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# Determination of anthocyanins in wine based on flow-injection, liquid–solid extraction, continuous evaporation and high-performance liquid chromatography–photometric detection

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**Abstract**

A continuous method, easy to automate, for the determination of anthocyanins in wine based on the coupling of continuous liquid–solid extraction, evaporation, HPLC individual separation and photometric detection is proposed. The target analytes are removed from the wine in a continuous fashion using a $C_{18}$ minicolumn and eluted with an aqueous solution (pH 2) with 16% acetonitrile. The eluted fraction is concentrated by solvent evaporation assisted by heat and dragging off the vapour using a flow of $N_2$. For in-line preconcentration, a continuous evaporation module was designed and located in the manifold between the solid-phase minicolumn and the injection valve of the chromatograph. In this way, injection of the sample into the dynamic system leads the plug through it for liquid–solid extraction of the anthocyanins, partial evaporation of the eluent (with a preconcentration factor as required) and transport to the high-pressure injection valve of the chromatograph, where individual separation and subsequent photometric detection take place. The method thus developed for the determination of malvidin-3-glucoside, cyanidin-3-glucoside and peonidin-3-glucoside anthocyanins in Spanish red wines is more sensitive than the batch manual method based on the same steps, has better linearity of the calibrations curves with lower detection limits and much wider determination range for the most abundant anthocyanins in wine. In addition, the method can be fully automated with low acquisition and maintenance costs. 

*Keywords:* Wine; Food analysis; Automation; Anthocyanins; Phenolic compounds; Glucosides

## 1. Introduction

The interest in phenolic compounds has grown in the last decade mainly due to their antioxidant character, but also to their vasodilation capacity and bactericide action [1–4]. Anthocyanins belong to the phenolic family, within which they are characterised by the presence of two aromatic rings linked by a heterocycle and containing glucose groups. There are three main types of anthocyanins, namely: anthocyanin-3-glucoside, where the aglycon is identified as delphinidin, cyanidin, petuniding, peonidin and malvidin; anthocyanin-3-(6-acetyl)glucoside and anthocyanin-3-(6-coumaroyl)glucoside. The most important of these compounds in red wine, because of its higher concentration, is malvidin-3-glucoside.

Overall separation of anthocyanins from the wine matrix is generally performed by liquid–solid extraction on either $C_{18}$ [5–9] or silica gel [10,11] with

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subsequent evaporation of the eluent. Previously either tangential filtration [12] or lyophilisation [13] have also been carried out. No data about continuous approaches for the development of these steps have been reported in the literature so far.

After matrix removal and preconcentration, anthocyanins in wines are individually separated and determined by high-performance liquid chromatography (HPLC) with spectrophotometric [7,8,10,12, 14–16], MS [6] or matrix-assisted laser desorption ionisation time-of-flight MS [9] detection. Only one method for the individual separation of these compounds based on capillary electrophoresis has been proposed [17,18].

Non-chromatographic continuous separation techniques are usually implemented with the help of dynamic manifolds based on flow injection (FI) [19]. These approaches have demonstrated their reliability and versatility for the development of automated methods, with clear advantages over their conventional counterparts. Based on their previous experience on the use of techniques such as liquid–solid extraction [20,21], dialysis [22] and pervaporation [23,24], the authors planned to design a coupled dynamic approach where the steps involved in the conventional, manual method (namely, sampling, liquid–solid extraction, preconcentration by evaporation, individual separation and detection) were all developed in a continuous fashion. With this aim both previously used devices, such as in-line minicolumns, and new modules, such as a continuous evaporator, were connected to a liquid chromatograph for the overall dynamic development of the process. Three anthocyanins whose concentration in red wines is low (cyanidin-3-glucoside, peonidin-3-glucoside) or high (malvidin-3-glucoside) were selected for development of the method in order to demonstrate its capability.

