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Autoanalyzer for continuous fractionation and quantitation of the polyphenols content in wines

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Abstract

A simple continuous flow autoanalyzer for the on-line fractionation of the polyphenols content in wines is proposed. The target compounds are isolated from the matrix by solid-phase extraction on an RP-C_{18} sorbent column, using selective solvents for the sequential elution of each polyphenol family. Moreover, evaporative light scattering detection (ELSD) is used for the first time for the on-line monitorization of the three polyphenol fractions present in the wine samples. Thus, a single sample injection is required to determine the global concentration of the three selected polyphenol fractions and the whole analysis is completed within a few minutes. Three calibration graphs were constructed for quantitative analysis of the global compounds concentration in every fraction and covered the range 5–300 mg l^{-1} (expressed as gallic acid). Average repeatability, expressed as relative standard deviation, was 4%. The proposed autonalyzer was applied to the analysis of a variety of commercial wine samples. The results obtained were compared with those provided by the Folin–Ciocalteau method, being similar in all instances.

Keywords: Polyphenol content; Red wines; Automatic fractionation; Solid phase extraction; Evaporative light scattering detection

1. Introduction

The presence of wine in human culture goes back to 6000 years, carrying out very important social and religious functions [1,2]. The chemical composition of wine is too complex, containing more than 500 different compounds in a wide range of concentrations [3]. It has been proved that a moderate consumption of wine is related to a decrease in the risk of cardiovascular disease [4], what is summarized in the so-called French Paradox [5,6]. This positive influence may be due to various factors, mainly alcoholic and phenolic contents.

Phenols are characterised by the presence of hydroxyl groups in its structure, linked to aromatic rings. They are present in the grape (skin and seeds), but they may be produced by yeast metabolism and could be extracted from the oak barrels in which the wine is stored. They are also affected by the climatic conditions and vinification process. From the point of view of quality , this family of compounds affects directly to the sensorial properties of wine, as colour, astringency or bitterness [7]. Moreover, they are involved in a protective effect on cardiovascular [8] and neurodegenerative disease. Phenolic compounds have high antioxidant capacity [9] and they are excellent free radical scavengers [10]. Some researches have demonstrated that these type of compounds reduce the peroxide concentration in plasma, LDL oxidation and thrombosis risk. The determination of this group of compounds can help to identify variations in wine types and differences in winemaking and maturation processes as well.

Polyphenols can be divided into different families according to either the polarity or the molecular weight. Most of the methodologies proposed for the determination of phenolic compounds in wine involve liquid chromatographic [11–14] or electrophoretic [15,16] separation of the target analytes using diode array (DAD) or fluorimetric detection.

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To improve the resolution of the separation, a previous solid phase extraction (SPE) step is usually required. The main disadvantage of these methods is that they are tedious and time-consuming. In order to avoid these inconveniences, different fractionation models have been established in routine analysis as those proposed by Glories [17], Oszmianski et al. [18] and, Di Stefano and Cravero [19]. In these models the first step is the retention of analytes in a sorbent material, followed by a sequential elution with solvents of different polarity. Within the protective effects of phenolic compounds, synergetic properties have been demonstrated; in this manner, the quantification of total concentrations of each family could be interesting for further studies.

In this paper a fractionation method, proposed by Oszmianski et al., is automated, carrying out the determination of three families: (I) procyanidins, catechins and anthocyanin monomers, (II) flavonols and (III) anthocyanin polymers. The method uses a continuous flow manifold, which includes an on-line SPE step, directly coupled to an evaporative light scattering detector (ELSD). Its quasi-universal response provides a global signal for each of the polyphenol fractions and thus avoids chromatographic separation. For this purpose, dilute wine samples adjusted to pH 7 are pumped through an RP-C_{18} minicolumn. After a washing step with water, three sequential and selective elutions were performed, each fraction being sequentially monitored by the ELSD using a single sample aliquot.

