FLOW INJECTION DETERMINATION OF TOTAL CATECHINS AND PROCYANIDINS IN WHITE AND RED WINES

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An easily automatable flow-injection (FI) method for the determination of total catechins is reported. The method is based on the reaction of vanillin in acid medium to yield a coloured product with maximum absorption at 500 nm. After optimisation by the univariate and multivariate approaches as required, the linear range was established (between 10 and 90 mgL^{-1} and 10 and 250 mgL^{-1} for white and red wines, respectively). Then, the assessment of the proposed versus the reference method was studied in terms of repeatability (2.57 mgL^{-1}), reproducibility (3.56 mgL^{-1}) (no significant differences were found), detection and quantification limits (not far from those of the reference method and always sufficient for the determination of catechins in any type of wine), traceability (excellent correlation under all conditions) and sample throughput (23 samples h^{-1} for the proposed method versus 3 samples h^{-1} for the reference method).

Introduction

Grapes contain non-flavonoid compounds in the pulp while flavonoid compounds are essentially located in the seeds and skin. An in-depth knowledge of the structures of the latter is of interest to explain their role in the evolution of wine during the vinification and aging processes. The main 3-flavonoid monomers in grape are (+) catechin and its isomer (-) epicatechin. The former (Fig. 1a) has two benzene cycles bound by a saturated oxygen heterocycle. This structure contains two asymmetrical carbons (C_2 and C_3) that gave rise to four isomers. Catechins may not be considered as tannins, as their molecular weight is too low [290.3 for (+) catechin] and they have very restricted properties as compared with tannins, which have molecular weights ranging from 600 to 3500. The molecular weight of catechins in their dimer form is high enough for binding to proteins. Catechins degradation in synthetic wine containing tartaric acid and iron traces yields dimers in which the two catechin units (the simplest procyanidin) are bound by an ethane group (Fig. 1b). These dimers are colourless, but they undergo a fast evolution to yellow forms with higher absorption than the products formed by either enzymatic oxidation or catechin autooxidation (Flanzy, 2000). This mechanism seems to play an important role in the non-enzymatic browning of white wines, which is directly corre-lated with flavanoid contents (Flanzy, 2000).

(a)
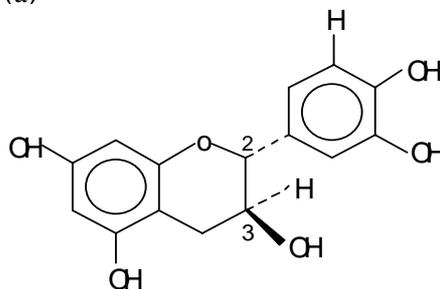
(b)
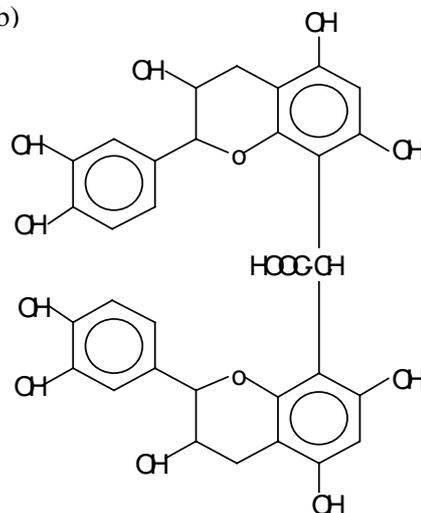

Figure 1. (a) Catechin estructure. (b) Catechin dimer involving an ethane bound.

There are two common methods for determining catechin contents in wines. The first method, proposed in 1959 by Swain and Hillis for the catechin assays in wines and also applied to vinegars

(García-Parrilla, Heredia, Troncoso & González, 1997), is based on chemical derivatisation using vanillin to yield a quinoid product whose colour ranges from pale to intense pink-red with an absorption maximum at 500 nm. The second method is based on HPLC with different detectors to obtain levels of individual catechins and procyanidins compounds (Carando, Teissedre, Pascual-Martínez & Cabanis, 1999). Capillary electrophoresis is also being used at present (Prasongsidh & Skurray, 1998). There are no references in the literature on flow injection methods for the determination of catechins.

