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Over-evaluation of total flavonoids in grape skin extracts containing sulphur dioxide

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ABSTRACT

Sulphur dioxide (SO_2) proved to increase absorbance at 280 nm of grape skin and seed extracts containing it, diluted with ethanol–HCl to assess total flavonoids and anthocyanins in the same analysis. Additional absorbance at 280 nm was also observed in acetone:H_2O extracts, if the acetone had not completely evaporated before the extracts were diluted with a solvent. Flavonoids were correctly quantified in the extracts when SO_2 or acetone were removed by solid-phase extraction with a C_{18} RP as sorbent and methanol as eluting solvent.

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1. Introduction

Solid–liquid extraction, usually but not always (Janik, Cozzolino, Dambergs, Cynkar, & Gishen, 2007), precedes qualitative and quantitative determination of phenols in vegetal tissues, and may be carried out with organic, aqueous or mixed organic-aqueous solvents. Acetone:H_2O (2:1, v/v) (Kennedy & Jones, 2001), neutral methanol or methanol acidified with strong acids (Revilla, Ryan, & Martin-Ortega, 1998), acidified ethanol (Downey, Harvey, & Robinson, 2003) and formic acid:water:methanol (2:28:70, v/v) (Jeong, Goto-Yamamoto, Hashizume, & Esaka, 2008) are some of the solvents used for polyphenol extraction from grape skin and seeds. In our laboratory, we use a pH 3.2 hydroalcoholic tartaric buffer containing 2 g L^{-1} of $Na_2S_2O_5$ (HATB + SO_2) as extraction solvent. Besides its good extraction efficiency, this buffer does not produce hydrolytic artifacts (due to its relatively high pH), allows skin and seed extracts to be stored in a solid state (−20 °C) for long periods before analysis, without previous treatment (Squadrito, Corona, Ansaldi, & Di Stefano, 2010) and, during berry peeling, can reduce to phenols the quinones produced by polyphenol oxidase (PPO) due to SO_2 (Danilewicz, 2007). The finer the plant material is crushed, the more its extraction with HATB + SO_2 or organic solvents gives similar results (our unpublished results). The HATB + SO_2 extracts of grape skin and seeds can be used for spectrophotometric and chromatographic analyses after sample preconcentration by solid-phase extraction (SPE) or dilution with appropriate buffers, depending on the class of compounds to be determined (Ferrandino, Carra, Rolle, Schneider, & Schubert, 2012; Squadrito et al., 2010). For example, for spectrophotometric determination of anthocyanins, extracts are diluted with ethanol–HCl (Squadrito, Corona, Ansaldi, & Di Stefano, 2007). Recording the absorption spectrum of this solution in the range 230–700 nm also allows evaluation of total flavonoid content (Corona, Squadrito, Borsa, & Di Stefano, 2010) from absorbance at 280 nm, mainly due to the contribution of anthocyanins and flavanols. In a previous work, Corona et al. (2010)

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observed that absorbance at 540 nm (λ_{max} of B-ring trisubstituted anthocyanins in ethanol–HCl) was consistent with the anthocyanin contents (as malvidin-3-glucoside equivalents) in the sample, but the total flavonoid contents (as (+)-catechin equivalents) determined by absorbance at 280 nm exceeded the value obtained by both the Folin Ciocalteu reagent and the Bathe-Smith reaction (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2004).

Corona et al. (2010) also found that absorbance at 280 nm of HATB + SO_2 grape skin extracts diluted with ethanol–HCl was greater than the sum of that at 280 nm of the two fractions of the same extracts passed through a C_{18} RP cartridge, eluted respectively with 5×10^{-3} M H_2SO_4 (non-adsorbed polar compounds) and methanol (non-polar or weakly polar adsorbed compounds). The absorbance at 280 nm was measured directly on the non-adsorbed polar fraction and after dilution with ethanol–HCl of the adsorbed non-polar fraction eluted with methanol. The very small absorbance at 280 nm of the polar fraction indicated that almost all substances with λ_{max} at 280 nm in grape skin extracts – mainly phenols, according to literature reports (Naczka & Shahidib, 2004; Ribéreau-Gayon et al., 2004) – were adsorbed on the C_{18} RP. However, why some of the substances adsorbed were not determined as total flavonoids by measurement of E'_{280} (Corona et al., 2010), after elution with methanol and dilution of the eluate with ethanol–HCl, still remained to be explained.