2. Experimental

2.1. Reagents and samples

All reagents were of analytical grade or better. HPLC gradient grade organic solvents (acetonitrile, ethyl acetate, methanol and ethanol) were supplied by Scharlau (Barcelona, Spain). Gallic acid and sodium hydroxide from Sigma–Aldrich (Madrid, Spain) and Milli-Q ultrapure water (Millipore Corp., Madrid, Spain) were also used.

A total of 18 wine samples, commercially available were analyzed. Once opened, wine samples were transferred to two 100 ml amber glass bottles (no headspace volume was left in order to prevent analyte losses) and stored in the dark at 4 °C. Replicated analysis were carried out within a few days to avoid storage damage of the samples. Aliquots were filtered through a 0.45 μm nylon filter and diluted if necessary. Samples were adjusted manifold to pH 7 with sodium hydroxide prior to their injection into the flow.

Folin–Ciocalteu reagent and sodium carbonate decahydrate and anhydrous (all from Merck, Darmstadt, Germany) were also used. The sodium carbonate saturated solution for the Folin–Ciocalteu method was prepared as follows: 35 g of Na_2CO_3 was dissolved in 100 ml of water by heating at 70–80 °C; the solution was allowed to cool overnight and the supersaturated solution was seeded with crystals of $Na_2CO_3{\cdot}10H_2O$ and filtered through glass wool after crystallization.

2.2. Apparatus

The flow system consists of a Hewlett Packard 1050 high pressure quaternary pump for solvents [water, acetonitrile 16% (v/v) in water, ethyl acetate and methanol] delivery; a six port LC injection valve (Knauer 6332000) fitted with a 1 ml PTFE sample loop and a DDL 31 evaporative light scattering detector (Eurosep, Cergy-Pontoise, France) for monitoring of analytes. The temperature of the ELSD evaporation chamber was set at 65 °C and compressed air (at 2 bar) was used as nebulizing gas. Gain detector was set at 700, 550 and 650 V (depending on the polyphenol fraction being analyzed) and was changed during the analysis. The sample loop was filled by means of a syringe, using on-line filtration through a commercial nylon filter (0.45 μm pore size). PTFE tubing of 0.5 mm I.D. for coils, and standard connectors were also employed. The flow system was connected to the ELSD by means of a 50 cm × 0.1 mm I.D. PEEK tubing. For retention of analytes, a laboratory-made RP-C_{18} column was constructed by packing 40 mg of the sorbent into a 3 cm × 4 mm I.D. PTFE tube using small cotton beads to prevent material losses. Signals were acquired using a Radiometer (Copenhagen, Denmark) REC 80 Servograph recorder and peak height was used as analytical signal.

2.3. Official method

The standard method for polyphenols determination in wines was implemented in accordance with the AOAC's recommendation [20]. A working solution of 40 mg l^{-1} of gallic acid (in water) was used for the construction of the calibration curve. Different volumes of this solution were placed in a 5 ml volumetric flask, and 250 μl of Folin–Ciocalteu reagent and 1 ml of sodium carbonate saturated solution were added; the volumetric flasks were made up to the mark with Milli-Q water. The calibration curve was run for solutions containing 0–9 mg l^{-1} of gallic acid ($n = 12$). The product was monitored at 750 nm 30 min after sample preparation.

2.4. Autoanalyzer functioning

The autoanalyzer, based on the Oszmianski et al. fractionation model (Fig. 1), operates in a sequential fashion. Initially the loop of the injection valve (1 ml) was filled with the dilute wine sample adjusted to pH 7, while a distilled water stream at a flow rate of 1.4 ml min^{-1} was directly introduced into the ELSD to obtain the baseline. Then the injection valve was switched to the inject position and the sample, carried by a Milli-Q water stream, passed through the RP-C_{18} sorbent column at a flow rate of 1.4 ml min^{-1}. Polyphenols were quantitatively retained while other matrix components (phenolic acids and sugars) were driven to the detector; the aqueous stream was allowed to pass through the column for