The objective of this work was to develop a flow injection method for determining catechins in their usual range of concentrations in Montilla-Moriles white wines (Sherry type) (15-35 mgL^{-1}) (Fabios, López-Toledano, Mayen, Mérida & Medina, 2000) and red wines (10-100 mgL^{-1}) (Mayen, Mérida & Medina, 1995), showing a precision as required for wineries and faster than the methods used at present.

Experimental

Apparatus and instruments
The manifold used is outlined in Fig.2. 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 (one of them acting as selection valve) (Elkay, Galway, Ireland), and PTFE tubing of 0.5 mm i.d. (Scharlau, Barcelona, Spain). A PU 8625 spectrophotometer (Cambridge, UK) equipped with a Hellma 138-QS flow-cell (Hellma, Jamaica, NY) and connected to a Knauer recorder were also used. Statistical treatments were made using Statgraphics™ plus for windows 2.1.

Reagents and solutions
For both the reference and proposed method, a 0.25 gL^{-1} catechin solution (purity 98%) from Merck (Darmstadt, Germany) in ethanol, 37% hydrochloric acid, 1 and 2,5% vanillin solutions (puity> 99%), respectively, and absolute ethanol from Panreac (Barcelona, Spain) were used.

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

Reference procedure
Determination of catechin (Swain and Hillis method). A 0.25 gL^{-1} catechin standard solution was prepared. 0, 1, 2, 3, 5 and 10 mL of standard solution, 10 mL of 37% hydrochloric acid and 5 mL of 1% ethanol solution of vanillin were mixed in 25-mL flasks. After 20 min the absorbance at 500 nm was measured in cuvettes of 10-mm pathlength. Ten milliliters of white wine and aliquots of 10 mL of 1:10 diluted red wine were poured instead of the standard solutions and the procedure was applied as described before. Blank samples contained all reagents but vanillin.

Proposed method
Figure 2 shows the manifold used for catechins determination. The sample without dilution was introduced into the

loop of the injection valve (IV) and injected into an 18% HCl aqueous solution which merged with solution 1 (2.5% vanillin in 35% HCl).
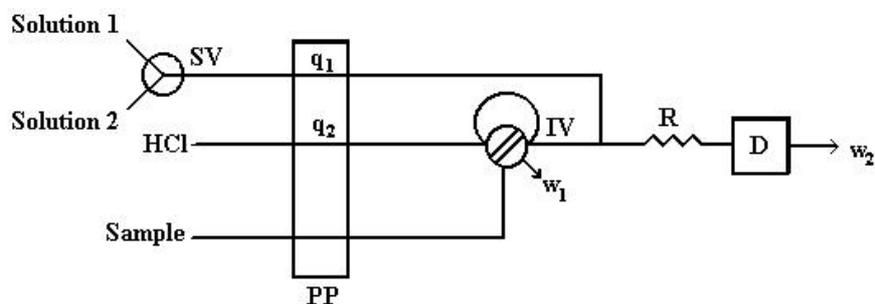

Figure 2. Manifold for the determination of catechins. PP= peristaltic pump, q= flow-rate, SV= selection valve, IV= injection valve, R= reactor, D= detector, w= waste.

The pink product of the derivatisation reaction was monitored at 500 nm. After this, the selection valve (SV) was switched to the position for introducing solution 2 (35% HCl) and a new sample aliquot was injected. The difference of absorbance between the two measurements at 500 nm was related to the catechins and procyanidin contents.

Results and discussion

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

Chemical variables
Figure 3 shows a plot of the absorbance values versus both the hydrochloric acid and vanillin concentrations in solution 1. As the P-value in the ANOVA table is less than 0.05, there is a statistically significant relationship between the variables at 95% confidence level. The R-squared statistic indicates that the model explains 98,87% of the absorbance variability. The equation of the fitted model is: $A = 0.0470 - 7.68 \times 10^{-3}$ [HCl] $+ 0.0228$ [vanillin] $+ 3.36 \times 10^{-4}$ [HCl]^2 $+ 1.46 \times 10^{-3}$ [HCl][vanillin] $- 0.071$ [vanillin]^2, where A is expressed in milli-absorbance units and the concentration of vanillin in % (m/v).