## 2. Experimental

### *2.1. Instruments and apparatus*

The manifold used is shown schematically in Fig. 1. It was built using a four-channel Gilson (Villiers le Bel, France) Minipuls-3 peristaltic pump fitted with a rate selector, a Rheodyne 5011 (Elkay,

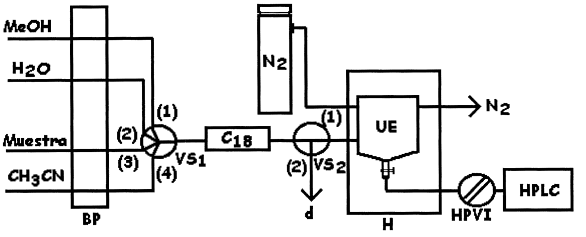


Fig. 1. Flow injection manifold for the determination of anthocyanins in wine. PP, Peristaltic pump; SV, selection valve; O, oven; EU, evaporation unit; HPIV, high-pressure injection valve, w, waste; HPLC, liquid chromatograph.

Galway, Ireland) six-position rotary selection valve and a Rheodyne 5041 injection valve acting as a valve for selecting between two channels, PTFE tubing of 0.5 mm I.D. (Scharlau, Barcelona, Spain) and a PTFE minicolumn of 4 cm×3 mm I.D.

The evaporation unit, shown in Fig. 2, was designed by the authors with the aim of concentrating to a small volume the eluate from the minicolumn, then allowing its evacuation towards the high-pressure injection valve of the chromatograph (HPIV in Fig. 1). The continuous evaporator, made of PTFE, consisted of two circular parts of 3 cm diameter. The upper part was provided with inlet and outlet orifices in order to allow circulation of gas for dragging the evaporate eluent. The lower part (2 ml volume) was provided with a lateral inlet orifice for entrance of the liquid phase and an outlet orifice,

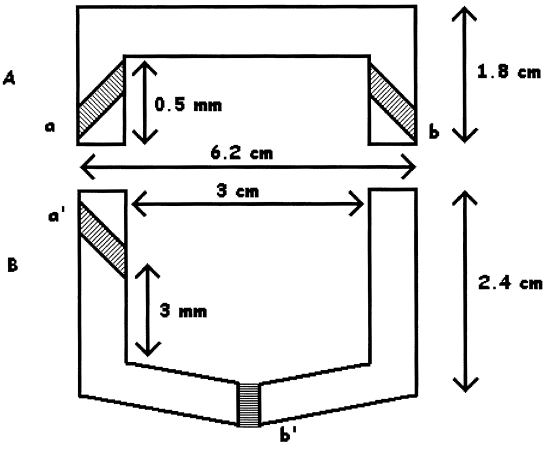


Fig. 2. Cross-sectional view of the evaporation unit. A and B= Upper and lower parts, respectively; a, a′=inlets; b, b′=outlets.

closed by a 016-002400 (Scharlau) two-way connector with valve located centered at the bottom. A slight slope from the walls to the central orifice facilitated complete and fast evacuation when the bottom valve was open. Both parts were in close contact by screwing the evaporation unit between aluminium supports with four screws. PTFE spacers of different thickness allowed one to change the volume of the lower chamber. A Selecta (Barcelona, Spain) Model 210 oven with heating range between 20 and 200°C was also used.

A modular 1100 Hewlett-Packard liquid chromatograph (Pittsburgh, PA, USA), consisting of a G1311A high-pressure quaternary pump, a G1322A vacuum degasser, a 7725 Rheodyne high-pressure manual injection valve (HPIV) and a G1315A diode array detector, was used for separation/detection of the target analytes.

A vacuum pump (Vac Elut SPS 24, Varian, P.S. Analytical, UK) and a rotary evaporator were used for development of the batch method.

