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Analytical Methods

Development and validation of a high-throughput analysis of glutathione in grapes, musts and wines by Stable Isotope Dilution Assay and LC–MS/MS

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ABSTRACT

For the first time, we proposed a high-throughput method to quantify glutathione in grapes, musts and wines for all grape varieties using Stable Isotope Dilution Assay (SIDA). Indeed, the use of SIDA as a quantification method is essential to overcome the chemical instability of glutathione. In practice, glutathione was derivatized *in-situ* with N-ethylmaleimide to block the cysteine residue and to enhance its lipophilic properties. After quenching with acetic acid, samples were directly analyzed by LC–MS/MS (run of 13 min) in Multiple Reaction Monitoring mode using labeled glutathione as internal standard. The validation according to the International Organization of Vine and Wine recommendations demonstrated the high sensitivity (LOD = 45 μg L^{-1}), accuracy (recovery = 112%) and intermediate reproducibility (RSD = 12%) of the method. This high-throughput method that requires only 1 mL of matrix, allowed us to analyze 70 samples per day for a moderate cost.

1. Introduction

Glutathione (GSH) is a tripeptide occurring in grapes, musts and wines at different levels of concentration and under different oxidized states that is to say reduced or oxidized forms.

Highly reactive, glutathione can participate in different mechanisms along the winemaking process. From chemical point of view, it is always occurring in equilibrium with its oxidized form whatever the winemaking step. In addition, GSH naturally occurring in grapes at concentrations ranging from 10 to 70 mg L^{-1} is essentially found combined with *trans*-caffeoyltartaric acid in musts to form the so-called Grape Reaction Product in presence of oxygen and polyphenoloxidase (Cheynier, Trousdale, Singleton, Salgues, & Wylde, 1986). More recently, it has been reported that GSH in musts was also involved in the biogenesis of some varietal thiols precursors (Fedrizzi, Pardon, Sefton, Elsey, & Jeffery, 2009; Roland, Schneider, Razungles, Le Guernevé, & Cavelier, 2010) during the crushing step. Finally, GSH occurred in wine at lower concentrations up to 10 mg L^{-1} and from a technical point of view, it can act as a competitive anti-oxidant by trapping quinones and preventing the loss of fruity aroma (Nikolantonaki & Waterhouse, 2012).

Even if all the roles of glutathione have not been yet elucidated, it has recently received significant scientific and commercial attention. Indeed, number of publications dealing with glutathione in wine has considerably increased since 2006 reaching more than 50 papers in 2012 against less than 5 in 1994. The principal reason was that glutathione can act as an anti-oxidant and could help decreasing the loss of aroma observed in wines during the storage. Since supplementation of glutathione in must is not yet allowed by the International Organization of Wine and Vine regulations, its preservation is crucial along the winemaking. So, to monitor the glutathione evolution all along winemaking process, several analytical methods have been developed for 10 years. Many publications in literature reported the quantification of glutathione in biological matrices but, up to now, only 10 were dedicated to grape or wine samples as recently reviewed by Kritzinger, Bauer, and du Toit (2013). Some of them enabled to quantify the oxidized form (Burseg & de Jong, 2009; Du Toit, Lisjak, Stander, & Prevoo, 2007; Kritzinger, Stander, & Du Toit, 2012; Mattivi et al., 2012) or both reduced and oxidized glutathione that is often called the "total glutathione" (Andujar-Ortiz, Pozo-Bayón, Moreno-Arribas, Martin-Alvarez, & Rodriguez-Bencomo, 2012; Cheynier, Souquet,

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Abbreviations: SIDA, Stable Isotope Dilution Assay; MRM, Multiple Reaction Monitoring; HPLC-Chip/MS, high performance liquid chromatography hyphenated with a chip cube and a mass spectrometer; ESI, electrospray ionization; RSD, residual standard deviation; GSH, glutathione; ISTD, internal standard.

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& Moutounet, 1989). Most of the reported methods were time consuming due the long sample preparation. Indeed, a derivatization step was often required to introduce on glutathione either a chromophore moiety (Elmann's reagent (Cheynier et al., 1989), parabenzoquinone (Yin et al., 2004)) or a fluorescent one (monobromobimane (Lavigne, Pons, & Dubourdieu, 2007), *o*-phthalaldehyde (Janes, Lisjak, & Vanzo, 2010; Park, Boulton, & Noble, 2000) or 2,3-naphthalenedialdehyde (Marchand & de Revel, 2010)) permitting its detection by a diode array or fluorescence detector. In a second extend, derivatization increased the lipophily of glutathione making easier its analysis under reverse phase (RP) conditions. Concerning the analytical productivity, only the method developed by Cheynier et al. (1989) reported the possibility of analyzing until 40 samples per day; the others remained difficult to be used as a routine analysis. If we focused on the quantification procedure, the only method using an internal standard was developed by Janes et al. (2010) which used the N-acetylcysteine whereas the others were based on external standardization which did not prevent from glutathione loss during sample preparation and induced a systematic analytical bias.

As detailed above, the quantification of GSH remains challenging for three reasons: (i) the lower level of concentrations especially in wines requires very sensitive and specific methods, (ii) the important hydrophilic behaviour gives the RP analysis very complicated and (iii) the high chemical reactivity generates a systematic analytical bias that only an adequate internal standard can avoid. For this purpose, we developed a new analytical method to quantify reduced glutathione in grapes, musts and wines by Stable Isotope Dilution Assay and LC–MS/MS.

