METHOD FOR MONITORING UREA AND AMMONIA IN WINE AND MUST BY FLOW INJECTION-PERVAPORATION

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An easy to automate flow-injection-pervaporation method for monitoring urea and ammonia in must and wine was developed. The method is based on separation of the ammonia from the sample matrix by pervaporation followed by its reaction with salicylate, hypochlorite and nitroprusside to form a diazonium salt with maximum absorption at 647 nm. Conversion of urea into ammonia catalysed by urease was mandatory before pervaporation. After optimisation by either the univariate or multivariate approaches as required, the linear range was established (between 0 and 25 mg l^{-1}) for both analytes. Then, the assessment of the proposed method versus a reference one for urea and ammonia was studied in terms of repeatability (0.52 mg l^{-1} and 0.43 mg l^{-1}, respectively), reproducibility (1.34 mg l^{-1} and 1.21 mg l^{-1}, respectively), detection and quantification limits (LOD=0.9 and 0.6 mg l^{-1}, LQ=1.02 and 0.67 mg l^{-1}, respectively) and traceability. The sample throughput was 16 samples h^{-1}. The method can be applied to the monitoring of the target analytes in must and young wine in order to control their contents, preventing formation of ethyl carbamate.

Keywords: wine, must, pervaporation, flow injection, spectrophotometry, urea, ammonia.

Introduction

Ethyl carbamate (EC), produced naturally during storage and/or aging of wines, is usually monitored as it becomes a serious problem for trading wines due to its potential carcinogenic effects on humans [1]. Thus, Canadian laws concerning imported wines have restricted the level of EC to values lower than 30 μg l^{-1} in table wine and 100 μg l^{-1} in dessert wine. Regulations about the content of EC in wines are being established at present in the European Community. The official method of the OIV (Office International de la Vigne et du Vin) for the determination of EC [2] is based on gas chromatography-electron capture detector. The method is tedious and time-consuming as it requires a previous extraction step and preconcentration by evaporation at low pressure. The main precursor of EC in wine is urea [3] as demonstrated by the correlation found between EC formation and urea content in wines stored for several years [4]. Kinetic studies developed by Kodama et al. [5] and subsequent application of the equations obtained have shown that the urea content in wine should be less than 2 mg l^{-1} in order to keep the EC content lower than the maximum contents allowed at present. So, the determination of urea before and during fermentation of young and stored wines, and also of wine subject to the aging step, is mandatory. Wines with urea content close to 2 mg l^{-1} should be subject to conversion of urea into ammonia catalysed by urease.

The usual method for determining the urea content in must and wine is based on enzymic commercial urease kits with subsequent spectrophotometric monitoring of the reduced form of the coenzyme. Recent methods in the literature for determination of urea in wines are based on enzymic analysis and monitoring of the pH change using two capillary glass electrodes capable of quantifying changes of pH of 0.001 units produced in the hydrolysis by urease [6]; on HPLC with a previous derivatisation reaction for facilitating separation [7,8] or on derivatisation with 1-phenyl-propan-1,2-dione 2-oxime in ethanol and photometric monitoring at 540 nm [9].

There are no Flow Injection (FI) methods in the literature for the determination of urea in wine or must, although methods for the determination of this analyte in milk using gas diffusion as a separation technique and enzymic conversion into ammonia followed by potentiometric [10] or photometric [11] detection has been described. Also, the NH$_3$ produced from urea in an enzymic urease reactor has been monitored by fluorescence (λ_{em} 450 nm, λ_{ex} 450 nm) after treatment with phthalaldehyde and 2-mercaptoethanol [12]. Only one method exists in the literature for the determination of urea and ammonia in biological fluids, using a FI-pervaporation approach with potentiometric detection [13]. The aim of this work was to develop a versatile, inexpensive, fast

and accurate method for the determination of urea and ammonia in wine and must in order to monitor the urea concentration during fermentation and storage for proper control of this parameter, avoiding the formation of ethyl carbamate and thus the problems it causes.