Table 1. Optimisation study of the variables affecting the FI method for total catechins and procyanidins

Variable	Tested range	Optimum value
Chemical		
% HCl (v/v)	0-37	37
% Vanillin (m/v)	0.1-3	2.5
FI		
q_1 (mLmin^{-1})	0.4-1.3	0.7
q_2 (mLmin^{-1})	-	0.4
Sample volume (μl)	50-500	100
R (cm)	50-175	75

The response surface shows that the most critical variable is the concentration of HCl which clearly affects the sensitivity of the determination. In order to increase the sensitivity, a 37% HCl concentration was chosen for further experiments. The range of vanillin concentrations studied showed that there were small differences in the absorbance when concentrations higher than 0.5% were used. A value of 2.5% was chosen in order to increase the instrument response. The optimum concentration of HCl in the carrier stream was 18 % (v/v).
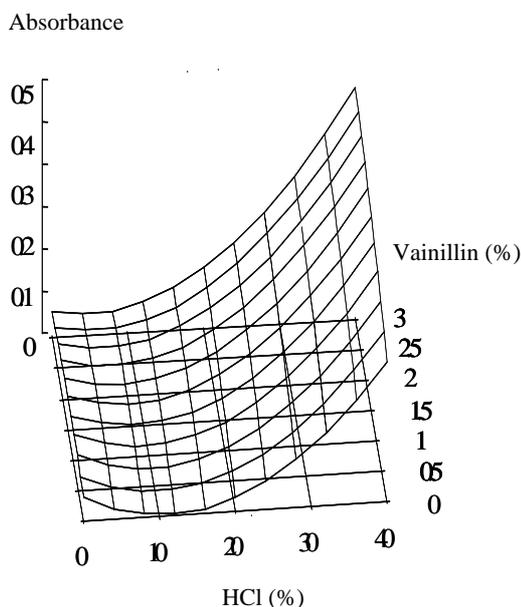

Figure 3. Response surface of the multivariate analysis: evolution of absorbance versus hycrochloric acid (%, v/v) and vanillin concentration (%, m/v) in solution 1.

Flow injection variables

The function of reactor (indicated as R in Fig. 2) was to provide both appropriate reagent-sample mixture and enough development of the derivatisation reaction before reaching the detector. A length of 75 cm was sufficient for these purposes. The flow-rate of the reagents was studied in the range 0.4-1.3 mL min^{-1} at constant carrier flow-rate of 0.4 mL min^{-1}. An optimal value of 0.7 mL min^{-1} was found for q_1 (see Fig.2). A flow rate of 0.4 mL min^{-1} was selected for the carrier channel (q_2) in order to minimise dispersion. The sample volume was tested from 50 to 500 μL and no significant differences in the absorbance response were found above 100 μL.

Characterisation of the method

Calibration curves

Calibration curves were run using as standard solutions white and red wine samples spiked with known concentrations of catechin (between 10 and 40 mg L^{-1} and between 10 and 250 mg L^{-1} for white and red wines, respectively) in order to minimise matrix effects. Each point in the curves was obtained as the average of a triplicate injection. Linear responses were found in the range of concentrations studied. In addition, standard solutions were analysed in triplicate by the reference method.

Assessment of the proposed method

Thirty different white and red wines samples were used in the assessment study. Each result was the average of a triplicate injection. The protocol for assessment consisted of studying parameters such as traceability with a reference method, repeatability, reproducibility, detection and quantification limits and sample throughput. A robustness study was also developed.

Repeatability (r). The F-test was applied in order to establish if the differences in repeatability of the 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 $\alpha=0.05$ (P=95%). As can be observed in Table 2, $Fobs < F_{1-\alpha}$, so the repeatabilities of the FI and reference method are similar. It is worth to noting that the repeatability of the flow injection method is better than that of the reference method.

Reproducibility (R) (30 days). Table 2 also shows the R values obtained as well as the results from the application of the F-test. The reproducibility of the FI method is better than that of the reference method. Both methods are statistically equal.

