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Investigation of liquid chromatography quadrupole time-of-flight mass spectrometry performance for identification and determination of hydroxylated stilbene antioxidants in wine

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ABSTRACT

The performance of liquid chromatography (LC) followed by quadrupole time-of-flight (QTOF) mass spectrometry (MS) for the determination of hydroxylated stilbene compounds in red and white wine samples is assessed. When combined with a solid-phase extraction step, LC–QTOF-MS allowed the selective determination of five target compounds (*trans*- and *cis*-resveratrol, *trans*-piceatannol, *trans*-piceid and *trans*-pterostilbene) attaining limits of quantification between 3 and 20 ng mL^{-1} and providing linear responses up to 4000 ng mL^{-1}. Recoveries, established against standards prepared in methanol, varied between 93% and 115%. The distribution of the above species in wine is illustrated with the analysis of 15 samples. *Trans*-pterostilbene remained undetected in samples, whereas *trans*-piceid and *trans*-resveratrol maximum concentrations exceed the 6000 ng mL^{-1} level. Values for *trans*-piceatannol and *cis*-resveratrol ranged from non detected to 600 ng mL^{-1}, and from 11 to more than 3200 ng mL^{-1}, respectively. Accurate MS and MS/MS scan spectra were used to investigate the existence of reduced (dihydro) and oxidized (dehydro) forms of resveratrol and picetannol in the processed samples. Dihydro derivatives appeared, as free compounds, in 100% (dihydro-piceatannol) and 40% (dihydro-resveratrol) of the samples. On the other hand, dehydro derivatives were noticed as conjugated (glycosylated) species, with detection frequencies of 100% and 47% for dehydro-glucosyl-resveratrol and dehydro-glucosyl-piceatannol, respectively. Above findings confirm the suitability of LC–QTOF-MS for the comprehensive study of hydroxylated stilbene antioxidants in wine samples.

1. Introduction

Hydroxylated stilbenes are bioactive compounds with putative health-preventive effects, particularly with regards to diseases derived from oxidative stress and cardiovascular problems [1]. Given that wine is one of the most important dietary sources of these antioxidants, a number of studies have addressed the determination of stilbenoids in this matrix; however, most of them have been focused on a single compound: resveratrol (3,5,4′-trihydroxystilbene) [2]. In vitro and in vivo research has revealed that the bioactivity and the bioavailability of stilbenes vary depending on a number of parameters such as: isomeric form (*trans*- or *cis*-), free versus glycosylated species, number of hydroxyl substituents in the stilbene skeleton and existence of dihydro and methylated derivatives [3–6]. Consequently, there is a need for analytical methodologies able to provide a comprehensive insight of hydroxylated stilbenes distribution in wine matrices.

Gas chromatography (GC) based methods permit the sensitive determination of free (non-conjugated) forms of hydroxylated stilbenes after a relatively elaborated sample preparation process, based on the use of exhaustive extraction [7,8], or equilibrium microextraction techniques [9–13], combined with a derivatization reaction [7,14]. When GC is applied to determine the total (free plus conjugated) concentration of these compounds an additional hydrolysis step must be introduced in the analytical methodology. Obviously, the resulting methods become time-consuming and do not allow discriminating between free and conjugated forms in the same run [15].

Liquid chromatography (LC) combined with tandem mass spectrometry (MS/MS) permits the simultaneous determination of free and conjugated stilbenes, without the need of either derivatization or hydrolysis reactions [16,17]. Despite these advantages, a sample preparation step, aiming to reduce the complexity of wine samples (particularly red wines), is still advisable to prevent changes

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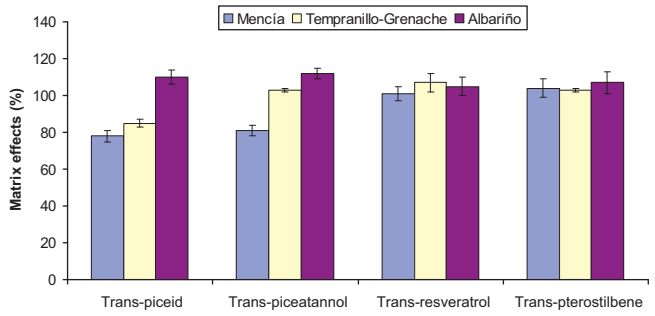

Fig. 1. Matrix effects evaluation for wine samples (2 mL volume) using OASIS MAX cartridges.

in the efficiency of electrospray ionization (ESI) between standard solutions and wine samples [18]. Obviously, such variations can be also prevented by wine dilution [2]; however, this choice leads to increased limits of quantification (LOQs). Usually, LC–MS/MS methods rely on triple quadrupole (QqQ) instruments due to their excellent selectivity and sensitivity when operated in the multiple reaction monitoring (MRM) mode. On the other hand, the MRM mode is blind to possible stilbenoids analogues, for which commercial standards are not available, and the sensitivity of QqQ instruments in scan is too reduced for trace species identification.

