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Sequential Injection System for the Enzymatic Determination of Ethanol in Wine

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A sequential injection system was developed for the enzymatic determination of ethanol in wine. The spectrophotometric determination is based on the enzymatic reaction catalyzed by alcohol dehydrogenase in the presence of NAD^+. The system was applied to the determination of ethanol in a range of 0.008−0.024% (v/v) with good repeatability; $RSD_{n=10} < 2.3\%$. The results obtained with the developed system showed good agreement with those obtained by using the reference method. The determination rate was 25 h^{-1}; 1 μmol of NAD^+, 1.1 units of enzyme, and 50 μL of sample were consumed per determination; and the waste produced was 2.2 mL per assay.

KEYWORDS: Sequential injection analysis; alcohol dehydrogenase; ethanol; wine; spectrophotometry

INTRODUCTION

The tendency for miniaturization apparent from the recent scientific literature is highly justified by the elevated costs of the reagents involved in enzymatic and immunoassays and by the often limited amount of sample available. The possibility of performing biochemical assays within integrated manifolds through automation of sample and reagent handling has received increased attention in recent decades.

Flow injection analysis (FIA) (*1*) is a well-established flow-based technology that has found applications in diverse fields such as pharmaceutical assays, environmental monitoring, process control, and agricultural and food analysis. In a conventional FIA procedure, continuous forward flow is used to transport the injected sample zone to mix with the reagents and to carry the reaction product into a flow cell for detection. This strategy allows high sampling frequency but generates more waste and consumes more reagents as a result of the continuous pumping.

Sequential injection analysis (SIA), based on a multiposition valve, uses the programmable flow approach (*2*) to mix and transport injected zones of samples and reagents and to carry them and the reaction products to the flow cell for detection. The flow programming, including flow reversal and stopped flow, offers versatility for the systems and makes it possible to develop multipurpose flow manifolds with a significant decrease in reagent consumption and waste production.

At the same time the parallel dynamic development in the area of bioengineering and biotechnology gave the basis for the increasing interest in reliable and fast methods for monitoring concentrations of substrates, metabolites, and inhibitors in culture media and control of bioreactors as well as in food product quality control (*3*). The high specificity of enzyme-based assays combined with the advantages of flow systems meets the requirements for fast and accurate process monitoring.

Substrate concentrations are almost always measured spectrophotometrically by monitoring the color of indicators coupled to products formed by substrate degradation. The most often used enzymes in FIA are the oxidases and dehydrogenases. For oxidases, which give rise to hydrogen peroxide, as well as for dehydrogenases, which require the presence of suitable coenzymes (NAD^+ or $NADP^+$), optical as well as electrochemical detection can be used. Hydrogen peroxide can be detected optically by coupling to appropriate chromogenic agents, by chemiluminescence via reaction with luminol, or by amperometry. The coenzymes are usually detected by spectrophotometry or fluorometry or by different electrochemical techniques (amperometry and detection at modified electrodes) (*4*−*6*).

The determination of ethanol is not only a key parameter in terms of quality and stability for alcoholic beverages but also an important factor for fermentation monitoring (*7*). The official methods (*8*, *9*) for the determination of ethanol in wines and other alcoholic beverages are laborious and complex and require in most cases the separation of the analyte from the sample matrix by distillation. The enzymatic determination of ethanol requires only a sample dilution step before the analysis. Automated flow procedures have been applied to the determination of this analyte, and most of them are based on the use of immobilized enzymes with the aim of reducing enzyme consumption. The efficiency of the enzyme immobilization procedure is reported in a wide range (from 5 to >90%), and the stability of the enzyme reactors is generally taken to be >3 weeks; however, an estimation of enzyme consumption per assay is scarcely given (*10*−*17*). The process of enzyme immobilization is laborious and involves the manipulation of toxic and allergen reagents. The development of automated sample