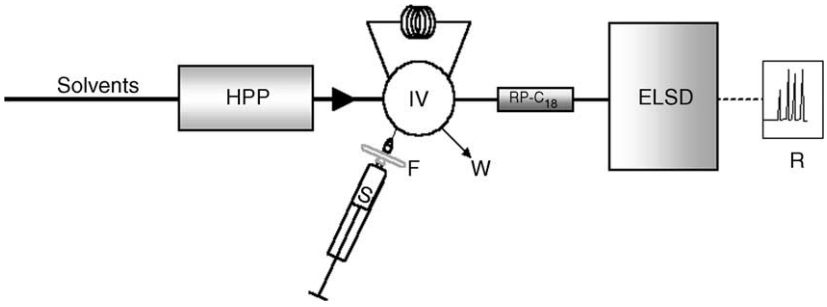

Fig. 1. Schematic diagram of the continuous manifold designed for the on-line fractionation and quantitation of polyphenols in wine. HPP, high pressure pump; IV, injection valve; S, sample; F, filter; W, waste; ELSD, evaporative light scattering detector; R, recorder.

3 min (washing step). Next, the high-pressure pump delivers the solvents in a sequential fashion. First, an acetonitrile stream [16%, v/v in Milli-Q water] passes through the column at a flow rate of 1.4 ml min^{-1} for 4 min, eluting the first polyphenol fraction, composed of procyanidins, catechins and anthocyanin monomers. Secondly, flavonols (second fraction) were eluted by means of an ethyl acetate stream also at a flow rate of 1.4 ml min^{-1} (1 min). The third fraction, containing anthocyanin polymers was removed from the RP-C_{18} column by using pure methanol stream at 1.0 ml min^{-1} (2 min). All the fractions were monitored in the ELSD and the sequential determination of the three parameters was completed in ca. 10 min. After each analysis, a water stream was passed through the system at a flow rate of 1.4 ml min^{-1} (3 min) for clean-up and sorbent column conditioning.

3. Results and discussion

3.1. Selection of the instrumental conditions

The instrumental conditions were optimized by using a dilute red wine sample as the standard and a flow configuration similar to that depicted in Fig. 1. Each sample was properly diluted in water (pH 7, adjusted with NaOH), and 1 ml was injected into the autoanalyzer. The instrumental parameters affecting the determination of analytes were the evaporative chamber temperature, the nebulizing gas pressure and the photomultiplier gain [21]. An univariant optimization model was used for this purpose. The evaporation chamber temperature must be selected as a compromise between uniformity of particle size generated and complete solvent evaporation (giving negligible noise). It was studied in the interval 55–85 °C, of 65 °C being the optimum value. The nebulizing gas pressure (air flow rate) affects the uniformity and size of the droplets formed, and was studied in the range 0.5–2 bar. Peak heights for the phenolic fractions increased up to 1.7 bar, remaining constant over this value; a working pressure of 2 bar was therefore selected. The critical parameter for this application was the photomultiplier gain, taking into account the different concentration level of the analytes in the three fractions. For this reason different optimum values were selected for each fraction (700 V for procyanidins, catechins and anthocyanin monomers; 550 V for flavonols and 650 V for anthocyanin polymers) and the photomultiplier gain was varied during/between analyses.

The nebulizer was cleaned monthly by passing an acetone stream through the detector at a flow rate of 2.0 ml min^{-1} for ca. 10 min, keeping the air pressure at 2.5 bar and the evaporation chamber temperature at 100 °C.

3.2. Optimization of chemical and flow variables

Initially, the chemical variables proposed in the manual alternative [18] were adopted, namely: samples adjusted to pH 7, RP-C_{18} as sorbent material, 16% acetonitrile at pH 2, ethyl acetate, and methanol for the elution of first, second and third fractions, respectively. A laboratory-made sorbent column packed with 50 mg of solid was inserted into the flow manifold for optimization purposes. It is well known that the RP-C_{18} material is not stable under strong acid or alkaline conditions, being a serious inconvenience when the sorbent column is coupled on-line to the detector as the degradation products can affect baseline stability and block the detector; the sorbent properties are changing between successive analysis as well. Therefore, the composition of the first eluent was studied. For this purpose, different aliquots of the standard red wine were introduced into the flow system and the signal corresponding to the first polyphenol fraction was evaluated using acetonitrile 16% (v/v) in water with and without pH adjustment. As negligible difference was obtained under the experimental conditions, no hydrochloric acid was added to prevent sorbent degradation, and therefore, increasing the stability of the column.

