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Original Article

HPLC analysis of diverse grape and wine phenolics using direct injection and multidetection by DAD and fluorescence

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Abstract

This paper proposes an HPLC method for analysing a great variety of phenolic compounds from vine and wine products with UV-Vis photodiode array (DAD) and fluorescence detection. In order to obtain a good chromatographic separation, a ternary solvent gradient that combines changes in polarity and pH was used. The pH changes from 2.6 to 1.5 to promote the elution of anthocyanins in their flavylium cationic form. The fluorescence detector enables the limit of quantification of flavan-3-ols to be reduced, and also prevents interferences caused by other phenolics that force the use of time-consuming fractionating protocols. With this method, it was possible to separate and quantify up to 48 phenolic compounds in a single, direct injection: 2 benzoic acids (DAD set at 280 nm), 9 hydroxycinnamic acids and GRP (DAD set at 320 nm), 7 flavan-3-ols (5 with fluorescence detector set at excitation/emission of 280/320 nm, and 2 with DAD set at 280 nm), 12 flavonols (DAD set at 360 nm), 15 anthocyanins (DAD set at 520 nm), and 2 stilbens (DAD set at 320 nm). Studies were performed to determine the precision, accuracy, and detection limits and data are provided on the concentration of the phenolics found in grape seed and skin extracts, as well as in wines prepared from the *Vitis vinifera* cultivar Cencibel.

Keywords: HPLC; Grape; Wine; Anthocyanins; Flavan-3-ols; Flavonols; Hydroxycinnamic acids; Benzoic acids

1. Introduction

Phenolic grape and wine compounds can be divided into two groups: non-flavonoid (hydroxybenzoic and hydroxycinnamic acids and stilbenes) and flavonoid compounds (anthocyanins, flavan-3-ols and flavonols). Anthocyanins are a family of polyphenols that are directly responsible for colour in grapes and young wines. Flavan-3-ols (monomeric cathechins and proanthocyanidins) are another large family of polyphenolic compounds, which are mainly responsible for the astringency, bitterness and structure of wines (Singleton and Essau, 1969; Gawel, 1998). The last group of flavonoids are flavonols (quercetin, myricetin, kaempferol, isorhamnetin and their glycosides), which seem to contribute to bitterness, affect red wine colour (Boulton, 2001; Schwarz et al., 2005), and display antioxidant activity (Plumb et al., 1999).

The concentration of phenolic compounds in grapes depends on the grape cultivar and is influenced by viticultural and environmental factors such as maturity stage, seasonal conditions, production area and fruit yield (Mazza, 1995; Cheynier et al., 1998; Broussaud et al., 1999; Ojeda et al., 2002).

Flavan-3-ols are also responsible for browning reactions in grapes and wine (Macheix et al., 1991) and react with anthocyanins, leading to the stabilisation of colour in red wines. From the onset of must fermentation, wine anthocyanins develop into forms that may or may not be pigmented. Known pigmented forms include oligomers of anthocyanins and flavan-3-ols, that could also be mediated by different aldehyde bridges (e.g. acetaldehyde, propionaldehyde). Some anthocyanin-aldehyde-flavanol pigments have also been characterised in model solutions (Dallas et al., 1996; Francia-Aricha et al., 1997; Saucier et al., 1997; Pissarra et al., 2003, 2004) and recently detected in real

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wines (Revilla et al., 1999; Mateus et al., 2002; Hayasaka and Kennedy, 2003; Zimman and Waterhouse, 2004).

Polyphenols, particularly certain phenolic acids and flavonols, participate in the phenomenon of copigmentation. For this reason, anthocyanins display far greater colour than would be expected from their concentration (Boulton, 2001). Besides, hydroxycinnamic acids are involved in the formation of a new class of low molecular weight anthocyanin-derived pigments, the so-called pyranoanthocyanins (Schwarz et al., 2003), which were initially related with certain secondary yeast metabolites, such as acetaldehyde or pyruvic acid (Fulcrand et al., 1996; Hayasaka and Asenstorfer, 2002).

Phenolic compounds of wine have also attracted much interest due to their antioxidant properties and their potentially beneficial effects for human health (Gardner et al., 1999; Shirkande, 2000; Fernández-Pachón et al., 2004; de Beer et al., 2005). For this reason, grape seed extract has become popular in recent years as a nutritional supplement (Waterhouse et al., 2000).