### 2.2. Materials

Malvidin-3-glucoside (Mv), cyanidin-3-glucoside (Cy) and peonidin-3-glucoside (Pe) (Extrasynthèse, Genay, France) were the target anthocyanins. Methanolic solutions [50 mg/l of each anthocyanin, containing one drop of HCl (Panreac, Barcelona, Spain) in order to obtain total dissolution] were prepared. From these and by appropriate dilutions in water containing 16% acetonitrile (Merck, Darmstadt, Germany) were prepared the solutions of the required concentrations. The solutions were stored at 4°C. $C_{18}$ HPLC sorbent (Sigma, St. Louis, MO, USA) and $C_{18}$ Bond Elut cartridges (Varian, Harbor City, CA, USA) were also used for the proposed and batch method, respectively.

The nitrogen gas used for dragging was supplied by Carburos Metálicos (Barcelona, Spain).

Acetonitrile and 10% formic acid (both HPLC grade and supplied by Merck) aqueous solution were used as mobile phases. Methanol and ultrapure water at pH 7.0 were used for conditioning and regeneration of the chromatographic column.

Ultrapure water obtained from a Millipore (Bedfore, MA) Milli-Q plus system was also used.

### 2.3. Chromatographic separation/photometric detection

The individual separation of the anthocyanins was performed on a 250 mm×4.6 mm I.D., 5 μm particles, Ultrabase $C_{18}$ analytical column (Scharlau) using an injection volume of 20 μl and a flow-rate of 1 ml/min of the mobile phase composed of acetonitrile–10% aqueous formic acid solution. The programme of the gradient elution allowed the change from 5 to 9% $CH_3CN$ in 5 min, 9 to 15% $CH_3CN$ in 25 min and 15 to 40% $CH_3CN$ in 10 min. The total chromatogram time was 40 min, recorded at 520 nm.

### 2.4. Batch method

The batch method involved three steps: liquid–solid extraction of the anthocyanins, concentration in a rotary evaporator and HPLC separation with photometric detection.

After conditioning of the $C_{18}$ Bond Elut cartridges with ultrapure water at pH 7.0, the liquid–solid extraction was carried out with the aid of a vacuum pump. A 7-ml volume of each, methanol and water, were passed through the cartridge for conditioning and then, 2 ml of the sample (wine), followed by 8 ml of 16% aqueous acetonitrile solution for elution of the retained species. The 8 ml of eluate was collected and introduced into a rotary evaporator until total dryness. A 2-ml volume of methanol was then added for reconstituting the dry extract. This methanolic solution was injected into the chromatograph under the previously described conditions.

### 2.5. Proposed method

Fig. 1 shows the manifold used and Table 1 the values of the variables. Initially, and in order to avoid both the solutions for conditioning and sample matrix reaching the evaporation unit, the selection valve $SV_2$ was in position (2) or waste position. The $N_2$ stream was continuously circulated through the evaporation unit and the bottom valve was closed. Meanwhile, valve $SV_1$ was switched from position (1) to (2) and (3) keeping 3 min in positions (1) and (2) for regeneration and conditioning of the mini-column and 1 min in position (3) for circulation of

Table 1
Optimisation of variables

| Step | Variable | Tested range | Optimum value |
|---|---|---|---|
| Liquid–solid extraction | $Q$ retention (ml/min) | 1.5–4.0 | 2.0 |
| | $Q$ elution (ml/min) | 2–4 | 2 |
| | $V$ sample (ml) | 1–10 | 2 |
| | $V$ eluent (ml) | 2–8 | 8 |
| | $\Delta t_1$ (min) | 0–20 | 8 |
| | $\Delta t_2$ (min) | – | 1 |
| Evaporation | Temperature (°C) | 100–140 | 140 |
| | $t$ evaporation (min) | 2–15 | 5 |
| | $Q$ $N_2$ (ml/min) | 0–600 | 600 |
| | $V$ eluate (ml) | 2–4 | 2 |
| Chromatographic separation | Injection volume (HPIV) (μl) | – | 20 |
| | Flow-rate (10% aqueous formic acid–$CH_3CN$) (ml/min) | – | 1 |
| | $\lambda$ (nm) | – | 520 |
| | Elution (see text) | | |