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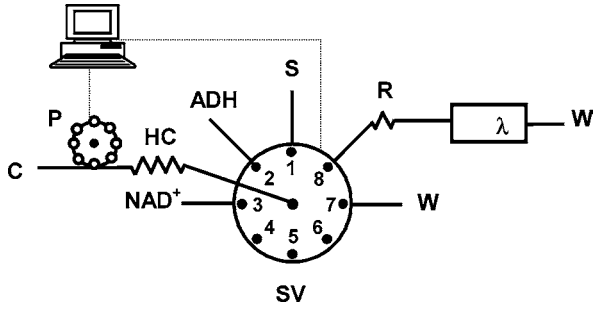

Figure 1. Sequential injection manifold for the determination of ethanol: S, samples or standards; ADH, alcohol dehydrogenase; NAD^+, cofactor 20 mM; C, carrier phosphate buffer, pH 9.5; P, peristaltic pump; HC, holding coil; W, waste; R, reactor (100 cm); λ UV–vis. spectrophotometer (340 nm).

handling, like sequential injection, for the delivery of small solution volumes urges the possibility of using enzymes in solution.

The objective of this work was to develop a sequential injection procedure for the determination of ethanol using the alcohol dehydrogenase (ADH; EC 1.1.1.1) enzyme in solution. Reaction rate measurement is generally considered to be the more suitable tool for enzymatic assays as it provides a readout based on the linear section of a rate curve, less biased by the sample matrix and the sample color (*10*, *17*, *18*). However, for the enzymatic determination of ethanol in alcoholic beverages, an extensive dilution is needed (1000−3500 times), resulting in a very much reduced matrix effect. Therefore, in this work an approach based on a two-point detection (peak height measurement) will be presented and compared to the existing flow procedures in terms of precision, accuracy, reagent consumption, and waste production.

MATERIALS AND METHODS

Solutions. All chemicals were of analytical grade, and Milli-Q water was used throughout the work. The carrier buffer was prepared by dissolving 22.3 g of $Na_4P_2O_7 \cdot 10H_2O$ in 1 L of water. To adjust the pH of the solution to 9.5, phosphoric acid 8% (v/v) was used. The enzyme buffer was prepared by dissolving 0.178 g of $Na_2HPO_4 \cdot 2H_2O$ in 100 mL of water. The pH was adjusted to 7.5 with 1 M phosphate buffer.

The NAD^+ solution was prepared daily by dissolving 0.0995 g of NAD^+ (NAD^+, free acid grade III, ~90%, 710113, Roche) in 10 mL of water. To prepare the enzyme solution, 1 mg of ADH (EC 1.1.1.1, from baker's yeast, A7011, Sigma; 303 units mg^{-1}) was dissolved in 1 mL of enzyme buffer, and 800 μL of this suspension was further diluted to 10 mL.

The working standard solutions of ethanol were prepared in a range between 2.4×10^{-3} and 2.4×10^{-2}% (v/v) by rigorous dilution of the stock standard solution (ethanol absolut pro analysis, 1.00983.1011, Merck). The samples were diluted with water.

For the dispersion studies, a 25 mg L^{-1} bromothymol blue solution was prepared as described by Ruzicka and Hansen (*19*).

Samples. Wine and other alcoholic beverages (bottled or boxed) were purchased in a local supermarket. A total of nine samples were analyzed, using the same bottle or box of the beverage for the reference and for the developed method. A certified reference sample of low alcohol level wine was also analyzed (CRM 653, wine, nominal 0.5% vol). No sample treatment other than dilution was applied before sample analysis. One of the main difficulties of spectrohotometric wine analysis is the considerable background absorbance; to overcome this problem, the system was designed to use a single and high dilution factor (1000 times) that can be applicable for all of the wine samples.

Apparatus. System components are arranged as shown schematically in **Figure 1**. Solutions were propelled by a Gilson Minipuls 3 (Villiers-le-Bel, France) peristaltic pump with a Gilson pumping tube, connected to the central channel of an eight-port electrically actuated selection valve (VICI, Houston, TX). All tubing connecting the different components of the sequential injection system was made of PTFE (Omnifit, Cambridge, U.K.) of 0.8 mm i.d.