Experimental

Apparatus and instruments

The manifold used is depicted in Fig.1. It was built using a four-channel Gilson Minipuls-3 peristaltic pump (Villiers le Bel, France) fitted with a rate selector, two Rheodyne 5041 injection valves (Elkay, Galway, Ireland) forming a laboratory-built dual injector, and PTFE tubing of 0.5 mm i.d. (Análisis Vínicos, Tomelloso, Spain). A Philips PU 8625 spectrophotometer (Cambridge, UK) equipped with a Hellma 138-QS flow-cell (Hellma, Jamaica, NY) and connected to a Knauer recorder was used.

Two SBS model TFB-1 Selecta (Barcelona, Spain) thermostats, a laboratory-made pervaporation module, described else-where [14], and PTFE membranes of 47 mm diameter and 1.5 mm thickness (Trace, Braunschweig, Germany) were used. Statistical treatments were made using Statgraphics™ plus 2.1 for Windows.
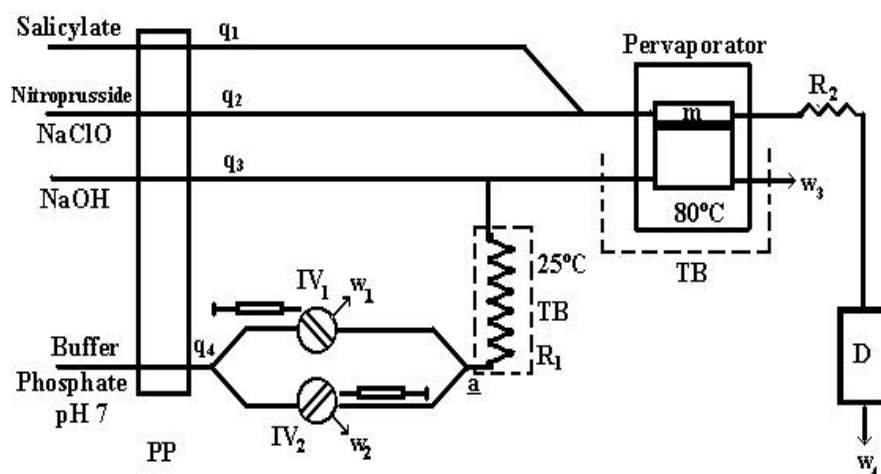

Figure 1. Manifold for the determination of urea and ammonia. PP= peristaltic pump, IV= injection valve, a= merging point, R= reactor, D= detector, w= waste, q= flow-rate, m=membrane, TB= thermostatic bath.

Reagents and solutions

Standard solutions both for optimisation studies and characterisation of the method were prepared from urea and ammonium nitrate, both from Panreac (Barcelona, Spain).

Boehringer Mannheim (Mannheim, Germany) kits for the determination of ammonia and urea were used for applying the reference method.

The proposed method used as acceptor stream in the pervaporation module an aqueous solution of 200 g l^{-1} sodium salicylate and 1 g l^{-1} sodium nitroprusside, both from Panreac, at pH 10 and sodium hypochlorite solution with 7.5 % of active chlorine from Merck (Darmstadt, Germany). A 10^{-4} M di-sodium hydrogen phosphate (Merck) buffered solution of 1.45 g l^{-1} of urease, type III from jack beans at pH 7 (Sigma, St. Louis, MO, USA) (EC 3.5.1.5) was used. A 1 M sodium hydroxide (Panreac) solution was used to be merged with the sample in order to guarantee alkaline medium (pH=9) of the latter when the mixture reached the lower chamber of the pervaporation module.

Doubly distilled water of high purity obtained from a Millipore Milli-Q plus system (Bedford, MA) was used thoroughly.

Reference procedure

Urea is hydrolysed to ammonia and carbon dioxide in the presence of the enzyme urease. Ammonia reacts with 2-oxoglutarate and the reduced form of nicotinamide adenine dinucleotide (NADH) in the presence of glutamate dehydrogenase (GIDH) yielding L-glutamate and an amount of NAD$^+$ stoichiometric to the amount of ammonia or to half the amount of urea. The depletion of NADH is monitored at 340 nm. Urea and ammonia are determined adding or not urease to the sample, respectively.

