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Analytical Methods

Development of a simple fluorescence-based microplate method for the high-throughput analysis of proline in wine samples

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ABSTRACT

This paper presents a simple, accurate and multi-sample method for the determination of proline in wines thanks to a 96-well microplate technique. Proline is the most abundant amino acid in wine and is an important parameter related to wine characteristics or maturation processes of grape. In the current study, an improved application of the general method based on sodium hypochlorite oxidation and *o*-phthaldialdehyde (OPA)-thiol spectrofluorometric detection is described. The main interfering compounds for specific proline detection in wines are strongly reduced by selective reaction with OPA in a preliminary step under well-defined pH conditions. Application of the protocol after a 500-fold dilution of wine samples provides a working range between 0.02 and 2.90 g L^{-1}, with a limit of detection of 7.50 mg L^{-1}. Comparison and validation on real wine samples by ion-exchange chromatography prove that this procedure yields accurate results. Simplicity of the protocol used, with no need for centrifugation or filtration, organic solvents or high temperature enables its full implementation in plastic microplates and efficient application for routine analysis of proline in wines.

1. Introduction

Amino acids in grapes and wines have various origins and their nature and concentration are of great importance on the wine-making process and quality of the final product (Arrieta & Prats-Moya, 2012; Hernandez-Orte, Ibarz, Cacho, & Ferreira, 2005; Soufleros, Bouloumpasi, Tsarchopoulos, & Biliaderis, 2003). Indeed, they can influence the growth of yeasts during fermentation, the fermentation rate or the production of aromatic compounds (Dukes & Butske, 1998). Among the amino acids pool, proline has been described as the most abundant in wine, accounting for 30–85% of the total amino acid content (Lehtonen, 1996). Moreover, it is generally acknowledged that proline is not used by yeasts as nutrient, and hence not metabolized during the fermentation process (Etievant, Schlich, Bouvier, Symonds, & Bertrand, 1988), although this assumption has been called into question in recent years and requires more thorough study (Peinado, Moreno, Maestre, & Mauricio, 2005; Salmon & Barre, 1998). Globally, proline is considered as a potentially interesting parameter for distinguishing wines according to grape variety and specific growth conditions (temperature, sun-exposed time, amount of rainfall, grape maturity), and it is thus related to origin identification, possible adulteration and quality of wines (Son et al., 2009). Furthermore, proline is also the major amino acid found in other food or beverage products such as beer or honey (Gorinstein et al., 1999; Mayadunne, Nguyen, & Marriott, 2005), and has been suggested as a key compound in stress adaptation of many plants (including grapevines) to conditions such as water and nutrient deficiency or temperature (Bertamini, Zulini, Muthuchelian, & Nedunchezhian, 2006; Verbruggen & Hermans, 2008).

Given these interests in proline determination, several analytical methods have been devised in the past decades for accurate measurement of this amino acid. Chromatographic methods such as gas chromatography-mass spectrometry (GC–MS) or pre-column derivatization high-performance liquid chromatography with fluorimetric detection enable determination of proline together with other amino acids (Heems, Luck, Fraudeau, & Verette, 1998; Mayadunne et al., 2005). Flow injection analysis (FIA) methodology and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) have also been reported for proline determination in wines (Cataldi & Nardiello, 2003; Costin, Barnett, & Lewis, 2004). Nevertheless, these methods are too cumbersome and expensive when high sample throughput is required for fast assessment of proline concentration in many samples, as it could be necessary within a broader study on the relationship between proline levels and wine characteristics. In this context, fast, simple and cost-effective methods in terms of instruments and reagents are required for the direct determination of proline.

A molecularly imprinted polymer-based chemiluminescence array sensor has recently been proposed, although selectivity

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towards other amino acids was limited (Chang, Zhang, & Yang, 2010). Direct spectrophotometric methods have also been described, based on reaction with ninhydrin (Chinard, 1952) or isatin (Grainger & Aitken, 2004). The ninhydrin method suffers from possible interferences from other amino acids or other compounds and requires quartz microplates for high sample throughputs (extraction with toluene) (Ringel, Siebert, & Wienhaus, 2003). Derivatization with isatin has proven to be more specific, although interferences have occurred at high sugar concentrations, and use of a microplate method could be strongly hindered by the need for boiling and evaporation to dryness (Long et al., 2012). Finally, spectrofluorometric measurement of proline after derivatization with *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol has been described and used successfully (Gorinstein et al., 1999; Yokotsuka, 1988; Yokotsuka, Kato, Ogbonna, & Amano, 1989). Nevertheless, the method is rather tedious and requires two filtration or centrifugation steps (use of activated carbon as pretreatment and final filtration of a yellow precipitate) that strongly prevent its application for microplate handling and for a high sample throughput.

With this aim in mind, we decided to devise a new simple spectrofluorometric method that could be fully and directly implemented in microplates. Similarly to Yokotsuka's procedure (Yokotsuka, 1988), our method involves oxidation of proline (secondary amino acid) with NaOCl to the corresponding primary amine, followed by formation of the corresponding fluorescent isoindole with OPA and a thiol compound. The main novelty of this work lies in the simple and selective quenching of other primary amines or amino acids that could otherwise strongly interfere in proline determination. To minimize these interferences, a preliminary reaction with OPA under neutral pH conditions is implemented. Development of the global procedure for microplate handling (depicted in Fig. 1) and validation on real wine samples are presented herein.

