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Original Research Article

Determination of phytosterols in oenological matrices by liquid chromatography-atmospheric pressure chemical ionization and ion-trap mass spectrometry

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ABSTRACT

The aim of the present study is the identification of plant sterols and the development of an analytical method that allows for the quantification of such family of compounds in oenological matrices. The application of liquid chromatography-atmospheric pressure chemical ionization ion-trap mass spectrometry (LC-APCI-ITMS) to sterol characterization is a useful tool and was selected to perform this research. Sterol separation was achieved using a C8 column with a mobile phase consisting of water and acetonitrile under gradient conditions and column temperature of 35 °C, which leads to analyte elution in less than 25 min. Retention times, precursor ions and MRM transitions of analytes allowed for the identification and sensitive quantitative determination of phytosterols in oenological matrices at trace levels. The method showed a dynamic linear range over the concentration ranges from 0.02 to 320 mg kg^{-1} for the different parts of grapes and from 8 to 100 ng mL^{-1} in case of wine. The most abundant phytosterol in all samples was β-sitosterol. The seeds are the richest source of phytosterols having a great amount of β-sitosterol, 314 mg kg^{-1} fresh berry mass, followed by stigmasterol, fucosterol and campesterol at much lower concentrations (ranging from 3 to 10 mg kg^{-1}).

1. Introduction

For years, grape berries and red wine have been a subject of investigation with respect to their chemical composition. Regular and moderate wine consumption, particularly red wine, has been associated with health benefits, in the same way as consumption of certain fruits such as blueberries or grapes, due to their constituents such as polyphenols that are believed to contribute to antioxidant, lipid regulating, and anti-inflammatory effects (Rodriguez-Cabo et al., 2014), as well as protection against neurodegenerative diseases.

Phytosterols (plant sterols) have been described as bioactive molecules. The nutritional role of these compounds to reduce circulating cholesterol levels in humans have been known since the 1950s (Pollak, 1953), a finding that has led to the development of various food products enriched with these compounds (Moreau et al., 2002). There are some publications about the content of phytosterols in the skin of grape berries (Jiménez-Escrig et al., 2006; Orban et al., 2009; Zhang et al., 2004) and grape musts (Delfini et al., 1993). Conversely, the available studies on such a

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family of compounds in red wine are relatively scarce (Ruggiero et al., 2013), and, taking into account the large number of molecular species in wine matrix, the measurement of phytosterols in wine is challenging. These compounds are an essential constituent of cell membranes that control both its fluidity and permeability. They are only available to humans through plant foods such as vegetable oils, nuts, seeds, cereals, legumes, fruits, and vegetables or industrial supplements from plant origin (Piironen et al., 2000).

The average daily intake of plant sterols in Western countries ranges from 150 to 400 mg, depending on dietary habits and geographic region (Han et al., 2008; Jiménez-Escrig et al., 2006; Normen et al., 2007; Sioen et al., 2011; Valsta et al., 2004), mainly by ingestion of vegetable oils, cereals, fruits and vegetables (Lagarda et al., 2006; Moreau et al., 2002; Piironen et al., 2002; Piironen et al., 2003; Valsta et al., 2004). Nevertheless, the amounts of plant sterols consumed daily are often not large enough to have significant cholesterol-lowering effects (Racette et al., 2009; Rodriguez-Cabo et al., 2014). A number of clinical trials have established that the consumption of 1.5–2.0 g/day of phytosterols can result in a 10–15% reduction in LDL cholesterol in as short as a three-week period in hyperlipidemic populations (EFSA, 2008).

Plant sterols are steroid alcohols derived from hydroxylated polycyclic isopentenoids containing a 1,2-cyclopentanophenanthrene structure and contain a total of 27–30 carbon atoms. They are similar in structure to cholesterol but differ in the side chain and ring saturation (MacKay and Jones, 2011; Ostlund, 2002). The basic skeleton of sterol is 5α-cholestane. Although there are more than 250 identified plant sterols, the most abundant in nature are β-sitosterol, stigmasterol and campesterol. Sterols in foods occur as free sterols (alcohol form) and as bound conjugates in which the 3β-hydroxyl group is esterified to a fatty acid, or glycosylated with hexose (usually glucose) or a 6-fatty acyl hexose (Figure S1, Supplementary data). By convention, the total sterol content is defined by the sum of all constituents. Due to their structural complexity and similarity, the analytical techniques for sterol isolation, separation and quantification need to be both accurate and precise. Therefore, an ideal sample preparation procedure for free or total sterol determination would include sterols from all possible conjugates, which is one of the most critical steps in the analysis. Some protocols are described in the literature (Azadmard-Damirchi et al., 2005; Harrabi et al., 2008; Verleyen et al., 2002). Regarding the different techniques for sterols analysis (Abidi, 2001; Lagarda et al., 2006), GC with flame ionization detection (FID) or GC with mass spectrometry detection (MS) to confirm peak identity can be considered typical analytical methods to determine phytosterols in food (Clement et al., 2010; Santos et al., 2007; Toivo et al., 2001). In the case of oenological matrices, phytosterols have been determined in grape-seed oils (Crews et al., 2006; Matthäus, 2008; Rubio et al., 2009) or in berry, seed tissues and wine (Ruggiero et al., 2013) in all cases using GC-FID.

