagent zones, a study of the influence of the chemical parameters (pH, NAD^+ and ADH concentration) on the sensitivity of the enzymatic reaction was studied. Univariate procedure was applied and the sensitivity achieved in the range of 0.00–0.040% (v/v) of ethanol was monitored using the peak height measurement mode.

Initial conditions were established as diphosphate buffer pH 9.5 and 20 mM and 24 U mL^{-1} as NAD^+ and ADH concentrations, respectively, based on the previously published work [13] for the same determination using a SIA manifold.

The NAD^+ concentration was studied in a range between 10 and 30 mM. The sensitivity increased about 56% with the increase of the concentration from 10 to 20 mM. For higher concentrations the sensitivity decreased by 10%; therefore the concentration was set to 20 mM.

The effect of the enzyme concentration was studied for 14, 24 and 56 U mL^{-1}. The sensitivity increased 85% when the concentration was raised from 14 to 24 U mL^{-1}. By further increasing the concentration, the sensitivity did not show any further improvement, for that reason the concentration used was 24 U mL^{-1}.

The working pH is essential to assure the activity of the enzyme. This parameter was studied in a range between 8.5 and 10. The pH selected was 9.5 since it presented a higher sensitivity and was close to the reported optimum pH for this enzyme [21].

One way to enhance the sensitivity of enzymatic reactions is to increase the reaction time before detection. In a LOV system this approach is quite easily accomplished by introducing a stop period in the program sequence. A stop time (time elapsed between flow reversal of the stacked zones and propulsion to the detector) between 10 and 40 s was studied. The sensitivity increased with the stop time in the range of 10–30 s, but decreased about 57% when the stop time was 40 s. Thus the stop time selected was 30 s.

Initial rate measurement mode

When samples exhibit either an intrinsic absorption or potential interferents with reaction kinetics different from the analyte, the initial reaction rate measurement could be the most efficient way to overcome these difficulties. The LOV format is particularly suitable for kinetic-based measurements due to the easy manipulation of time sequences and the low volumes involved. In this case the stacked zones in the holding coil can be sent to the flow cell and the rate of product formation can be monitored during a pre-set time period. In this measurement mode, not only the length of the stop period is important for achieving adequate sensitivity and linearity, but also the volume used to propel the reagent zones to the flow cell. This volume will define what portion of the dispersed reagents/sample zone will be monitored during the initial rate measurement [18,19,22]. This volume will also define the relative concentrations of the reagents and the sample inside the flow cell. These flow reversal volumes were studied between 90 and 120 μL, with 5 μL increments. A higher initial rate ($\Delta A/\Delta t$) was achieved using a 100 μL volume; therefore this volume was set.

The stop time in the flow cell was studied in the range of 10–40 s. When this stop time was higher than 15 s the total number of recorded data (spectrophotometer specifications allow a reading frequency of 2 Hz) did not give a linear relationship. Therefore the results of the kinetic method were obtained from initial reaction rate using the data collected during the first 10 s of the stop period. Fig. 3 illustrates the increase of the initial rate with the increase of the ethanol concentration. It can be concluded that, besides the previously mentioned advantages concerning spectral and chemical interferences, the initial rate approach also presents a higher determination rate.

Figures of merit

The performance of the proposed methods was evaluated in terms of reagent consumption, application range, determination rate, repeatability and accuracy (Table 3). Both peak height mea-

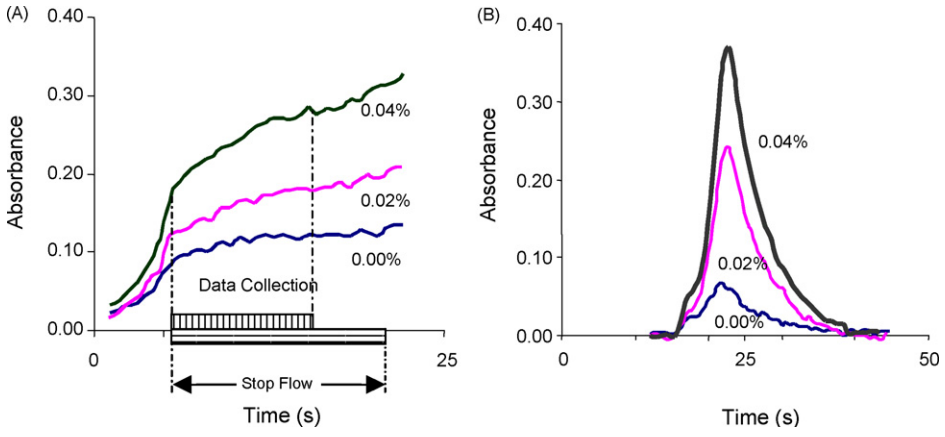

Fig. 3. Variation of the absorbance with the increase of the concentration of ethanol by (A) initial rate measurements and (B) peak height measurement.