the sample. Then, $SV_2$ was switched to position (1), thus allowing passage of the eluate to the evaporation unit when channel (4) was selected. After 1 min of passage of the eluate to the evaporator (located in the oven at 140°C), $SV_2$ was turned to position (2) and the acetonitrile solution was allowed to pass for 3 min more in order to remove any rest of retained compounds. The evaporation time was 5 min, after which the bottom valve was open and the concentrated acetonitrile solution was allowed to fill the HPIV of the chromatograph. The delay time between switching the bottom valve and the HPIV valve was 5 s; after which the chromatographic step was developed for 40 min. During this interval regeneration of the $C_{18}$ minicolumn cartridge was carried out by circulating for 3 min each, water and methanol by selecting positions (2) and (1), respectively, in $SV_1$.

## 3. Results and discussion

First, and in order to have a reference value, the figures of merit of the batch method were calculated. Calibration curves for the three anthocyanins (Cy, Pe, Mv) were run using a series of six standards with concentrations between 0.5 and 16, 0.5 and 16 and 1.0 and 60 mg/l of Cy, Pe and Mv, respectively. The linear ranges obtained and the figures of merit for each anthocyanin are summarised in Table 2, where the RSD values were calculated from six samples of

Table 2
Features of the method

| Method | Equation[a] | $r$ | Range (mg/l) | RSD (%) | LOD (mg/l) |
|---|---|---|---|---|---|
| *Batch* | | | | | |
| Mv | $A=1121.5+24.43x$ | 0.9994 | 10–50 | 15 | 0.4 |
| Pe | $A=296.1+36.25x$ | 0.995 | 0.5–16 | 15 | 0.3 |
| Cy | $A=80.65+43.43x$ | 0.98 | 0.5–16 | 15 | 0.3 |
| *Continuous* | | | | | |
| Mv | $A=675+56x$ | 0.993 | 1.0–60 | 10 | 0.2 |
| Pe | $A=315.6+109.1x$ | 0.99 | 0.5–16 | 15 | 0.2 |
| Cy | $A=373+57.5x$ | 0.99 | 0.5–16 | 10 | 0.2 |

[a] Concentration expressed in mg/l; $A$ indicates the peak area.

red wine injected in triplicate and the limit of detection (LOD) was calculated using the regression standard deviation of the instrument signal [25].

Then, the optimisation study for development of the continuous method was performed from the last to the first step; thus is, from the chromatographic separation to the liquid–solid extraction. The previous steps were developed in batch under the working conditions of the batch method as detailed under Experimental. All these studies were developed using red wine except identification and chromatographic separation of the target analytes which were performed with standard solutions of the analytes. The univariate method was used in all instances.

### *3.1. Optimisation of the chromatographic step*

The usual concentrations of cyanidin-, peonidin- and malvidin-3-glucoside in wines range between 1 and 6, 0.001 and 10 and 6 and 60 mg/l, respectively. These wide ranges are a consequence of the changes in concentration these compounds undergo as a function of both the elaboration step of wine and the type of grape involved.

For identification and optimisation of the retention time of each analyte, solutions of 6 mg/l of Cy, 5 mg/l of Pe and 32 mg/l of Mv were prepared in 16% aqueous acetonitrile solution from the standard methanolic solutions described under Experimental. The chromatographic conditions found previously [7] (namely: flow-rate, 1 ml/min: $\lambda$=520 nm; mobile phases, 10% aqueous formic acid aqueous–acetonitrile; elution programme, 5–9% acetonitrile in 5 min, 9–11% acetonitrile in 10 min, 11–15% acetonitrile in 25 min, 15–20% acetonitrile in 10 min and 20–30% acetonitrile in 15 min and 30–40% acetonitrile in 5 min) were used as the starting point. After location and proper separation of the analyte peaks using standards, a red wine subjected to the batch liquid–solid extraction and evaporation steps was used for subsequent studies. More than 15 peaks appeared in addition to those of the analytes when the concentrated, reconstituted eluate was injected. Due to the shorter retention time of the target analytes with respect to those of other co-eluted species, the chromatographic step was shortened by changing the programme gradient for a fast and common elution of the co-eluted species after obtaining the peaks of the analytes. Foreseeably these co-eluted species are anthocyanins, as these must be the only compounds in the extract after pretreatment. The retention times obtained for the analytes were: 11.68, 19.88, 22.28 min for Cy, Pe and Mv, respectively. The chromatogram, obtained under the optimum working conditions as stated under Experimental and summarised in Table 1, is shown in Fig. 3.