Proposed method

Figure 1 shows the manifold used for the sequential determination of both analytes. The sample and the buffered solution of urease were introduced into the loops of valves IV$_1$ and IV$_2$, respectively, using syringes in order to save both reagent and sample. Both valves were switched simultaneously to the injection position and their contents merged at a, then circulating through R$_1$ at the optimum working temperature of the biocatalyst. After that and before the mixture reached the low chamber of the pervaporation unit, ammonium was transformed into ammonia gas by merging with a basic stream. Ammonia was pervaporated and collected into the acceptor stream reacting with the reagent mixture to yield the diazonium salt whose absorbance (A$_1$) was monitored spectrophotometrically at 647 nm. This absorbance corresponds to the contribution of both the ammonium initially present in the sample and that formed in the enzymic reaction from urea. The urease transformed the urea into ammonium at a rate of 1 μmol of ammonium per unit of urease and minute at 25ºC. In the meanwhile, the injection valves were switched and filled with a new aliquot

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of the sample and phosphate buffered solution (IV_1 and IV_2, respectively). When the baseline in the detector was reached, the valves were switched to the inject position and the content of the loops were merged. After pervaporation and development of the derivatisation reaction, the detector provided an absorbance (A_2) which was the concentration of ammonium present in the sample. The difference between A_1 and A_2 yielded the content of urea in the sample.

Results and discussion

Optimisation of the method

The variables which affect the method for the determination of urea and ammonia were studied by the univariate and multivariate approaches, depending on their interdependence, and the optimum values obtained are summarised in Table 1.

Chemical and biochemical variables

The chemical variables studied were the concentrations of sodium salicylate and sodium hypochlorite (both interrelated), nitroprusside, NaOH, phosphate buffer and urease. Figure 2 (a) shows a plot of the absorbance values versus both the hypochlorite and salicylate concentrations in the acceptor stream. Since the P-value in the ANOVA table is less than 0.1, there is a statistically significant relationship between the variables at 90% confidence level. The equation of the fitted model is : Abs= -0.0132 + 0.00327[NaClO]+0.00157 [Salicylate].

The optimum values were those providing the highest signal with the less reagent consumption. They were selected from the response surface. The rest of the variables were studied using the univariate method.

The criterion for optimisation of Na_2HPO_4 and NaOH was the minimum concentration providing a pH for optimum development of the enzymic reaction, which was pH=7.0 and a subsequent basic medium (pH higher than 9) for conversion of the ammonium ion into ammonia, respectively.

Concentrations of sodium nitroprusside from 0 to 1 g l^{-1} increased the analytical signal, which levelled off from the latter value. Concerning urease catalysis, one unit of enzyme liberates one μmol of ammonium from urea per min at pH 7.0 and 25ºC. Taking into account the concentration range of urea in wines and the residence time of the sample-biocatalyst mixture in reactor R_1, the range of enzyme concentration studied was 1-3 g l^{-1} and an optimum value of 1.45 g l^{-1} was found, which corresponds to 50 units of urease per millilitre. The uncatalysed reaction did not progress under these conditions.

Flow injection and pervaporation variables

The length of reactor R_1 was fixed at 2 m as a compromise between sensitivity, which increased by increasing the reactor length (longer time for development of the enzymic reaction) and the sampling rate, which decreased when the residence time of the reacting plug

Table 1. Optimisation of variables

| Variable	Tested range	Optimum value
Chemical		
NaClO (g l^{-1})	0-150	75
Sodium salicylate (g l^{-1})	0-500	200
Urease (g l^{-1})	1-3	1.45
Na$_2$HPO$_4$ (mol l^{-1})	10^{-3}-10^{-5}	10^{-4}
NaOH (mol l^{-1})	0-1.5	1
Sodium nitroprusside (g l^{-1})	0-2	1
FI		
$Q_{2=}q_4$ (ml min^{-1})	1-2	1.5
$Q_{1=}q_3$ (ml min^{-1})	0.6-1.5	0.65
IV$_1$=IV$_2$ (ml)	1-3	2
R$_1$ (cm)	1-3	2
R$_2$ (cm)	0.5-0.75	0.75
Pervaporation		
T (ºC)	50-90	80

in the dynamic system increased.