As detection system, a Thermo-Spectronic (Cambridge, U.K.) Helios γ UV−vis spectrophotometer equipped with a Hellma (Mullheim/

Table 1. SIA Protocol

valve position	operation time (s)	volume (μL)	description
1	5	100	aspiration of standard/ sample
2	5	50	aspiration of the enzyme solution
3	5	50	aspiration of the NAD^+ solution
8	10	200	flow reversal and propelling to reactor
8	60	0	stop-time
8	60	1800	propelling toward detection

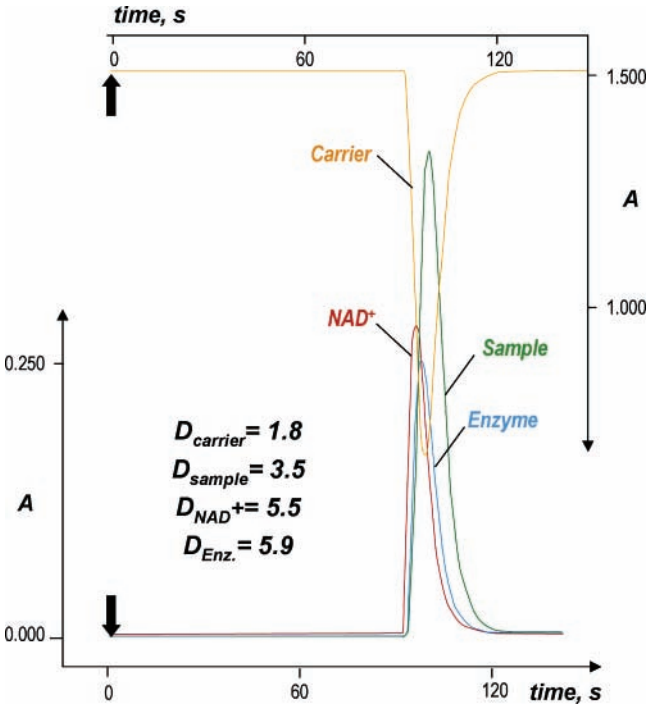

Figure 2. Recorded peak profiles obtained by the injection of bromothymol blue solution (25 mg L^{-1}). D_i, dispersion coefficient.

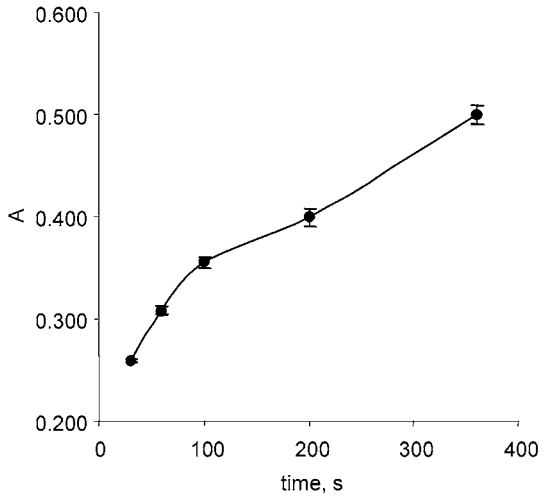

Figure 3. Study of the effect of assay time on the recorded peak height in absorbance of the developed system. Other conditions: 0.1% v/v ethanol; 0.06 unit of ADH per assay; 15 mM NAD^+; transport buffer, 1 M, pH 8.8.

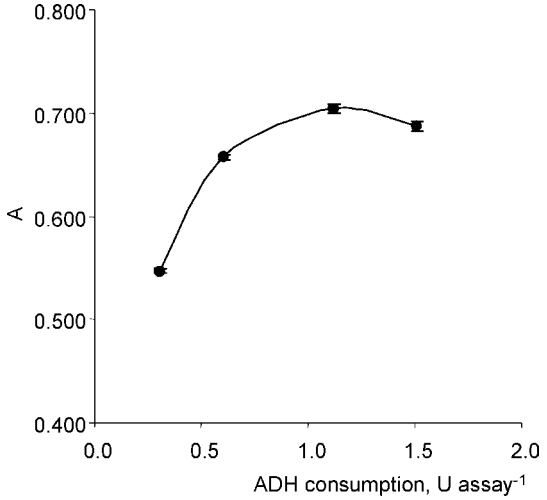

Figure 4. Study of the effect of enzyme quantity on the recorded peak height in absorbance of the developed system. Other conditions: 60 s stop time; 0.1% v/v ethanol; transport buffer, 1 M, pH 9.0; 10 mM NAD^+.