New generation, hybrid quadrupole time-of-flight (QTOF) MS systems provide acceptable sensitivity, accurate mass assignments and full scan MS and MS/MS spectra. The combination of above features permits the screening of acquired chromatograms to investigate new compounds, not considered during development of the quantitative method, and/or for which standards are not commercially available. The assignation of an empirical formula to an unknown compound is made taking into account (1) the exact mass of its molecular ion, (2) isotopic abundances and (3) spacing between signals in the cluster corresponding to the molecular ion. Further structural information can be obtained from accurate product ion MS/MS scan spectra [19]. Following the above chart-flow, LC–QTOF-MS has been recently applied to investigate the presence of oligomeric stilbenoids in red wine after a LLE extraction step [20]; however, in this study, the quantitative possibilities of the system were not evaluated. Also, the performance of a single TOF MS spectrometer was compared with that provided by a QqQ instrument for quantification of *trans*- and *cis*-resveratrol in diluted wine samples [21]. The LOQs rendered by LC–TOF-MS were 50 times higher than those achieved by LC–QqQ-MS [21].

Hence, the aim of this study was to investigate the performance of LC–QTOF-MS for the determination of hydroxylated stilbenes in wine samples. A quantitative procedure, based on solid-phase extraction (SPE) and MS/MS detection, was optimized to measure the levels of *trans*- and *cis*-resveratrol, *trans*-piceatannol (3′,4′,3,5-tetrahydroxy-*trans*-stilbene), *trans*-piceid (3-O-glucosyl-*trans*-resveratrol) and *trans*-pterostilbene (3,5-dimethoxy-4′-hydroxy-*trans*-stilbene) in red and white wines. In addition, the information provided by accurate MS and MS/MS scan spectra was used to investigate the existence of stilbene analogues in wine, with special attention focused on reduced (dihydro) and oxidized (dehydro) forms.

2. Experimental

2.1. Standards, solvents, reagents and samples

Trans-resveratrol (99%) was obtained from Aldrich (Milwaukee, WI, USA); *trans*-piceatannol (98%), *trans*-pterostilbene (98%) and *trans*-piceid (95%) were acquired from TCI Europe (Zwijndrecht, Belgium), *cis*-resveratrol was purchased from Cayman Chemical (Ann Arbour, MI, USA), $^{13}C_6$ *trans*-resveratrol (97%), used as internal surrogate (IS), was also provided by Aldrich. The chemical structures, octanol–water partition coefficients ($\log K_{ow}$) and pK_a values of these compounds are provided as supplementary information, Table S1. Individual solutions of target compounds and the IS were dissolved in methanol. Further dilutions and mixtures of them were prepared in the same solvent. Two different series of calibration standards were dissolved in methanol and synthetic wine, respectively.

Methanol and acetonitrile, HPLC-grade puritiy, formic acid (99%), acetic acid (99%), sodium acetate (99%) and ammonium acetate (99%) were supplied by Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q (Millipore, Billerica, MA, USA) system. OASIS HLB (divinylbenzene-*co*-*N*-vinylpirrolidone polymer) 60 mg and OASIS MAX (dimethylbutyl amine functionalized divinylbenzene-*co*-*N*-vinylpirrolidone polymer) 60 mg SPE cartridges were acquired from Waters (Milford, MA, USA). Graphitized carbon 250 mg cartridges were provided by Supelco (Bellefonte, PA, USA).

Red and white wine samples were obtained from local markets and farmers. Synthetic wine was prepared by addition of tartaric acid (3.5 g L^{-1}) to 13% ethanolic solutions in ultrapure water, followed by pH adjustment to 3.5 with NaOH (0.1 M).

2.2. Sample preparation

Sample preparation conditions were optimized with the aim of minimizing variations in the efficiency of electrospray (ESI) ionization between calibration standards (prepared in methanol or synthetic wine) and extracts from wine samples. Two different red wines (*Mencía* and *Tempranillo-Grenache* varieties) and a white wine (*Albariño* variety) were used during optimization of extraction conditions. Unless otherwise stated, matrix effects and SPE recoveries were investigated with samples spiked at 1000 ng mL^{-1} per compound.

Under optimized conditions, 2 mL of wine (spiked with the IS) were diluted with the same volume of ultrapure water and passed through a HLB (white wines) or a MAX (red wines) cartridge, previously conditioned with methanol followed by ultrapure water (3 mL each). Thereafter, cartridges were washed with 3 mL of a methanol:water (20:80) solution, 0.1 M in sodium acetate and adjusted at pH 6 with glacial acetic acid. Finally, cartridges were dried with a gentle stream of N_2 and eluted with 2 mL of methanol.

2.3. Determination conditions

Compounds were determined using a LC-ESI–QTOF-MS system acquired from Agilent (Wilmington, DE, USA). The LC instrument was an Agilent 1200 Series, consisting of an autosampler, two isocratic high pressure mixing pumps, a vacuum degasser unit and a chromatographic oven. The QTOF mass spectrometer was an Agilent 6520 model, furnished with a Dual-Spray ESI source.

Compounds were separated in a Zorbax Eclipse XDB C_{18} column (100 mm × 2 mm, 3.5 μm) acquired from Agilent and connected to a C18 (4 mm × 2 mm) guard cartridge from Phenomenex (Torrance, CA, USA). Ultrapure water (A) and acetonitrile (B), both 0.1% in formic acid, were used as mobile phases applying the following gradient: 0–2 min, 5% B; 18 min, 70% B; 19–22 min, 100% B; 23–30 min, 5% B. The mobile phase flow was 0.2 mL min^{-1}, the injection volume for standards and sample extracts was 5 μL and the column temperature was set at 30 °C.

Nitrogen (99.999%), provided by a high purity generator (Erre-Due srl, Livorno, Italy), was used as nebulizing (30 psi) and drying gas (350 °C, 7 L min^{-1}) in the ESI source. The QTOF instrument