Equal volumes of enzyme and sample were injected in all instances and both were changed at the same time between 1 and 3 ml. The signal obtained increased from 1 to 2 ml and levelled off for higher volumes; thus 2 ml was the volume selected for subsequent studies. The derivatising reaction between the pervaporated species and salicylate and hypochlorite occurred in the upper chamber of the pervaporation unit, so the function of reactor R$_2$ was only to connect this unit with the detector. The length required for this function was 75 cm.

After fixing the length of reactor R$_2$, an univariate study of the flow-rate was developed with the criterion that the overall flow-rate in the upper and lower chambers of the pervaporator were equal in order to avoid membrane bending ($Q=q_1+q_2 =q_3 +q_4$). The stability of the monitored product was affected by both the temperature and the residence time (namely, the time elapsed between formation of the product and monitoring), the latter depending on the flow-rate for a given length of reactor R$_2$. So, both the temperature of the thermosted bath and the flow-rate (Q) were the subject of a multivariate optimisation. Figure 2(b) shows the results of this study. The P-value in the ANOVA was less than 0.1, so the relationship between the variables at 90% confidence level was statistically significant. The equation of the fitted model is: Abs= 0.4636 + 0.00633Q+0.00367 T.
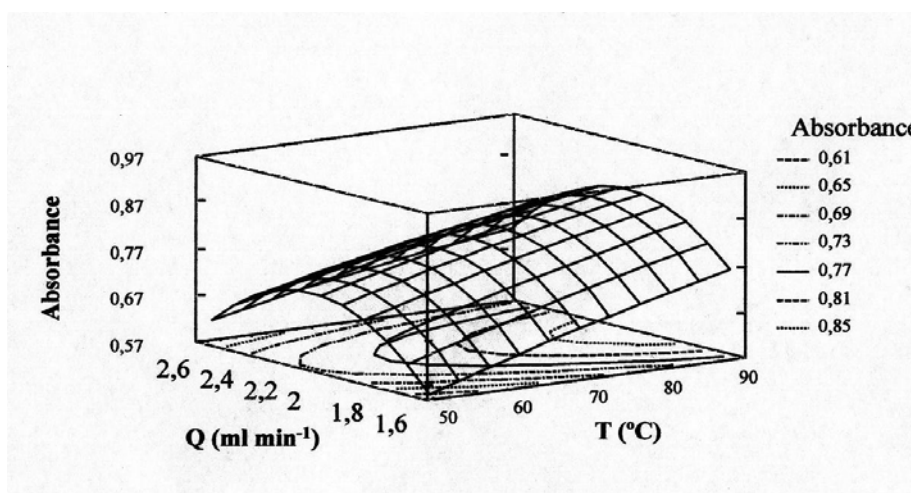
a)
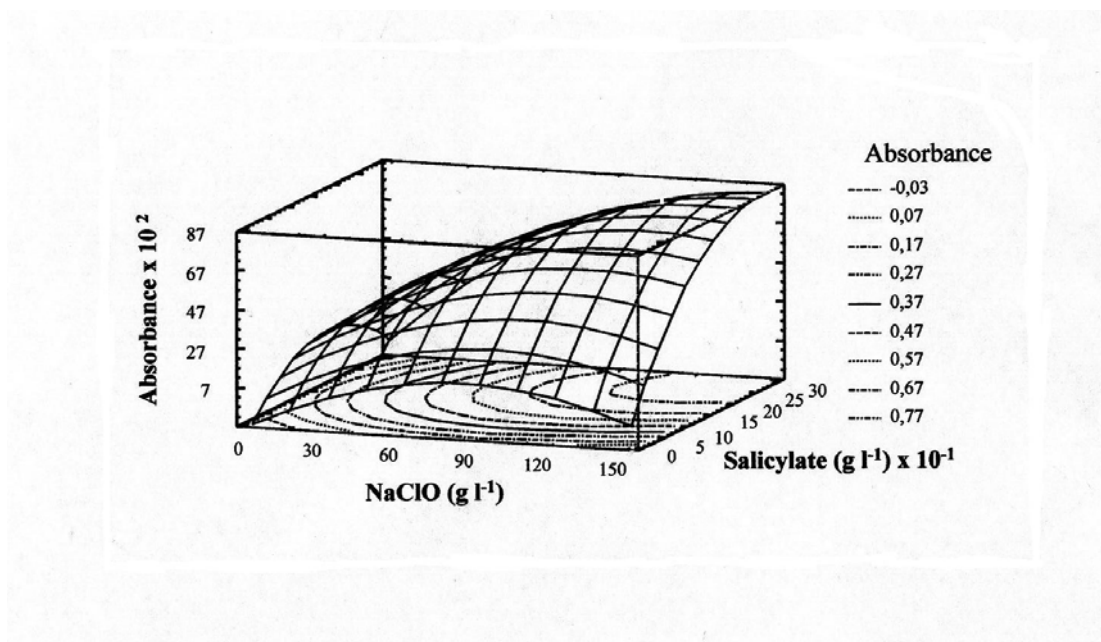
b)