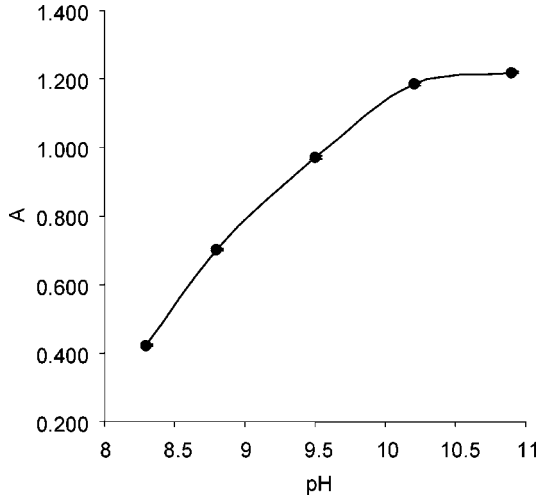

Figure 5. Study of the effect of pH of the transport buffer 1 M on the recorded peak height in absorbance of the developed system. Other conditions: 60 s stop time; 0.1% v/v ethanoll 1.1 units/assay ADH; 10 mM NAD^+.

Baden, Germany) 178.712-OS flow-through cell (internal volume = 80 μL) was used, and the wavelength was set at 340 nm. The analytical signals were recorded in a Kipp & Zonen (Delft, The Netherlands) BD 111 strip chart recorder.

A Samsung (Kyungki-Do, Korea) SD700 386 personal computer, equipped with an Advantech (Taipei, Taiwan) PCL818L interface card, running homemade software written in QuickBasic, controlled the selection valve, the pump rotation, and the flow direction.

Flow Procedure. The flow protocol is summarized in **Table 1**. The first three steps consisted in the aspiration of sample (1000 times diluted), enzyme, and NAD^+ solutions. In the fourth step the flow is reversed and the mixture is propelled to the reactor with the transporting buffer solution. After a stop-time, to increase the extent of the reaction, the last step is used to propel the reaction zone toward the detection.

Reference Procedure. For the reference ethanol determination (*8*), 250 mL of wine was distilled for each assay. A distillation apparatus and Denis Alcoholmeters were used, one in the range of 6−14% (v/v) and another in the range of 14−22% (v/v) alcohol content.

RESULTS AND DISCUSSION

Optimization of the Manifold. As a first approach, the SIA system (**Figure 1**) was set up to study the chemical conditions of the enzymatic reaction and the SIA process. Working conditions are represented in **Figure 1**.

The total volume of the injected solutions was set at 200 μL to provide a reaction zone with low dispersion. The total volume was a sum of 100 μL of sample, 50 μL of enzyme, and 50 μL of NAD^+ solutions to ensure the overlapping of the injected sample and reagent zones (*2*). In **Figure 2** the recorded peak profiles obtained by the injection of bromothymol blue solutions are presented. The dispersion number (*D*) of the developed system was determined as recommended by Ruzicka and Hansen (*19*). Using water as carrier, a 25 mg L^{-1} bromothymol blue solution was introduced into the system sequentially in each port involved in the determination, and the resulting peak profile