2. Materials and methods

2.1. Chemical and standards

Reduced glutathione (GSH), labeled Glutathione (GSH-Glycine-$^{13}C_2$,^{15}N, 99 atom%), N-ethylmaleimide, acetic acid, benzene sulfinic acid, sodium metabisulfite, glucose and tartaric acid were obtained from Sigma Aldrich (Saint Quentin en Fallavier, France). Ethanol was from Carlo Erba (Rodano, Italy) and potassium carbonate from Merck (Darmstadt, Germany). The LC–MS grade water, acetonitrile and formic acid were supplied by Biosolve (Valkenswaard, The Netherlands).

2.2. Sample preparation

For the analysis of grapes or musts, samples were chemically blocked as reported by Cheynier et al. (1989) using benzene sulfinic acid (1 mg mL^{-1}) and sodium metabisulfite (4.5 mg mL^{-1}) either during the crushing or at the sampling moment. Then, must samples were centrifuged (10,000 rpm, 10 min, 4 °C) prior to analysis.

2.3. Model must and wine preparation

Model must was composed of glucose (220 g L^{-1}) and tartaric acid (4 g L^{-1}). Model wine was composed of water (800 mL L^{-1}), ethanol (120 mL L^{-1}) and tartaric acid (3.5 g L^{-1}). The pH of both was adjusted to 3.5 using potassium carbonate.

2.4. Analysis of glutathione

2.4.1. Derivatization procedure

The derivatization procedure was adapted from the Iwasaki et al. method (Iwasaki, Hoshi, Ito, Saito, & Nakazawa, 2006). Briefly, 10 µL of the internal standard solution (GSH-Glycine-$^{13}C_2$,^{15}N, 2 mM) then 10 µL of N-ethylmaleimide solution (50 mM) in a NH_4HCO_3 buffer (100 mM) were added to the samples (1 mL of centrifuged must or wine). After 15 min at room temperature and under magnetic stirring, the reaction was quenched by the addition of 1 µL of iced acetic acid. Then, the samples were diluted at 1/10 in water before analysis.

2.4.2. HPLC-Chip/MS system and chromatographic conditions

In the HPLC-Chip/MS system, the liquid chromatograph consisted of an Agilent series 1260 infinity instrument including a capillary pump with a degasser, a thermostated microwell-plate autosampler (held at 4 °C) with a 8 µL injection loop, and a HPLC-Chip/MS interface (chip cube, Agilent Technologies, Waldbronn, Germany). Analysis was performed using an HPLC-polymeric chip that integrates a 500 nL enrichment column (25 mm) packed with 5 µm ZORBAX 300 SB-AQ 80A particles, a RP-LC separation column (43 mm × 75 µm) packed with 5 µm Reprosil-Pur ZB-AQ particles, and a nanospray emitter. The fabrication process of the HPLC-Chip/MS system has been described in detail previously (Yin et al., 2004). The HPLC-Chip is inserted into the HPLC-Chip/MS interface, which includes a miniature camera for spray visualization, an HPLC-Chip loading and ejection mechanism, a microvalve for flow switching, and fluid connection ports for the nano-LC and microwell-plate autosampler. The interface was connected to an Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany). The Agilent Mass Hunter ChemStation software (version B 04.01) was used for data acquisition and processing. The mobile phase components were water (A) and acetonitrile (B), both of them containing 0.1% (v/v) of formic acid. For the analysis of derivatized glutathione using the HPLC-Chip/MS system, the sample was loaded into the enrichment column in a mobile phase containing 100% of A using the capillary pump at a flow rate of 4 µL min^{-1}. Following the enrichment, analytes were backflushed into the separation column using the nano pump at 0.3 µL min^{-1}. The injection volume was 1 µL, and the glutathione was separated with the following gradient: from 0 to 3 min 0% of B, from 3 to 8 min 0–80% of B, and from 8 to 8.1 min 80–0% of B. The column was equilibrated with 100% A for 5 min and the total run time was 13 min per analysis. The flush volume was equal to 4 µL.

2.4.3. Mass spectrometry conditions

All the analyses were performed on an Agilent 6460 triple quadrupole. Purified nitrogen (Zefiro 35 nitrogen generator) was used as a drying gas. The analyses were carried out in the positive ionization mode. Prior to perform analysis, a standard micro electrospray ion source was employed to tune the mass spectrometer. The optimized conditions were as follows: the drying gas temperature was 325 °C, the drying gas flow rate was 2.5 L min^{-1}, and the capillary voltage was 2050 V. Then, the HPLC-Chip-MS system was installed to define the best ion source operating conditions. The position of the nanoESI tip of the HPLC-Chip system was optimized to achieve maximum sensitivity and a stable spray. Ionization and mass spectrometric conditions were optimized for each analyte using the calibration chip and the Optimizer software (Agilent Technologies). Derivatized glutathione was analyzed in dynamic MRM mode and detection conditions were reported in the Table 1. Quantification was performed using the following transitions: 433 $m/z \rightarrow$ 304 m/z for GSH and 436 $m/z \rightarrow$ 307 m/z for the internal standard (ISTD).

2.5. Validation procedure

Analytical method validation was performed by evaluating the following parameters: matrix effect, linearity, accuracy,