Concerning the hydrodynamic variables, the flow rates for the four streams were studied within the interval 0.5–2.0 ml min^{-1}. The signal behaviour was similar in all cases; the signal increased with the increasing flow rates reaching a steady-state over certain value, being optimum 1.4 ml min^{-1} for water, acetonitrile 16% (v/v) in water and ethyl acetate streams, and 1.0 ml min^{-1} for the methanol one. Finally, the amount of sorbent material was evaluated between 15 and 75 mg. The signal increased with the increasing amount of sorbent up to 30 mg, beyond which it remains virtually constant. A value 40 mg was adopted for further experiments.

Table 1
Figures of merit of the proposed autoanalyzer for the determination of polyphenol fractions in wine

Fraction	Regression equation[a]	Linear range ($mg\,l^{-1}$)	RSD (%)
I	$\log S = 1.58 \log C - 3.4$	30–300	2.8
II	$\log S = 1.49 \log C - 2.4$	6–60	3.8
III	$\log S = 1.86 \log C - 2.6$	5–50	5.1

[a] S: analytical signal (V); C: concentration ($mg\,l^{-1}$ expressed as gallic acid).

3.3. Analytical performance

The analytical figures of merit for the method were established by using the autoanalyzer depicted in Fig. 1. The calibration graphs for the three families of compounds studied were constructed by injecting dilute wine aliquots in the autoanalyzer (wine sample 14, Table 3), as previously analyzed by the Folin–Ciocalteau method (to determine the global concentration of polyphenols in each fraction). The calibration curve for each fraction was constructed by using 10 wine aliquots [diluted between 1:40 and 1:1, v/v] and analyzed by triplicate ($n = 30$). When the ELSD is used, it was assumed that in a large range of sample sizes, the measured analytical signal (S) can be related to the samples mass by the following relationship:

$$S = am^b$$

where a and b are coefficients that depend on droplet size, concentration and nature of solute, evaporation temperature, etc. In the present method, the peak height was selected as the quantitative signal, which was related to the concentration by a double logarithmic expression. The figures of merit for the calibration graphs are summarized in Table 1. The precision, expressed as relative standard deviation, calculated for 11 replicates of a dilute wine sample was acceptable in all instances.

3.4. Analysis of wine samples

To demonstrate the applicability of the proposed autoanalyzer, 13 commercial rosé and red wine samples were analyzed. Samples were diluted five times (to include the polyphenol concentration in each fraction within the respective calibration interval), adjusted to pH 7 and 1 ml was injected into the automated system. The results obtained for the three polyphenol fractions are listed in Table 2. Average concentrations were calculated from five individual amounts of each sample and all determinations were made in triplicate ($n = 15$). As expected, the lowest concentrations of polyphenols corresponded to rosé wine.

In order to validate the proposed autoanalyzer, a parallel set of five samples (wine samples 14–18) was also analyzed following the continuous fractionation procedure, and determining the total polyphenol contents in each fraction by the Folin–Ciocalteau method described under Section 2. Briefly, the on-line trace enrichment was carried out as follows. The wine sample to be fractioned (typically 1 ml of wine dilute five times and adjusted to pH 7 with NaOH) was applied to an RP-C_{18} column and after a washing step with water at pH 7 (to remove phenolic acids and sugars), the retained polyphenols were sequentially eluted by using the same solvents as in the automatic method. The organic solvents were collected in 5 ml volumetric flasks containing 2 ml of distilled water and evaporated to dryness under a nitrogen stream as they dropped into the flasks. Once the solvent changeover step was completed, the volumetric flasks were filled to the mark with distilled water. Different volumes of these solutions (depending on the polyphenol concentration) were transferred to 5 ml volumetric flasks and the Folin–Ciocalteau method followed. The results obtained for the five samples analyzed (four red and one rosé wines) are listed in Table 3. A direct comparison of the data reveals the usefulness of the proposed alternative for the on-line fractionation-detection of polyphenols in wine samples taking into account the similar concentrations found using either continuous or manual procedures.