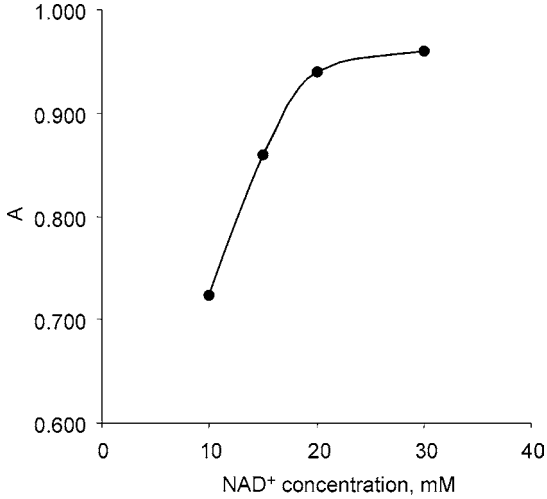

Figure 6. Study of the effect of NAD^+ concentration on the recorded peak height in absorbance of the developed system. Other conditions: 60 s stop time; 0.05% v/v ethanol; transport buffer, 1 M, pH 9.5; 1.1 units/assay ADH.

Table 2. Comparison of Results Obtained for Analysis of Different Wine Types According to the Developed Method and the Reference Procedure

	% ethanol (v/v)		
wine sample	developed method[a]	reference method[b]	RD %[c]
sangria	6.98 ± 0.04	7.00 ± 0.01	−0.3
young white, vinho verde type	8.4 ± 0.1	8.3 ± 0.2	1.2
young red, vinho verde type	9.0 ± 0.2	9.12 ± 0.03	−1.3
table, white	11.0 ± 0.1	11.02 ± 0.03	−0.2
table, red	11.78 ± 0.06	11.89 ± 0.02	−0.9
Port, white	20.1 ± 0.2	19.0 ± 0.2	5.8
Port, tawny	18.9 ± 0.3	18.8 ± 0.1	0.5
aromatized wine-based drink	13.2 ± 0.2	13.32 ± 0.03	−0.9
wine liquor Jeropiga	16.6± 0.1	15.6 ± 0.1	6.4

[a] Average and sample deviation corresponding to three replicate dilutions analyzed in duplicate. [b] Average and sample deviation corresponding to two replicate distillations and three alcoholmetric readings for each. [c] Relative deviation.

Table 3. Results Obtained in the Analysis of the Certified Reference Wine Sample, CRM 653

certified value[a]	found value[b]	recovery[c] (%)	t ($\alpha = 0.05$, $n_{cert} = 11$, $n_{SIA} = 3$)	t_{crit}
0.539 ± 0.0095	0.529 ± 0.006	98.1	1.75	2.179

[a] Laboratory mean and standard deviation of laboratory means. [b] Mean and standard deviation of three separate dilutions analyzed in triplicate. [c] Calculated as recommended by IUPAC (*22*).

Table 4. Comparison of Some Analytical Characteristics of the Developed System with Existing Flow Methodologies

parameter	developed system	FIA (*10*)	FIA (*11*)	FIA (*12*)	FIA[a] (*13*)	FS[b] (*14*)	SIA (*15*)	SIA[a] (*16*)	lab on valve (*17*)
matrix	wine	beverages	wine, beer	wine	wine	wine, sake	ferment. broth	wine	no application
enzyme	solution	solution	immobilized	immobilized	immobilized	immobilized	immobilized	immobilized	solution
reagent consumption									
ADH (units/assay)	1.1	225	not given	not given	not given	16.7	not given	not given	48
NAD^+ (μmol/assay)	1	2.4	3.2	0.6	14	0.52	0.5	0.3	0.7
sample (μL/assay)	100	30	30	25	3000	50	150	45	30
waste production (mL/assay)	1.8	1	1.8	11	8.1	5.2	not given	1.8	1
determination rate (h^{-1})	25	120	40	30	20	10	26	45	120
RSD (%)	<2.3	not given	±0.3	<2.2	<2.3	2	<6	<3.4	<3

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

was recorded overlapped as shown in **Figure 2**. Subsequently, the 25 mg L^{-1} bromothymol blue solution was used as carrier, and water was inserted through ports 1−3. It can be concluded that in the presented flow conditions, sufficient mixing was achieved within the introduced solutions.

The effect of the other parameters of the enzymatic reaction (reaction time, pH, and concentration of reagents) on the analytical signal was studied on the basis of a uniparametric optimization study. For the clarity of the presentation of the results only one concentration level is presented in **Figures 3**−**6**; however, complete calibration curves were performed (with at least five standard solutions) in all cases.