### *3.2. Optimisation of the continuous evaporation step*

As the evaporation unit used for the concentration of the anthocyanins was built of PTFE, checking of the behaviour of PTFE with the temperature in the presence of the organic solvents required for development of the method was a mandatory first step of this study. A PTFE piece (4 cm diameter and 5 mm thickness) was plunged into acetonitrile and introduced in the oven at different temperatures within 100–160°C for 30 min (sequentially, this repeated by plunging the piece into methanol and then into 10% aqueous formic acid solution). Up to 150°C the PTFE piece was unaltered; above this temperature less rigid texture was detected. Thus, the optimisation study of this variable for evaporation of the eluent did not surpass 140°C in order to avoid deterioration of the evaporation unit. After this preliminary test, the optimisation of variables affecting the evaporation system (temperature, $N_2$ flow-rate, evaporation time and volume of eluate in the unit) was carried out. Anthocyanins from red wines were extracted in a continuous fashion using the following conditions: flow-rate 3 ml/min; 2 min 30 s of methanol; 2 min 30 s of water; 40 s; 2 min 40 s of acetonitrile solution. The first 2 ml of eluate was used for manual injection into the evaporation unit.

The evaporation temperature was studied by fixing the oven temperature between 100 and 140°C. The 2 ml of eluate was manually injected into the chamber and subjected to different temperatures for 10 min with at a nitrogen flow-rate $Q(N_2)$=300 ml/min; then, the concentrated was collected in a vial and injected into the chromatograph. The analytical signal (peak height) increased when the temperature increased. A temperature of 140°C was selected as optimum.

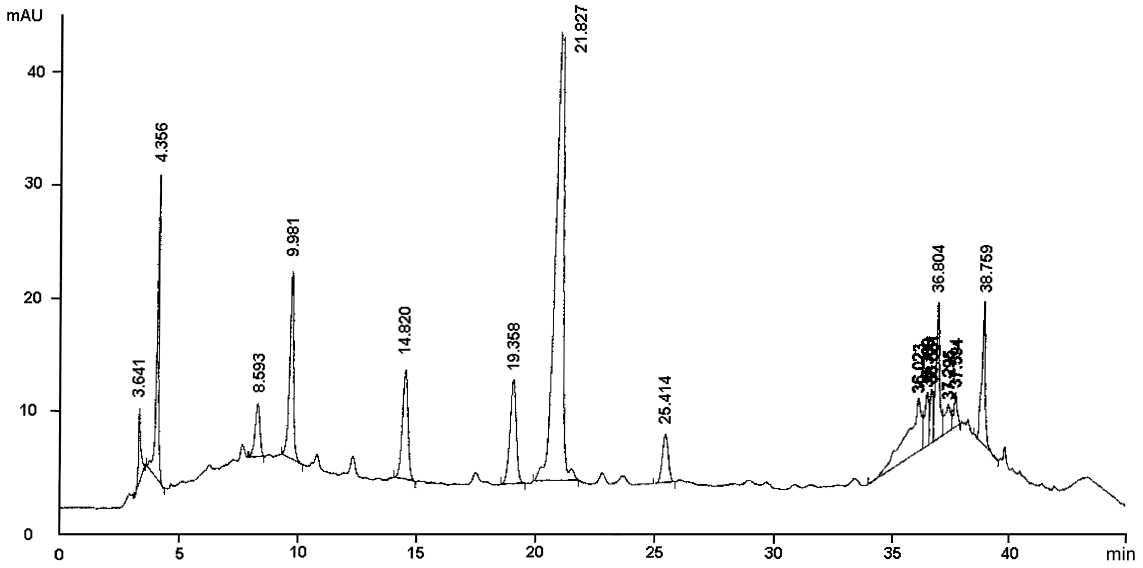


Fig. 3. Wine chromatogram obtained under the optimal conditions. Time scale in min.

