LWT - Food Science and Technology 71 (2016) 249–259

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Determination of polyphenols in grape-based nutraceutical products using high resolution mass spectrometry

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ARTICLE INFO

Article history:
Received 18 May 2015
Received in revised form
26 February 2016
Accepted 22 March 2016
Available online 26 March 2016

Keywords:
Grape-based nutraceuticals
Polyphenol compounds
Mass spectrometry
Orbitrap

ABSTRACT

The detection and quantification of polyphenols in grape-based nutraceutical products were performed using ultra high performance liquid chromatography coupled to single-stage Orbitrap high resolution mass spectrometry (UHPLC-Orbitrap-MS). The method is based on an extraction using a mixture of methanol:water (80:20, *v/v*), followed by two dilutions (10 or 50 times). The method was validated in terms of linearity, precision and trueness, and limits of detection and quantification ranged from 1 to 10 μg L^{-1} and 2–50 μg L^{-1} respectively. High variability of the amount of anthocyanins and flavanols were obtained in the analyzed samples, with values ranging from 2 to 27448 mg kg^{-1}, whereas the resveratrol was only detected in 6 out of 8 samples at concentrations ranging from 3 to 11107 mg kg^{-1}. Furthermore, other polyphenols belonging to other families like dihydrochalcones, flavanones, flavonols, isoflavonoids or phenolic acids (hydroxybenzoic and hydroxycinnamic acids) were quantified. Apart from this, a tentative identification of 8 compounds was performed.

1. Introduction

The search of products to increase longevity and improve health across the lifespan has been widely investigated in recent years. This has led to expand the market of nutraceuticals from plants, and the interest in grape based products has increased due to its high concentration in natural antioxidants (Xia et al., 2013).

Resveratrol has attracted considerable attention due to it has been suggested that it is responsible for health benefits associated with cardioprotective (Tomé-Carneiro et al., 2013) and anti-inflammatory effects (Tomé-Carneiro et al., 2012), as well as for atherosclerosis (Agarwal et al., 2013) and some kinds of cancer prevention (Juan, Alfaras, & Planas, 2012). In addition, it has been reported that it could reduce the risk of neurodegenerative disorders, especially Alzheimer's disease (Li, Gong, Dong, & Shi, 2012), and provide some kind of prevention against obese disease (Bhatt, Thomas, & Nanjan, 2012; Silk & Smoliga, 2014). The low bioavailability, rapid clearance from the circulatory system, and inter-individual variability in resveratrol treatments hamper its clinical potential (Silk, & Smoliga, 2014).

Other components present in grapes at high concentrations are anthocyanins. These compounds are the main coloring agents in red berries and wine (La Cruz et al., 2013). In particular, anthocyanins are mostly accumulated in skins, whereas procyanidins are located in the seeds (Yang, Martinson, & Liu, 2009). The most frequently detected anthocyanins in grape skins are the 3-*O*-glucosides of malvidin, cyanidin, delphinidin, peonidin and petunidin (Yang et al., 2009). Anthocyanins possess antioxidant activity, which is considered to be an important physiological function (Yang et al., 2009). Anthocyanins have been linked to health effects against cardiovascular diseases (Kruger, Davies, Myburgh, & Lecour, 2014) and cancer prevention (Feng et al., 2007; Wang & Stoner, 2008) between other properties. Moreover, this kind of products based on grape seeds is a rich source of catechins (Cavaliere et al., 2008).

Therefore, the occurrence of polyphenols in grapes skin (Priego Capote, Rodríguez, & Luque de Castro, 2007; Zhu, Zhang, & Lu, 2012), grapes skin and seed (Cavaliere et al., 2008; Rodríguez Montealegre, Romero Peces, Chacón Vozmediano, Martínez Gascueña, & García Romero, 2006), wines (Alonso Borbalán, Zorro, Guillén, & García Barroso, 2003; Yang et al., 2009) or grape juice (Wang, Race, & Shrikhande, 2003) has been more widely

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http://dx.doi.org/10.1016/j.lwt.2016.03.037
0023-6438/

studied than in nutraceutical products (Orlandini, Giannini, Pinzauti, & Furlanetto, 2008), and low resolution mass spectrometry analyzers have mainly been used for their determination in most cases (Ramirez-Lopez, McGlynn, Goad, & DeWitt, 2014; Wu & Prior, 2005).

Regarding the extraction of polyphenols in these matrices, several extraction procedures as solid–liquid extraction (SLE) using methanol acidified with hydrochloric acid (De Nisco et al., 2013; La Cruz et al., 2013) or formic acid (Cantos, Espín, & Tomás-Barberán, 2002a, 2002b) or a mixture of methanol with acidified water, using formic acid (Cavaliere et al. 2008; Rodríguez Montealegre et al., 2006) or acetic acid (Wu, & Prior, 2005; Zhu et al., 2012) at different percentages have been extensively used. In some investigations, two methods, one for the determination of anthocyanins and another one for the rest of polyphenols (Ramirez-Lopez et al., 2014; Zhu et al., 2012) are also applied. On the other hand, for the extraction of polyphenols from liquid samples as wine or grape juices, solid phase extraction (SPE) is typically used as extraction technique (Hashim et al., 2013; Matejícek, Klejdus, Mikeš, Šterbová, & Kubán, 2003).

High performance liquid chromatography (HPLC) coupled to fluorescence (Rodríguez Montealegre et al., 2006), diode-array detection (DAD) (Mattivi, Guzzon, Vrhovsek, Stefanini, & Velasco, 2006; Revilla, García-Beneytez, Cabello, Martín-Ortega, & Ryan, 2001), mass spectrometry (MS) (Alonso Borbalán et al., 2003; Ramirez-Lopez et al., 2014; Wu, & Prior, 2005), or coupled to two detectors as DAD and MS (Cantos et al., 2002a, 2002b; Wang et al., 2003) are usually used for the separation and detection of the target compounds.