Table 3
Figures of merit of the proposed methods

Parameter	Peak height measurement	Initial rate measurement
Reagent consumption per assay		
ADH	0.12 U	0.12 U
NAD^+	0.066 mg	0.066 mg
Sample solution	15 μL	15 μL
Buffer	150 μL	150 μL
Waste production per assay	1.2 mL	1.2 mL
Application range	Up to 0.04% (v/v)	Up to 0.04% (v/v)
Determination rate	27 h^{-1}	37 h^{-1}
LOD	0.003% (v/v)	0.004% (v/v)
LOQ	0.009% (v/v)	0.01% (v/v)
Repeatability (R.S.D.)	1.0% (9.1%, v/v) (n = 4)	5.0% (9.4%, v/v) (n = 6)
	0.7% (11.1%, v/v) (n = 4)	4.0% (11.0%, v/v) (n = 8)
	1.0% (10.4%, v/v) (n = 4)	4.0% (5.2%, v/v) (n = 7)
		2.5% (38.3%, v/v) (n = 10)
		4.6% (40.3%, v/v) (n = 10)

surement and initial rate measurement have the same reagent consumption, and they were linear up to 0.040%, v/v. The sampling rate was higher for the initial rate measurement. R.S.D.'s were below 5.0% in a range between 5.2% and 40.3% (v/v). The accepted precision for a reference method is established as ±0.1% (v/v) ethanol, referring to the measurement of different physical properties of the distilled samples. When looking at the results in Tables 3 and 4, the developed methods show worse repeatability, but it must be kept in mind that these assays were performed on the whole sample without distillation, and that the obtained precision is adequate for the control of the fermentation process [23]. The limit of detection and the limit of quantification were calculated as recommended by Miller and Miller [24].

The reproducibility of the initial rate measurement was evaluated by performing the calibration procedure under identical operation conditions during a working day. Applying a single factor

Table 4
Comparison of the results obtained for the analysis of different beverages according to the reference and the developed procedures

Sample	Reference methods %[a] ethanol (v/v)	%Ethanol (v/v)	
		Peak height measurement[b]	Initial rate measurement[b]
Red table wine	9.3 (±0.1)	9.1 (±0.1)	9.7 (±0.6)
Red table wine	12.4 (±0.1)	12.3 (±0.9)	12.3 (±0.2)
Red table wine	11.1 (±0.1)	11.1 (±0.1)	10.4 (±2.9)
White table wine	10.2 (±0.1)	10.4 (±0.1)	10.4 (±0.1)
Beer 1	5.2 (±0.1)	–	5.1 (±1.2)
Beer 2	4.6 (±0.1)	–	4.8 (±0.5)
Beer 3	4.7 (±0.1)	–	4.7 (±0.2)
Spirit 1	36.7 (±0.1)	–	36.7 (±3.7)
Spirit 2	36.6 (±0.1)	–	36.3 (±2.9)
Spirit 3	39.2 (±0.1)	–	39.8 (± 2.0)

[a] Mean and accepted precision for n = 3.
[b] Mean and standard deviation for n = 3.

Table 5
Results obtained in the analysis of the certified reference wine sample, CRM 653

Certified value, % ethanol (v/v)	0.539 ± 0.0095[a]
Peak height measurement, % ethanol (v/v)	0.537 ± 0.025[b]
t (α = 0.05)[c]	0.03
Initial rate measurement, % ethanol (v/v)	0.548 ± 0.026[b]
t (α = 0.05)[c]	0.15

[a] Laboratory mean and standard deviation of laboratory means.
[b] Mean and standard deviation for n = 7.
[c] t critical: 2.45.