However, the major disadvantage of GC is the requirement of both thermally stable compound and chemical derivatization before analysis, which makes the method complicated and time consuming. For this reason, other techniques such as HPLC with a variety of detectors (Careri et al., 2001; Zarrouk et al., 2010) have been more frequently and widely used for the separation of individual sterols. As sterols are highly lipophilic with few polar functional groups, liquid chromatography combined with a soft ionization technique, such as atmospheric pressure chemical ionization (APCI), is worthwhile. In fact, LC-APCI-MS has been widely used for sterol analyses in different plant matrices (Lu et al., 2007; Mezine et al., 2003) such as cereals (Rozenberg et al., 2003), vegetable oils (Carretero et al., 2008; Lerma-Garcia et al., 2010; Wewer et al., 2011; Zarrouk et al., 2009), and grape berries (Orban et al., 2009).

As little is known about the distribution of sterol content in oenological products, the aim of this work was to quantify sterols in seed, skin, pulp and related red wine obtained from Rioja vineyards (northern Spain). For this purpose, a method for extraction, separation and posterior identification and quantification of phytosterols in oenological matrices was developed. Free and conjugated sterols (quantified as free forms) were also determined.

2. Material and methods

2.1. Reagents and chemicals

Desmosterol (DES; purity ≥85%), ergosterol (ERG; purity ≥95%), brassicasterol (BRA; purity ≥98%), cholesterol (CHO; purity ≥99%), fucosterol (FUC; purity ≥93%), campesterol (CAM; purity ≥65%), stigmasterol (STIGM; purity ≥95%), β-sitosterol (β-ST; purity ≥95%), sitostanol (SITOST; purity ≥95%), cholesteryl n-decanoate (CHO-DECA; purity ≥95%) and β-sitosterol-3-O-β-D-glucoside (β-ST-GLU; purity ≥75%), were purchased from Sigma-Aldrich (Madrid, Spain). Isotopically labelled β-sitosterol (5-cholesten-24(RS)-ethyl-3-β-ol-25,26,26,26,27,27,27-d7; purity 98%) and labelled cholesterol (cholesterol-25,26,26,26,27,27,27-d7; purity ≥98%) were purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada) and Sigma-Aldrich, respectively, and were used as internal standards.

All organic solvents (acetone, chloroform, ethanol, hexane and diethyl ether) were of analytical or HPLC grade, and methanol and acetonitrile, both LC-MS grade, used as the organic mobile phase, were supplied by Scharlab (Barcelona, Spain). Potassium hydroxide purchased from Merck (Darmstadt, Germany) and hydrochloric acid S.G. 1.18 (37%) from Fisher Scientific (Loughborough, Leicestershire, UK) were used in sample treatment. Ultra-high purity (UHP) water, prepared from tap water, was pre-treated using Elix reverse osmosis cartridges before filtration by a Milli-Q system from Millipore (Bedford, MA, USA) and used throughout the study.

2.2. Stock standards, working solutions and fortified matrices

Stock standard solutions of each phytosterol (containing 1000 mg L^{-1}) were prepared in acetone and stored frozen at −42 °C. Individual solutions (100 mg L^{-1}) were prepared by dilution of the stock standards in acetonitrile and were kept in dark under refrigeration, at 4 °C. Further mixed dilutions in acetonitrile were prepared weekly to obtain working solutions at different concentrations.

To quantify phytosterol content in pulp, skin and seeds, calibration curves were constructed in matrix-fortified with the studied analytes, as an ideal blank was not available. First, each part of the grape was pooled, comprising all samples under study. Subsequently, increments of standard solution were added to pooled sample aliquots of 5 g (first addition levels were estimated according to previous analyses). The added concentration was compound dependent and representative of the expected study concentration, with ranges from 0.02 to 320 mg kg^{-1}. Labelled compounds were used as surrogates to compensate analytical errors during extraction and separation steps, and signal variation within the ionization procedure. All calibration levels contained both labelled cholesterol and labelled sitosterol at 0.1 mg kg^{-1} and 5 mg kg^{-1}, respectively, because it is expected that some phytosterols will be at higher concentration than others.

Focusing on wine samples, a matrix-matched calibration method was applied. Calibration standards in wine matrices were prepared in duplicate by adding the appropriate volume of the working solutions to diluted wine (1:5, to assure no signal of target