Nevertheless, high resolution MS (HRMS) analyzers have been scarcely studied for the detection of anthocyanins or other polyphenols in grape berries or derived products, though its efficacy has been demonstrated for the determination of several types of polyphenols in different matrices (Dias et al., 2013; López-Gutiérrez, Romero-González, Garrido Frenich, & Martínez Vidal, 2014; Mullen, Larcombe, Arnold, Welchman, & Crozier, 2010).

HRMS analyzers provide better mass resolution in relation to conventional analyzers as triple quadrupole (QqQ). In addition, they offer full scan acquisition mode, without limitations in the number of monitored compounds, and the information can be obtained in a single injection and retrospective analysis could be performed. Moreover, exact mass measurements can also be helpful to improve the knowledge of the composition of these kinds of products. These characteristics make them a suitable tool to identify and quantify polyphenols in complex matrices as nutraceutical products.

The aim of this study has been the use of ultra high performance liquid chromatography (UHPLC) coupled to Orbitrap-MS to investigate the composition of grape based nutraceutical products. In addition, the development and validation of a suitable method for the simultaneous analysis of several families of polyphenols present in grape nutraceuticals (tablets and capsules) has been performed.

2. Materials and methods

2.1. Reagents and apparatus

All phytochemical standards were obtained from four different suppliers: Extrasynthese (Genay, France), Sigma–Aldrich (Madrid, Spain), ChromaDEX (Irvine, CA, USA) and Fluka (Steinheim, Germany) as it is indicated in Table 1. All standards have a purity >95%, except chrysin, daidzin, kaempferol-7-*O*-glucoside, pelargonin chloride, quercetin-3-*O*-glucoside and theaflavin with a purity ≥90%. Apigenin-8-*C*-glucoside, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-gallocatechin and (−)-gallocatechin gallate were prepared in a mixture of methanol:water (50:50, *v/v*). Apigenin-7-*O*-neohesperidoside, luteolin-7-*O*-glucoside and luteolin-8-*C*-glucoside were prepared in ethanol, and glycitin, coumestrol, genistin and daidzein in dimethyl sulfoxide. Anthocyanins (Priego Capote, Rodríguez, & Luque de Castro, 2007) and the rest of standard solutions were prepared in methanol.

All standard solutions were prepared at concentrations ranging from 90 to 683 mg L^{-1}. All stock solutions were stored in amber bottles at −18 °C in the dark for no longer than 6 months except for anthocyanins, which was only stored for 3 months due to their fast degradation (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009).

Three working solutions (anthocyanins, catechins and the rest of polyphenols) were prepared in methanol by an appropriate dilution of aliquots of each individual stock standard solution to obtain final concentration of 5 mg L^{-1}. All solutions were stored under refrigeration (<−18 °C) in amber bottles in the darkness for 6 months except for anthocyanins, which were only stored for 3 months.

Acetonitrile (ACN) (LC-MS grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Dimethyl sulfoxide (HPLC grade) and methanol (MeOH) (LC-MS grade) were supplied from Sigma–Aldrich.

Ammonium acetate (purity 97%) and ethanol (HPLC grade) were obtained from Panreac (Barcelona, Spain). Formic acid (LC-MS grade) and water (LC-MS grade) were purchased from Scharlau (Barcelona, Spain). Hydrochloric acid was supplied from J.T. Baker (Deventer, The Netherlands).

The Orbitrap analyzer used a mixture of acetic acid, caffeine, Met-Arg-Phe-Ala-acetate salt and Ultramark 1621 (ProteoMass LTQ/FT-hybrid ESI positive), and a mixture of acetic acid, sodium dodecyl sulfate, taurocholic acid sodium salt hydrat and Ultramark 1621 (fluorinated phosphazines) (ProteoMass LTQ/FT-Hybrid ESI negative) from Thermo-Fisher (Waltham, MA, USA) in order to perform mass calibration.

Millipore Millex-LG filters (0.20 μm, Millipore, Carrightwohill, Ireland) were used to filtrate the extracts.

A Centronic BL II centrifuge (J.P. Selecta, Barcelona, Spain), a Reax 2 rotatory agitator from Heidolph (Schwabach, Germany), and vortex mixer WX from Velp Scientifica (Usmate, Italy) as well as a coffee grinder (Orbit, Hong Kong, China) were used to process all samples.

2.2. UHPLC-Orbitrap-MS

An UHPLC system Transcend (Transcend 600 LC, Thermo Fisher Scientific, San Jose, CA, USA) was used for chromatographic separations. Analyses were carried out using a Waters (Milford, MA, USA) Acquity C_{18} column (2.1 × 100 mm, 1.7 μm particle size).

A single-stage Orbitrap mass spectrometer (Exactive™, Thermo Fisher Scientific, Bremen, Germany) was used for identification and quantification purposes. The mass spectrometer was operated using a heated electrospray interface (ESI) (HESI–II, Thermo Fisher Scientific, San Jose, CA, USA), in positive (ESI+) and negative ionization (ESI−) modes.

Optimal conditions were as follows: sheath gas (N_2, >95%), 35 (adimensional); auxiliary gas (N_2, >95%), 10 (adimensional); spray voltage, 4 kV (−4 kV in ESI−); skimmer voltage, 18 V (−18 V in ESI−); capillary voltage, 35 V (−35 V in ESI−); tube lens voltage, 95 V (−95 V in ESI−); heater temperature, 305 °C; capillary temperature, 300 °C. The automatic gain control (AGC) was set at a target value of 1×10^6.

The system was operated employing four alternating acquisition functions: (1) full MS, ESI+, without fragmentation (the higher