It was initially hypothesized that substances with λ_{max} at 280 nm, apparently not eluted with methanol, were phenol compounds bonded to polysaccharide chains released from the cell walls of berry skins by the hydrolytic action of HSO_3^- (Ribéreau-Gayon et al., 2004). Their phenol component was deemed to have been adsorbed by the C_{18} RP and not eluted with methanol, as the polysaccharide moiety is insoluble in methanol. This hypothesis was rejected, because no turbidity or precipitate due to dilution of skin extracts with ethanol ("ethanol index"; Glories, 1978) was observed. In these conditions, phenol compounds bonded to polysaccharide chains or other polymers (proteins, glycoproteins) would be precipitated. The possibility that these substances were phenols linked to the structure of cutin was also rejected, since elution with chloroform after methanol did not recover compounds with λ_{max} at 280 nm from the C_{18} RP cartridge. Lastly, it was assumed that the substances apparently not eluted with methanol were intermediates in the biosynthesis of anthocyanins or proanthocyanidins. Like anthocyanins, they would bind to HSO_3^- during extraction of grape skins with solvents containing SO_2 and separated from HSO_3^- during adsorption on the C_{18} RP, forming carbocations with a positive charge located in position 4 of the C ring (Pfeiffer et al., 2006). Unlike anthocyanins (the positive charge of which is stabilized by resonance), the hypothesized intermediates would react with the proanthocyanidins adsorbed on the C_{18} RP. The incorporation of these intermediates in grape skin proanthocyanidins matched the almost unchanged reactivity with vanillin (vanillin assay; Sun, Ricardo-da-Silva, & Spranger, 1998) and the increased contents of proanthocyanidins (determined by the Bathe-Smith reaction; Di Stefano & Gentilini, 2002; Ribéreau-Gayon et al., 2004) of the flavanols eluted from the cartridge with respect to whole grape skin extracts. It also explained the decrease in absorbance at 280 nm of extracts after SPE on the cartridge. However, subsequent experiments (not described here) showed that, with respect to whole extracts, the increase in proanthocyanidins was due to the lower content of H_2O of the hydrophobic fraction eluted with methanol from the C_{18} RP, to which the Bathe-Smith reaction was applied.

Critical re-examination of the experimental attempts described above, as well as subsequent experiments (described here) finally enabled us successfully to identify the substance responsible for the non-phenol absorbance at 280 nm of HATB + SO_2 skin extracts diluted with ethanol–HCl.

2. Materials and methods

2.1. Chemicals

The following chemicals were used: Sodium metabisulfite ($Na_2S_2O_5$), ethanol, methanol, acetone, NaOH, H_2SO_4 95–97%, and H_3PO_4 85% (Merck, Milan, Italy); phloroglucinol (Fluka, Milan, Italy); malvidin-3-glucoside (Extrasynthese, Genay, France); and tartaric acid, HCl 37%, (+)-catechin (Sigma–Aldrich, Milan, Italy).

2.2. Sampling and extraction of phenols from grape skins and seeds

Briefly: groups of 50 berries with petioles were selected from a sample of 400 berries randomly collected from vineyard. After weighing, berries of each group were skinned, and the skins and seeds were placed for 4 h in flasks, containing 50 mL of extraction solvent Three different extraction solvents were used: (i) pH 3.2 tartaric buffer obtained dissolving 5 g tartaric acid, 22 mL NaOH 1 N, 2 g $Na_2S_2O_5$, 125 mL ethanol 95–96%, brought to 1 L with H_2O (hereafter HATB + SO_2); (ii) same as (i) but without $Na_2S_2O_5$ (hereafter HATB); (iii) acetone:water (70:30, v/v).

The samples were then homogenized and centrifuged (4000 rpm for 15 min), and the liquors were collected in 100-mL volumetric flasks. The pellets were re-suspended in 40 mL of the same solvent and centrifuged after 1 h (4000 rpm for 15 min). The second liquor was added to the first and brought to 100 mL with the same solvent.

2.3. Determination of total anthocyanins and flavonoids

0.5 mL of grape skin extract sample was diluted to 25 mL with ethanol:H_2O:12 M HCl, 70:30:1 v/v (hereafter ethanol–HCl), and the spectra from 230 to 700 nm and absorbances at 540 or 536 nm for cultivars in which respectively B-ring tri- or B-ring di-substituted anthocyanins prevailed were recorded, and E'_{280} was calculated. Total anthocyanins (as malvidin-3-glucoside equivalents) and flavonoids (as (+)-catechin equivalents) of grape skin extract were calculated according to the following equations:

$$\text{Total anthocyanins mg L}^{-1} = 16.17 \times E_{540} \times 50$$

$$\text{Total flavonoids mg L}^{-1} = 82.4 \times E'_{280} \times 50$$

MW/ε = 16.17 for malvidin-3-glucoside in ethanol–HCl was calculated from ε = 33,700 for malvidin-3-glucoside in methanol–HCl (Wulf & Nagel, 1979). The ratio of (+)-catechin concentration/E'_{280} determined on a 10-mg L^{-1} solution of (+)-catechin was 82.4. The dilution coefficient of the extracts was 50. E'_{280} was the length (in absorbance units) of the segment joining the peak at 280 nm of the spectrum of the skin extracts diluted in ethanol–HCl, with the intersection point between the perpendicular drawn from the 280-nm peak to the λ axis and the tangent to the spectrum in the UV region. For skin extracts, data in mg L^{-1} were converted to mg kg^{-1}.

To determine total flavonoids in seeds, the 230-400 nm spectra of grape seed extracts diluted 1:10 with ethanol–HCl were recorded, and absorbance at 280 nm (E'_{280}) was calculated as for skin extracts. Total flavonoids and anthocyanins of grape skin extracts and total flavonoids of seed extracts were also determined after absorption of extracts on a C_{18} RP cartridge and elution of polyphenols with methanol.

2.4. Purification of phenols on C_{18} RP cartridge

Samples of 0.5 mL grape skin or seed extracts, diluted to 5 mL with 5×10^{-3} M H_2SO_4, were passed through a 400-mg C_{18} RP car-

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