The evaporation time, studied between 2 and 15 min, showed an increased signal of the peaks obtained after chromatographic separation with the time. An evaporation time of 10 min led to an extract volume of 200 μl, not sufficient for ensuring reproducible automatic rinsing and filling of the high-pressure injection valve. An evaporation time of 5 min was selected as a compromise between sensitivity and sampling frequency.

The $N_2$ flow-rate was also optimised in the range 0–600 ml/min. The speed of the dragging increased with the $N_2$ flow-rate as a consequence of the displacement of the equilibrium of the eluent between the liquid and vapour state. The chromatographic signal obtained from the extracts drastically increased when the flow-rate of the dragging gas changed between 0 and 200 ml/min and the increase was slight for flow-rates between 200 and 600 ml/min.

The volume of eluate introduced into the evaporation unit was also studied. With this aim volumes of eluate between 2 and 4 ml from the minicolumn were collected and, after locating in the evaporation unit the spacers necessary for maintaining its inner volume, injected into this module. The evaporation time was doubled for volumes of eluate of 4 min and accordingly for volumes between 2 and 4 ml for comparable final volumes. Smaller chromatographic signals were obtained for volumes different from 2 ml as the concentrate from the 4 ml extract had a volume double that from the 2 ml extract and so on. This fact showed that the most concentrated eluate corresponded to the first portions, as was foreseeable.

### *3.3. Optimisation of the continuous liquid–solid extraction step*

The study of the solid-phase extraction was performed using both the overall manifold as it appears in Fig. 1 and the values of the variables previously optimised (see Table 1). First, variables such as retention and elution flow-rates were studied without connection between the minicolumn and the evaporation unit by manual collection the eluate from the column because of the difficult in collecting in the evaporation unit always the same portion of the eluate plug when the flow-rate changed.

In order to study the retention flow-rate, 2 ml of sample was aspirated to the $C_{18}$ minicolumn at flow-rates ranging between 1.5 and 4.0 ml/min. The chromatograms obtained showed an increase of the peaks when the flow-rate decreased from 4.0 to 2.0 ml/min, thus demonstrating the influence of the

retention kinetics. Flow-rates between 1.5 and 2.0 ml/min provided very similar results; so a flow-rate of 2 ml/min was selected for subsequent experiments. Lower flow-rates were not tested in order to achieve a not very low sampling frequency.

The elution flow-rate was studied in the same range of retention and the same behaviour was observed. Once more, the kinetics of the process, in this case those of elution, must be taken into account.

After the above study, the minicolumn and the evaporation unit were connected as shown in Fig. 1 and the rest of the variables (namely, the delay times $\Delta t_1$ and $\Delta t_2$ and the volumes of the sample and eluent) were optimised.

The delay times were $\Delta t_1$ [interval between switching valve $SV_1$ to position (4), or elution position, and switching valve $SV_2$ for sending the eluate to the evaporation unit] and $\Delta t_2$ [interval between the change of $SV_2$ to position (1) and switching again to position (2) for sending the rest of the eluate to waste]. The optimum values found are summarised in Table 1. Taking into account the optimum flow-rates and short connections between the sorption minicolumn, selection valves and evaporation unit (not more than 10 cm in each case), it can be concluded that, as foreseeable, the head portion of the eluate contained the highest concentration of retained species.

In order to determine the breakthrough of the $C_{18}$ minicolumn, the volume of sample aspirated to the sorption material was studied in the range between 1 and 10 ml. The signal increased up to 8 ml and remained constant for higher volumes. A 2-ml volume of sample was selected in order to not surpass the retention capacity of the minicolumn in the case of wines with higher content in anthocyanins.