Table 6
Comparison of some analytical figures of enzymatic (ADH) flow methodologies for ethanol determination in beverages

	Present work		FIA [7]	FIA[a] [10]	FIA[b] [12]	FIA[b] [14]	FIA [15]	SIA[a] [11]	SIA [13]	SIA [16]	Lab-on-valve [18]
Sample matrix	Table wine, beer and spirits		Beverages	Wine	Wine, saké	Wine, beer	Wine	Wine	Wine	Ferment. broth	No application
Measurement	Peak height	Initial rate	Peak height	Peak height	Initial rate	Peak height	Peak height	Peak height	Peak height	Peak height	Initial rate
Enzyme	Solution		Solution	Immobilized	Immobilized	Immobilized	Immobilized	Immobilized	Solution	Immobilized	Solution
Reagent consumptions											
ADH (U/assay)	0.12		225	Not given	16.7	0.5	≈0.4	Not given	1.1	Not given	48
NAD^+ (μmol/assay)	0.1		2.4	14	0.52	3.2	0.6	0.3	1	0.5	0.7
Sample (μL/assay)	15		30	3000	50	30	25	45	100	150	30
Waste production (mL/assay)	1.2		1	8.1	5.2	1.8	11	1.8	1.8	Not given	1
Range of applicability (%, v/v)	Up to 0.04	Up to 0.04	Up to 0.4	0.05–0.5	0.04–100 mM	Up to 50×10^{-3}	5–25	0.10–0.50	Up to 0.024	0.25–100 mM	50–250 mg L^{-1}
LOD (%, v/v)	0.003	0.004	Not given	2×10^{-3}	0.02 mM	2.5×10^{-3}	0.4	0.005	0.008	Not given	Not given
Determination rate (h^{-1})	27	37	120	20	10	40	30	45	25	26	120
R.S.D. (%)	<1	<5	Not given	<2.1	2	±0.3	<2.2	<3.4	<2.3	<6	<3

[a] With simultaneous determination of glycerol.
[b] With simultaneous determination of acetaldehyde.

ANOVA [24] treatment between and within day for the calibration curves revealed no significant differences for a 95% confidence level.

Application to beverage samples

To evaluate the accuracy of the proposed methods, a total of 10 beverage samples were analyzed. The reference procedure was also carried out using the same content of the bottle. The results obtained in the analysis are presented in Table 4.

The linear relationships ($C_{\text{peak height meas.}} = C_0 + SC_{\text{ref. meth.}}$) and ($C_{\text{initial rate meas.}} = C_0 + SC_{\text{ref. meth.}}$) were established ($n = 4$ and 10, respectively) and are described by the equations $C_{\text{peak height}} = -0.056\ (\pm 4.245) + 1.003\ (\pm 0.393) C_{\text{ref. meth.}}$, and $C_{\text{initial rate meas.}} = -0.037\ (\pm 0.446) + 1.003\ (\pm 0.021) C_{\text{ref. meth.}}$ [25]. Values presented in parentheses represent the limits of the 95% confidence intervals for the equation parameters. These values demonstrate that the obtained results were not statistically different.

A certified wine sample, CRM 653, with a low level alcohol (0.5%, v/v nominal value) was also analyzed. In this case the sample was 20 times diluted before introduction into the system, resulting in a much higher matrix to analyte ratio than in the case of the table wines. The results obtained for the peak height and the initial rate measurement presented in Table 5 show good agreement with the certified value [25].

Conclusions

The results obtained for the enzymatic determination of ethanol in beverages were comparable to those obtained by the reference method with good repeatability, minimum sample treatment and low reagent consumption. The low reagent consumption is an advantage when compared with some flow methodologies for the same determination (Table 6). The application range and the corresponding detection limits are comparable to those obtained by the other flow methodologies. The limit of detection for the proposed method is lower than the ones obtained with other flow methodologies using enzymes in solution.

The determination rate can be increased when the initial rate measurement is performed as only a 10 s period is used for data acquisition while in the peak height measurement the maximum absorbance must be achieved.

The easy manipulation of time sequences and the low volumes used in the LOV format system makes it proper for the kinetic-based assays. The initial rate measurement is the most efficient way when sample shows evidence of intrinsic absorption or contains potential interferents with a reaction rate different of the analyte. Additionally, the analytical response is not influenced by the schlieren effect in this type of measurement.

The use of a lab-on-valve format with reduced injection volumes, and strategic selected aspiration sequence proved to be an efficient way to overcome the deficient overlapping of sample and reagent zones frequently attributed to conventional SIA assays.

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