Figure 2. Response surface of the multivariate analysis of: (a) evolution of absorbance versus salicylate (g l^{-1}) and NaClO (g l^{-1}) concentrations in the acceptor stream of the pervaporation unit. (b) evolution of absorbance versus temperature of the pervaporation unit (°C) and the flow-rates (ml min^{-1}) in the upper and lower chamber of the pervaporation unit.

Despite the development of the derivatisation reaction was favoured by increased temperature, a value of 80ºC was selected instead 90ºC because the difference of absorbance was not so significant and the former is easy to maintain.

Characterisation of the method

Calibration curves

For the reference method, the linearity of the calibration plots was between 0.008-80 mg l^{-1} and 0.15-140 mg l^{-1} for ammonia and urea, respectively.

For the proposed method, calibration curves were run using standard solutions with known concentrations (between 0 and 25 mg l^{-1}) of ammonium (Y=6.43x10^{-3}±7.8x10^{-4} X + 1.2x10^{-3}±5.1x10^{-4}; r=0.998) and urea (Y=6.16x10^{-3}±8.23x10^{-4} X + 2.7x10^{-3}±9.1x10^{-4}; r=0.994). Each point in the calibration curve was the average of three determinations. This range can be extended by increasing the amount of urease if necessary.

Assessment of the proposed method

Ten different white wines and twenty must were used in the assessment study. The protocol for assessment consisted of studying analytical parameters such as linear range, traceability by the reference method, repeatability, reproducibility, detection and quantification limits and sample throughput. Robustness and costs studies were also developed.

Repeatability (r). The F-test was applied in order to establish if the difference of repeatability between the proposed and reference methods was significant. With this aim, the Fobs=S_r^2/S_{ref}^2 was compared with the $F_{1-\alpha}$ obtained from F tables for α=0.05 (P=95%). As can be observed in Table 2, Fobs^r<$F_{1-\alpha}$, so the repeatability of the FI and reference method is similar.

Reproducibility (R) (25 days). The R values as well as the results from the application of the F-test, in Table 2, show that the reproducibility of the urea FI-method is statistically equal to that of the reference method because Fobs^R< $F_{1-\alpha}$.

Detection (LOD) and quantification (LQ) limits. Table 2 shows that the best LOD (x_b±3σ_b) corresponds to the reference method but the values provided by the FI method for these parameters are not far from the reference ones, so they are adequate for the analysis of these analytes both in wine and must. The value of LQ (x_b±10σ_b) by the reference method has not been calculated.