The effect of the stop-time (**Figure 3**) in the reactor was studied between 30 and 360 s; although the sensitivity increased with time, a 60 s stop-period was chosen so as not to unnecessarily decrease determination rate. The sensitivity achieved in these conditions was enough for our purpose.

The enzyme concentration (**Figure 4**) was studied within the range of 0.30−1.5 units/assay; 1.1 units/assay was chosen as it provided the highest analytical signal.

The pH has an important effect on the enzyme activity. In this study the pH was studied in the range of 8.3−10.9. Although the enzyme activity increased over the range studied (**Figure 5**), pH 9.5 was chosen for further studies because of the proximity to the reported optimum pH of the enzyme (*20*).

The concentration of NAD^+ was also studied within the range of 10−30 mM (**Figure 6**). At >20 mM there was no significant increase in sensitivity; therefore, this concentration was chosen.

Figures of Merit of the Developed System. Under the optimized conditions an application range up to 0.024% (v/v) was achieved with a corresponding quantification limit (n = 10, σ = 3) of 8×10^{-5}% (v/v). Therefore, wines with a concentration of ethanol up to 24% (v/v) can be analyzed with a 1000 times dilution. Repeatability of the method was assessed at three different concentration levels, and relative standard deviations (n = 10) of 0.07, 1.2, and 2.3% were found corresponding to concentrations of 6.9, 11.7, and 18.5% (v/v) of ethanol, respectively.

The stability of the developed system was assessed by performing the calibration procedure under identical operational conditions, during a working day. The 95% confidence interval was estimated for the equation parameters; no significant difference was found between the calibration curves. The overall calibration curve is as follows: $A_{340\ nm} = -419.5\ (\pm 64.9)\ [\text{Et-OH}]^2 + 27.2\ (\pm 1.9)\ [\text{Et-OH}] + 0.205\ (\pm 0.012)$, $R = 0.997$; the values in parentheses are the limits of the 95% confidence intervals.

Analysis of Wine Samples. To validate the proposed methodology, the developed procedure was applied to the determination of ethanol in wine samples of different type and origin. The reference procedure was also carried out using the same bottle/box of wine. The results obtained in the analysis of the samples are presented in **Table 2**.

A linear relationship ($C_{SIA} = C_0 + SC_{ref.meth}$) was established ($n$ = 9), and the values for intercept (C_0), slope (S), and correlation coefficient were −0.66 (± 1.06), 1.069 (± 0.076), and 0.994, respectively, where the values in parentheses are the limits of the 95% confidence intervals (*21*). These figures demonstrate a good agreement between the developed and reference procedures.

A certified reference wine sample with nominal alcohol content of 0.5% (v/v) (CRM 653) was also analyzed. This sample contains a low level of alcohol and therefore allows the developed method to be validated in different dilution conditions. In this case the sample was diluted 100 times, resulting in a much higher matrix/analyte ratio. The results obtained are summarized in **Table 3**. These figures indicate agreement of the results obtained by the developed method and the certified value.

In conclusion, the results obtained by the developed system showed good agreement with those obtained by the reference method. **Table 4** shows a comparison of the developed system with some of the existing flow methodologies for the same determination. It can be concluded that the developed system presents advantages over the existing methods in terms of reagent consumption and repeatability. The developed system has an enzyme consumption of 1.1 units/assay; all of the other applications present higher enzyme consumption. The waste production in some methodologies is 5−10 times more. Compared with the reference procedure, the sample consumption is largely reduced (the reference determination uses 200−250 mL of sample); the determination rate is also highly improved because in the reference procedure only one determination can be carried out per hour.

LITERATURE CITED

(1) Ruzicka, J.; Hansen, E. H. Flow injection analysis. Part I. A new concept of fast and continuous flow analysis. *Anal. Chim. Acta* **1975**, *78*, 145−157.

(2) Ruzicka, J.; Marshall, G. D. Sequential injection: a new concept for chemical sensors, process analysis and laboratory assays. *Anal. Chim. Acta* **1990**, *237*, 329−343.