Table 2. Analytical characteristics of proposed method and the reference method

Parameters	Reference Method	Flow injection method
Repeatability (mg l^{-1})	3.10	2.57
S_r (mgL^{-1})	0.56	0.45
Reproducibility (mg l^{-1})	5.30	3.56
S_R (mg l^{-1})	1.02	0.95
Fobs^r	-	0.646
Fobs^R	-	0.867
$F_{1-\alpha}$ (n=30)	-	1.69
LOD (mg l^{-1})	2.2	4.5
LQ (mg l^{-1})	6.1	5.85
Sample throughput (h^{-1})	3	23

Where S_r and S_R are the deviation of repeatability and reproducibility, respectively. $Fobs = S_r^2 / S_{ref}^2$

Detection (LOD) and quantification (LQ) limits. Table 2 shows that the best LOD and LQ correspond to the reference method but the values of these parameters in the FI method are not far from those of reference method.

Traceability. The traceability of the methods was studied by comparing the results obtained from 30 samples of red and white wines analysed by both the reference and proposed methods. Fig. 4 shows the regression of the flow injection method versus the reference method for catechins in both red wines (Fig. 4a) and white wines (Fig. 4b). The regression equations, (A) y= 0.998 x + 0.462 (r= 0.993) and (B) y= 0.984 x + 0.451 (r= 0.993) show good correlation

between data from both methods. Confidence limits of 95% are shown in the figure in dotted lines. In both cases the traceability was assured using the t-test.
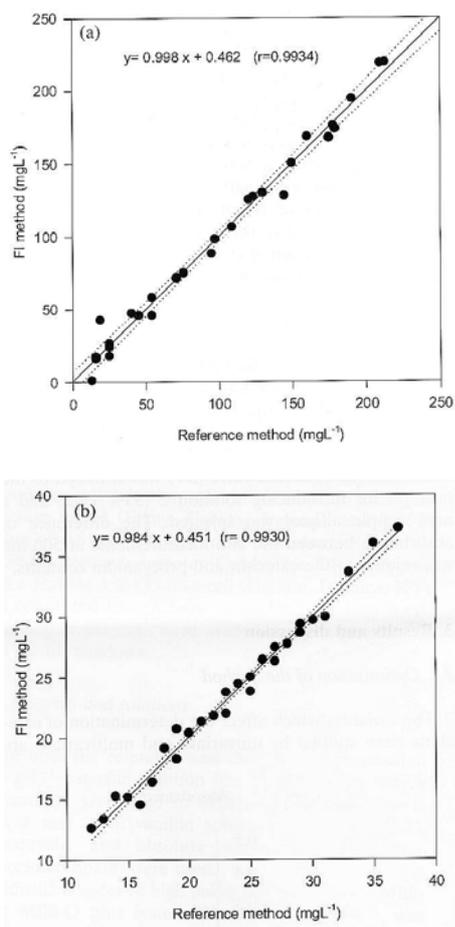

Figure 4. Correlation graphs of the reference method versus the FI method for the determination of catechins in: (A) red wine (B) white wine. Confidence interval: 95%.

Sample throughput. The results in Table 2 show that the FI method provides a sampling frequency much higher than that of the reference method. This is a consequence of the non-equilibrium but fixed-time conditions in which FI measurements are performed. Thus, there is not necessity for waiting until reaction completion.

Robustness study. The study was developed using the Younden-Steiner procedure. Critical variables of the system are hydrochloric acid, vanillin concentrations in solution 1 and flow-rate. These variables were modified $\pm 10\%$ of their optimum values. All errors were below 5%, except in the case of hydrochloric, which yields changes of 50% in the analytical signal in the range under study.

Conclusions

The proposed method enables the overall determination of catechins in red and white wine with characteristics similar or better than those of current existing methods. Thus, the proposed method is simple, inexpensive, presents a good correlation with the reference methods commonly used for these analytes and can be easily implemented in a winery for monitoring catechins in both white and red wines. Other advantages of the method are a sampling throughput higher than that of the methods routinely used in wineries and its capability for either partial or full automation.

This method constitutes the unique application of flow injection for the

determination of catechins in white and red wines proposed so far and offers the advantage of reducing time and costs.

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