Analysis of phenolic compounds from vine and wine products (grape seeds and skins, musts, and wines) is rather complicated due to their great diversity. High-performance liquid chromatography (HPLC) has become the method of choice for analysing phenolic compounds in grape skins and in wines (Wulf and Nagel, 1978). Depending on the nature of the phenolic compounds to be analysed, the general requirements for an HPLC analysis method comprise a previous purification step by liquid-liquid or solid phase (columns filled with RP silica gel C-18, Toyopearl gel HW-40 or polyamide) extractions, using for elution several solvents differing in pH and/or polarity (Gómez-Plaza et al., 2001; Mateus et al., 2001; Jordao et al., 2001; Peña-Neira et al., 2004). When analysing phenolic compounds from grape seeds and skins, an initial extraction step must be added; this can be performed using methods such as the milling of grape solid materials in the presence of the extraction solvent (Piergiovanni and Volonteiro, 1981; Gao et al., 1997).

Some phenolic compounds found in vine and wine products show characteristic absorbances in the UV-Vis region and can therefore be easily detected in HPLC by a photodiode array detector. Anthocyanins are red-coloured and show an absorbance maximum of around 520 nm; flavonols are yellow-coloured and display an absorbance maximum of around 360 nm; finally, hydroxycinnamic acids can be specifically detected by their high absorbance around 320 nm. Unfortunately, the main vine and wine polyphenols (flavan-3-ols) show no specific absorbance and have a maximum of around 280 nm, like all the above-mentioned phenolics. In contrast, flavan-3-ols have fluorescence properties that the other vine and wine polyphenols do not. This is the reason why the use of a fluorescence detector has been proposed for analysing flavan-3-ols (Viñas et al., 2000).

The aim of this study was to optimise a method for analysing vine and wine phenolics by HPLC, developed on the basis of the direct injection method described by Lamuela-Raventós and Waterhouse (1994), later modified by Vaadia (1997). This analytical method combines UV-Vis photodiode array (DAD) detection with a fluorescence detector (FD). Using the fluorescence detector, the limit of quantification of flavan-3-ols can be lowered, and the interferences due to the other phenolics requiring the use of time-consuming fractionating protocols can also be avoided. This optimised HPLC method has been applied in the analyses of phenolic compounds from grape seeds and skins of the grape cultivar Cencibel (also known as Tempranillo), as well as from some young red wines elaborated with this grape cultivar.

2. Materials and methods

2.1. Extraction method

An amount between 100 and 150 g of healthy grapes was finger pressed to remove the pulp and the seeds. The remaining skins and seeds were washed three times in water (Milli-Q) and dried twice by softly patting them between sheets of filter paper. An amount of 20 and 2 g of the dried skins and seeds, respectively thus obtained was weighed, and 150 ml of a mixture 50:48.5:1.5 (v/v) of $CH_3OH/H_2O/HCOOH$ was added (Gao et al., 1997). The mixture was immediately homogenised in a blender for 2 min and then centrifuged at 2500 *g* at 5 °C for 15 min. The supernatant was filtered through a 0.45-μm nylon membrane (Millipore, Bedford, MA, USA), discarding the first 5–6 drops, and injected directly onto the liquid chromatograph.

2.2. HPLC analysis

The analyses were performed on an HPLC Varian ProStar (Varian Inc., Walnut Creek, CA, USA) comprising a ProStar 240 ternary pump, a ProStar 410 autosampler, a ProStar 330 photodiode array detector and a ProStar 363 fluorescence detector. The column was thermostated at 20 °C in a MFE-01 oven (Análisis Vínicos, Tomelloso, Spain). After injecting 10 μL of sample, separation was performed in an Ace® 5 C18 250 × 4.6 mm column (Advanced Chromatography Technologies, Aberdeen, Scotland).

For detection and quantification of compounds, the chromatograms were recorded at 280, 320, 360 and 520 nm in the photodiode detector and the chromatogram corresponding to excitation at 280 nm and emission at 320 nm in the fluorescence detector.

Table 1 shows the solvent gradient used for separation. This was performed with a ternary mobile phase gradient in which, in addition to reducing the polarity of the solvent to elute the least polar compounds (flavonols), the pH of the mobile phase was reduced from 2.6 to 1.5 to separate the anthocyanins in their flavylium cationic form from the other phenols.