The volume of eluent (acetonitrile) necessary for total elution of the species retained in the minicolumn was tested between 2 and 8 ml. Portions of eluate from the head of the plug, medium zone and tail were led to the evaporation unit for checking. The tail portion of an eluent volume of 8 ml did not contain any retained species (baseline chromatogram); so, this volume was circulated through the minicolumn after each sample. From this the 2 ml of the head was led to the evaporation unit and the rest to waste. Then, the minicolumn was regenerated by circulating through it water and methanol for 3 min each.

### *3.4. Characterisation of the proposed method*

Calibration curves for each anthocyanin were run using similar standard solutions as for characterisation of the batch method. The figures of merit for each anthocyanin are summarised in Table 2, where a higher sensitivity of the proposed method is demonstrated from both the slope of the linear portion of the calibration curve and the detection limit for all the target analytes, and also for the lower quantitation limit in the case of malvidine. The linear ranges encompass the concentrations of the target analytes in red wines in all instances.

Under the optimal working conditions the sampling frequency was limited for the duration of chromatogram as the previous steps of each analysis can be developed during the chromatographic step of the previous sample.

### *3.5. Application of the method to natural samples*

In order both to validate the method and demonstrate its usefulness, red Spanish wines from different Apellations d'Origin were subjected to the proposed method and the results of the anthocyanins content were compared with those obtained by the standard addition, due to the lack of official methods for the determination of anthocyanins. Three additions were made to aliquots of each sample (with three replicates of each) and the concentrations of the three studied anthocyanins were in agreement with those found by interpolation in the calibration curve obtained from the standards, thus demonstrating the absence of matrix effects on the proposed method. The results obtained are shown in Table 3.

## 4. Conclusions

The assembly of three continuous separation techniques (namely, sorption, evaporation and chromatography) is proposed for the first time and applied

Table 3
Application of the method to wines

| Wine[a] | Amount of anthocyanins found | | | | | |
|---|---|---|---|---|---|---|
| | By the calibration curve (mg/l) | | | By standard addition (mg/l) | | |
| | Cy | Pe | Mv | Cy | Pe | Mv |
| 1 | 1.8±0.2 | 1.10±0.09 | 12±0.9 | 1.7±0.2 | 0.9±0.1 | 11.0±0.8 |
| 2 | 1.4±0.2 | 0.61±0.03 | 5.3±0.2 | 1.2±0.2 | 0.63±0.06 | 5.1±0.2 |
| 3 | 2.8±0.2 | 0.92±0.06 | 9.0±0.2 | 2.6±0.2 | 0.9±0.1 | 8.8±0.3 |
| 4 | 3.1±0.2 | 0.50±0.02 | 4.5±0.2 | 3.0±0.2 | 0.52±0.06 | 4.4±0.2 |
| 5 | 1.1±0.2 | 0.52±0.03 | 5.1±0.2 | 0.9±0.2 | 0.51±0.05 | 5.0±0.3 |
| 6 | 0.54±0.02 | 0.15±0.02 | 3.2±0.2 | 0.5±0.1 | 0.10±0.02 | 3.1±0.2 |

[a] Wines from Rioja, 1, 2; Valdepeñas, 3,4; Valladolid, 5; Navarra, 6.

to the determination of anthocyanins in red wine, thus achieving development of the overall process in a continuous fashion, which can be easily automated.

The method thus developed shows a better sensitivity than that of the batch method (both higher slope of the linear portion of the calibration curve and better detection limit), with smaller relative standard deviation and wider linear range and lower quantitation limit for the anthocyanin with higher concentration in wines (malvidin-3-glucoside). The steps previous to the chromatographic separation are shorter in the proposed method; nevertheless, the duration of the chromatogram is similar in both methods.

The use of in-line evaporation is proposed for the first time and a module for continuous evaporation was designed and built with this aim. The excellent performance of the evaporator calls for an extension of its use to other continuous methods which require a preconcentration step of non-thermolabile analytes.

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