Traceability. The traceability of the method was studied by comparing the results obtained from 10 samples of different white wines and 20 must samples analysed by both the reference and proposed method. Figure 3 shows the regression of the flow injection method for urea and ammonium on the reference method. The regression equations are Y= 0.986±0.024 X + 0.077 ± 0.022 (r= 0.991) and Y= 0.904 ± 0.031X + 0.857 ± 0.211 (r=0.989) for

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urea and ammonium, respectively. Both regression plots show good correlation between the data from the two methods and their reference counterparts.

Confidence limits of 95% are shown in Figure 3 in dotted lines. In both cases, the traceability was assured using the t-test.

Sample throughput. The results in Table 2 show that the FI method has a sampling frequency higher than that of the reference method (16 vs 9 h.$^{-1}$). The reference method required sample pretreatment while the FI method did not.

Table 2. Analytical characteristics of the proposed method as compared with the reference method

Parameters	Urea		Ammonia	
	Reference method	FI method	Reference method	FI method
Repeatability (mg l^{-1})	0.1854 [1]	0.523	0.1232 [2]	0.432
S_r (mg l^{-1})	0.066 [1]	0.098	0.0412 [2]	0.065
Reproducibility (mg l^{-1})	0.2145 [1]	1.345	-	1.212
S_R (mg l^{-1})	0.076 [1]	0.132	-	0.12
Fobs^r	-	2.205	-	2.489
Fobs^R	-	3.017	-	-
$F_{1-\alpha}$ (n=25)	-	4.24	-	4.24
LOD (mg l^{-1})	0.15 [1]	0.9	0.08	0.6
LQ (mg l^{-1})	-	1.02	-	0.67
Sample throughput (h^{-1})	9 [3]	15-16	9 [3]	15-16
Costs (€) [4]	3.7	0.28	3.47	0.05

S_r and S_R are the deviation of the repeatability and reproducibility, respectively. Fobs = S_r^2 / S_{ref}^2
[1] Obtained from the technical information provided for the enzyme test for an urea concentration of 0.611 mg l^{-1}.
[2] Obtained experimentally in a double determination for an ammonia concentration of 0.9 mg l^{-1} .
[3] Eight in series determinations including sample pretreatment.
[4] Only reagent cost.

Robustness study. The study was developed using the Younden-Steiner [15] procedure. The most significant variables of the system (namely, flow-rate, temperature and concentrations of NaClO, sodium salicylate, urease, Na_2HPO_4, NaOH and sodium nitro-prusside were modified ±10% from their optimum values. Errors lower than 5% were observed in all cases.
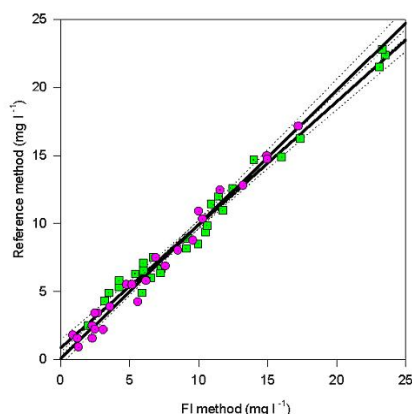
Figure.3. Correlation graph of the reference method with the FI method for urea and ammonia. Interval of confidence: 95%. (o) urea; (□) ammonia.

Cost. The cost per analysis concerning reagent consumption is also summarised in Table 2. The FI method is significantly cheaper than the reference one (thirteen times more). In both methods the most expensive reagent was the enzyme. No instrumental, personal nor energy costs have been included. Time and personal are also aspects significantly diminished in the FI method.

Conclusions

The FI method for the determination of urea and ammonia in wine and must here proposed fulfils the requirements for monitoring urea in wine during storage, must or fermented wine in order to prevent formation of ethyl carbamate. The linear range, detection and quantification limits and trace-ability with the reference method guarantee the capability of the new method. The method is robust and has a sample throughput higher than that of the reference method and its cost is lower. An additional advantage of the method reported here is its easy automation and potential for on-line monitoring. The use of active interfaces for controlling the peristaltic pump and the injection valves is an easy task, as is the use of a passive interface for collecting data from the spectrophoto-meter, which would convert the present mechanised method into automated.

Acknowledgement

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