2. Experimental

2.1. Reagents and solutions

All chemicals were of analytical reagent grade and used without further purification. NaOCl solutions were prepared by dilution of a commercial sodium hypochlorite solution (5% w/v active chlorine) obtained from Acros Organics (Illkirch, France). OPA was obtained from Acros Organics and *N*-acetyl-L-cysteine (NAC) from Sigma–Aldrich (Saint-Quentin Fallavier, France). The OPA/NAC solutions were prepared by dissolving pure compounds in 0.05 M borate buffer (pH 10.5). The borate buffer was prepared by dissolving sodium tetraborate decahydrate ($Na_2B_4O_7.10\ H_20$) (Sigma–Aldrich) in ultrapure water (Millipore, resistivity > 18 MΩ cm) and pH was adjusted with 48% (w/v) sodium hydroxide. OPA solutions were prepared by dissolving appropriate amount of OPA in ethanol-0.2 M phosphate buffer (20:80, v/v). The phosphate buffers were prepared by dissolving potassium dihydrogen phosphate (Sigma–Aldrich) in ultrapure water. 10 mM proline (Sigma–Aldrich) stock standard solutions and working solutions were prepared in ultrapure water.

2.2. Microplate instrumentation

Microplate fluorescence measurements were carried out on a microplate reader (Infinite M200, Tecan France SAS, Lyon, France) equipped with an excitation and emission double monochromator (bandwidths of 9 and 20 nm for excitation and emission monochromator, respectively) and controlled by i-control™ software (Tecan). Fluorescence detection was performed from above the microplate wells (top configuration), at λ_{ex} = 330 nm and λ_{em} = 455 nm. Operating temperature was set to 30 °C. Other parameters were as follows: gain: 80; number of flashes: 5; integration time: 20 µs. Fluorescence intensities were expressed in arbitrary units (a.u.). Polystyrene black 96 flat-bottom well microplates (Fisher Scientific, Illkirch, France), with a maximum capacity of 375 µL for each wells were used.

2.3. Optimized protocol for proline determination in wine samples

Given high levels of proline in wines and potential interference due to the color of wine, samples were firstly diluted 500-fold in ultrapure water. 100 µL of these diluted wine samples or proline standard solutions were dispensed into the wells of the microplate, where 30 µL of OPA 30 mM in ethanol-0.2 M phosphate buffer pH 7.75 (20:80, v/v) were then added. The plate was shaken for 4 min, and 25 µL of NaOCl 2 mM in 0.2 M phosphate buffer (pH 7.75) was added in each well of the plate. After another shaking period of 2 min, 50 µL of a 3 mM OPA/ 6 mM NAC solution in 0.05 M borate buffer (pH 10.5) was added in each well and the mixture was shaken for 4 min. Fluorescence intensity was then recorded, with excitation and emission wavelengths set at λ_{ex} = 330 nm and λ_{em} = 455 nm, respectively. Proline concentrations in wine samples were determined using the linear calibration curve obtained with standards. All experiments were performed in duplicate.

2.4. Ion-exchange chromatography determination of proline

Proline concentration together with other primary amino acid compounds in selected wines was determined by an external laboratory using an automatic amino acid analyzer (Biochrom 30+, Cambridge, England). Selected wines were six red wines, seven white wines and two rosé wines all originating from different regions of France (more information about the wines in Table 1). Analysis were performed using manufacturer standard protocols. Wine samples were initially diluted in a sodium citrate buffer (pH 2.2). All amino acids were detected after post-column derivatization with Ninhydrin reagent. Elutes were spectrophotometrically monitored at 570 nm for primary amino acids and 440 nm for proline. Concentrations of amino acid compounds in unknown samples were determined by comparison with standard peak areas (Sigma–Aldrich amino acid standard kit) and by using norleucine as internal standard. All analyses were performed in duplicate. Ion-exchange chromatography analyses on real wine samples were

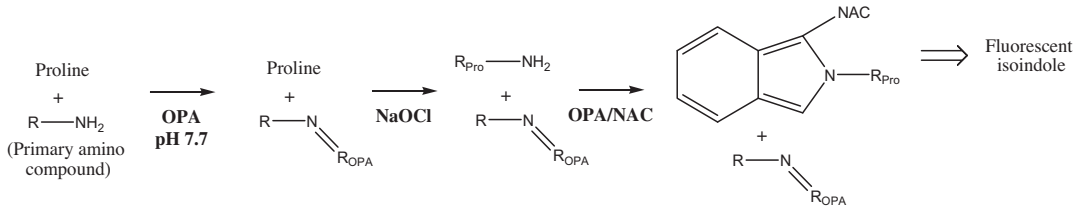

Fig. 1. Schematic depiction of the protocol for selective determination of proline. OPA = *o*-phthaldialdehyde; NAC = *N*-acetyl-L-cysteine.