(3) Schugerl, K. Progress in monitoring, modeling and control of bioprocesses during the last 20 years. *J. Biotechnol.* **2001**, *85*, 149−173.

(4) Prodromidis, M. I.; Karayannis, M. I. Enzyme based amperometric biosensors for food analysis. *Electroanalysis* **2002**, *14*, 241−261.

(5) Mello, L. D.; Kubota, L. T. Review of the use of biosensors as analytical tools in food and drink industries. *Food Chem.* **2002**, *77*, 237−256.

(6) Girelli, A. M.; Mattei, E. Application of immobilised enzyme reactor in on-line high performance liquid chromatography: a review. *J. Chromatogr. B* **2005**, *819*, 3−16.
(7) Amerine, M. A.; Ough, C. S. *Methods for Analysis of Must and Wines*; Wiley: New York, 1980.
(8) Office International de la Vigne et du Vin (OIV). *Recueil des Methodes Internationales d'Analyse des Vins et des Mouts, Titre Alcoométrique Volumique*; OIV: Paris, France, 2005; MA-F-AS312-01-TALVOL.
(9) Association of Official Analytical Chemists (AOAC). *Official Methods of Analysis*, 16th ed.; AOAC: Gaithersburg, MD. 1997; pp 28.1−28.3.
(10) Worsfold, P. J.; Ruzicka, J.; Hansen, E. H. Rapid automated enzymatic method for the determination of alcohol in blood and beverages using flow injection analysis. *Analyst* **1981**, *106*, 1309−1317.
(11) Lázaro, F.; Luque de Castro, M. D.; Valcarcel, M. Individual and simultaneous determination of ethanol and acetaldehyde in wines by flow injection analysis and immobilised enzymes. *Anal. Chem.* **1987**, *59*, 1859−1863.
(12) Rangel, A. O. S. S.; Tóth, I. V. Determination of ethanol in wines by flow injection spectrophotometry using gas-diffusion and an immobilised enzyme reactor. *Am. J. Enol. Vitic*. **1999**, *50*, 259−263.
(13) Rangel, A. O. S. S.; Tóth, I. V. Enzymatic determination of ethanol and glycerol by flow injection parallel multi-site detection. *Anal. Chim Acta* **2000**, *416*, 205−210.
(14) Mori, H.; Sekine, Y.; Takahashi, Y. Simultaneous determination of ethanol and acetaldehyde in liquor using a flow system composed of two enzyme reactors and an octadecylsilica column. *J. Health Sci.* **2003**, *49*, 55−58.
(15) Hedenfalk, M.; Mattiasson, B. Sequential injection analysis of ethanol using immobilised alcohol dehydrogenase. *Anal. Lett.* **1996**, *29*, 1109−1124.
(16) Segundo, M. A.; Rangel, A. O. S. S. Sequential injection flow system with improved sample throughput: determination of glycerol and ethanol in wines. *Anal. Chim Acta* **2002**, *458*, 131−138.
(17) Chen, Y.; Ruzicka, J. Accelerated micro-sequential injection in lab-on-valve format, applied to enzymatic assays. *Analyst* **2004**, *129*, 597−601.
(18) Segundo, M. A.; Rangel, A. O. S. S. Kinetic determination of L-(−)malic acid in wines using sequential injection analysis. *Anal. Chim Acta* **2003**, *499*, 99−106.
(19) Ruzicka, J.; Hansen, E. H. In *Flow Injection Analysis*, 2nd ed.; Wiley: New York, 1988; p 301.
(20) http://www.sigma-aldrich.com, consulted July 12, 2005.
(21) Miller, J. C.; Miller, J. N. In *Statistics for Analytical Chemistry*, 3rd ed.; Ellis Horwood: New York, 1993; pp 120−124.
(22) Burns, D. T.; Danzer, K.; Townshend, A. Use of the terms "recovery" and "apparent recovery" in analytical procedures (IUPAC recommendations 2002). *Pure Appl. Chem*. **2002**, *74*, 2201−2205.

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