Table 2
Analysis of the three phenolic fractions of rosé and red wine samples using the proposed autoanalyzer ($n = 15$)

Sample	Variety	Alcohol content (%, v/v)	Fraction I ($mg\,l^{-1}$)[a]	Fraction II ($mg\,l^{-1}$)[a]	Fraction III ($mg\,l^{-1}$)[a]
1	Tempranillo (95%)	12.5	448 ± 14	153 ± 6	83 ± 5
2	Tempranillo (100%)	13.0	579 ± 18	241 ± 10	114 ± 6
3	Tempranillo (85%)	12.0	482 ± 15	162 ± 6	79 ± 5
4	Tempranillo (100%)	13.0	486 ± 15	183 ± 9	101 ± 5
5	Tempranillo (95%)	12.5	591 ± 20	173 ± 8	92 ± 5
6	Tempranillo (80%)	12.5	526 ± 20	161 ± 6	100 ± 6
7	Tempranillo (100%)	12.5	568 ± 20	156 ± 6	92 ± 5
8	Tempranillo (100%)	13.5	695 ± 25	225 ± 10	172 ± 10
9	Tempranillo (100%)	13.0	690 ± 25	212 ± 10	153 ± 9
10	Tempranillo (85%)	13.0	650 ± 20	190 ± 10	134 ± 8
11	Merlot (100%)	13.0	710 ± 30	270 ± 12	182 ± 10
12	Cabernet Sauvignon (100%)	13.0	800 ± 30	211 ± 10	172 ± 10
13[b]	Garnacha (100%)	12.5	180 ± 5	46 ± 2	23 ± 1

[a] Concentration expressed as gallic acid.
[b] Rosé wine samples.

Table 3
Analysis of the three phenolic fractions of rosé and red wine samples using the Folin Ciocalteau method ($n = 5$)

Sample	Variety	Alcohol content (%)	Total polyphenol (mg l^{-1})[a]	Fraction I (mg l^{-1})[a]		Fraction II (mg l^{-1})[a]		Fraction III (mg l^{-1})[a]	
			FC[b]	Autoanalyzer	FC[b]	Autoanalyzer	FC[b]	Autoanalyzer	FC[b]
14	Cabernet Sauvignon (100%)	13.5	1130 ± 50	690 ± 20	700 ± 30	244 ± 10	255 ± 15	184 ± 10	175 ± 12
15	Tempranillo (95%)	12.0	687 ± 30	465 ± 15	470 ± 20	120 ± 5	127 ± 10	94 ± 5	90 ± 6
16	Tempranillo (100%)	12.5	777 ± 40	540 ± 18	550 ± 25	121 ± 5	115 ± 8	108 ± 6	112 ± 8
17[c]	Garnacha (100%)	13.0	219 ± 10	158 ± 5	149 ± 8	37 ± 2	40 ± 3	27 ± 1	30 ± 2
18	Tempranillo (95%)	13.0	772 ± 40	506 ± 15	500 ± 20	187 ± 9	185 ± 13	91 ± 5	87 ± 6

[a] Concentration expressed as gallic acid.
[b] Folin–Ciocalteau method.
[c] Rosé wine sample.

4. Conclusion

The autoanalyzer developed permits the continuous fractionation of polyphenols present in wines into three main groups with specific chemical properties. The method provides a global response for each fraction making unnecessary chromatographic separation. The on-line coupling of a solid-phase extraction configuration to an ELSD is rather simple and robust with the inherent advantages of the automate devices. The proposed autoanalyzer surpassses the manual alternative in terms of sample and reagents (sorbent material and organic solvents) consumption and sample throughput. The performance of this alternative was validated by direct comparison of the data obtained with those provided by the conventional methods for the same samples with satisfactory results.

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