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Phytosterols in grapes and wine, and effects of agrochemicals on their levels

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ABSTRACT

To improve the knowledge on the chemical diversity and complexity of grapevine, we investigated the plant sterol content of berry and seed tissues at pre-*véraison* and *véraison* stages in 2009 and 2010. We also assessed the effects of benzothiadiazole and chitosan elicitors on content of sterols in grapes and their levels in the corresponding experimental wines. β-Sitosterol was the most abundant component in berry tissues, in both growth stages and years, with the highest amounts in the flesh and skin at pre-*véraison* and *véraison*, respectively. Stigmasterol and campesterol were present in lower concentrations in both phenological stages and vintages. During the transition from pre-*véraison* to *véraison*, phytosterols decreased in all tissues, in both years, apart from stigmasterol in seeds. In addition, the results showed that the plant activators were more effective than conventional fungicides in rising the levels of sterols, particularly β-sitosterol, both in grapes and in microvinificates.

1. Introduction

Health benefits of grape products have been emphasised during the last decade, focusing on the chemical diversity of grapevine secondary metabolites (Iriti & Faoro, 2009). As a major component of the traditional Mediterranean diet, the regular and moderate consumption of red wine at main meals has been associated to a significantly reduced risk of cardiovascular diseases and other chronic, degenerative disorders (Costanzo, Di Castelnuovo, Donati, Iacoviello, & de Gaetano, 2010). Healthy properties of wine have been mainly attributed to polyphenols, a class of phytochemicals with a plethora of biological effects, including antioxidant, anti-inflammatory, antiproliferative, vasorelaxant, immunomodulatory and (phyto)estrogenic activities. Polyphenols are divided into three classes, stilbenes and flavonoids, with resveratrol and anthocyanins as main compounds, respectively, and proanthocyanidins or condensed tannins (Iriti et al., 2009). However, the knowledge on complexity of grape chemistry has been increasing over the past few years. Melatonin, an indoleamine traditionally considered a vertebrate neurohormone with a powerful antioxidant activity, and its isomers have been recently reported in grapes and wine, thus contributing to elucidate the healthy potential of these foods (Vitalini, Gardana, Simonetti, Fico, & Iriti, 2012; Vitalini, Gardana, Zanzotto, Simonetti, et al., 2011).

Noteworthy, both endogenous and environmental factors regulate the biosynthesis of secondary metabolites *in planta*. In addition to the genetic traits of grape varieties, climatic, agrometeorological and edaphic conditions, as well as biotic and abiotic stresses, agricultural practises and winemaking interact modifying the content of bioactive phytochemicals in grapevine products (Lachman, Šulc, Faitová, & Pivec, 2009). In particular, it has been reported that plant activators, a class of agrochemicals able to stimulate systemic acquired resistance (SAR), a complex plant defence system, elicited the synthesis of polyphenols and melatonin in grapevine, as well as of different metabolites in other plant species, thus contributing to improve the biological activities of plant products (Bavaresco, Mattivi, De Rosso, & Flamini, 2012; Iriti, Mapelli, & Faoro, 2007; Vitalini, Gardana, Zanzotto, Fico, et al., 2011). Benzothiadiazole (BTH), a functional analogue of the plant hormone salicylic acid, and chitosan (CHT), a deacetylated chitin derivative, are among the most studied resistance inducers (Iriti et al., 2011; Vitalini et al., 2011).

In this view, and in order to improve the knowledge on the chemical diversity and complexity of grapevine, we investigated, in this work, for the first time, the phytosterol (plant sterol) content of different berry and seed tissues at two different phenological stages (pre-*véraison* and *véraison*) and in 2 years (2009 and

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2010). We also assessed the effects of elicitors, BTH and CHT, on content of sterols in grapes and the levels of these metabolites in the corresponding experimental wines.

Phytosterols are ubiquitous in plants and they are similar in structure to cholesterol, sharing the same basic cyclopentanoperhydrophenanthrene ring structure (the steroid nucleus), but differing in the alkyl side chain. As sterols, they arise from the isoprenoid biosynthetic pathway, from acetyl coenzyme-A via squalene (see Fig. in Burlini et al., 2011), and they are structural components of the plasmalemma, where they regulate membrane fluidity and permeability. Though the term phytosterols refers to more than 200 different compounds up to now identified, β-sitosterol, stigmasterol and campesterol predominate in higher plants and typical human diets (Fig. 1). Appreciable amounts of phytosterols are found in lipid-rich plant foods, i.e. vegetable oils, nuts, legumes and other edible seeds, whereas cereal grains, fruits and vegetables contribute to a certain extent to the daily intake of these phytoconstituents (Jiménez-Escrig, Santos-Hidalgo, & Saura-Calixto, 2006). Health benefits of plant sterols have been emphasised in the last decade, mainly because of their serum cholesterol-lowering effect and anticancer properties (Jones & Abumweis, 2009; MacKay & Jones, 2011). In particular, because of their structural similarity to cholesterol, phytosterol partially inhibit the intestinal absorption of both dietary and biliary (endogenously produced) cholesterol, thus lowering their circulating levels and exerting anti-atherogenic and cardioprotective effects (Trautwein & Demonty, 2007).

2. Materials and methods

2.1. Phytoiatric campaign

Open-field treatments with plant activators (Table 1) were performed in 2009 and 2010 on an autochthonous grapevine (*Vitis vinifera* L.) cultivar of Lombardia, Groppello, cultivated in an experimental vineyard located at Raffa di Puegnago (Azienda Agricola San Giovanni, Brescia, Italy). The treatments were: (i) 0.03% (w/v) chitosan (CHT, 76 kDa molecular weight and 85% deacetylation degree; Sigma–Aldrich, St. Louis, MO, USA), (ii) 0.03% CHT in combination with 150 g/h L copper hydroxide (Kocide 3000; Du Pont, Wilmington, DE, USA) (CHT/Cu) and (iii) 0.3 mM benzothiadiazole [benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester, BTH, trade name Bion®, Syngenta, Basilea, CH]. Untreated grapevines were used as negative control, while plants treated with conventional fungicides (penconazole and methyldinocap) were used as positive control (Table 1).

The trial was set up as a complete randomised block design in four replications, with 10 vines (a parcel) per treatment in each block. Plants were sprayed every 10 days approximately with a spray lance, according to the meteorological conditions, from the beginning of grape susceptibility to fungal diseases until the complete *véraison* (i.e. approximately from the half of April to the end of July). Sampling was scheduled at two phenological phases: pre*véraison* ('pea-size' stage, before the end of the berry's growth cycle) and 100% *véraison* (stage when berry turn colour and soften). Bunches were randomly collected from plants during the morning and stored at −20 °C. Different tissues (berry fleshes and skins, and seeds) of frozen berries, were randomly selected from bunches and carefully separated with a chilled scalpel and then lyophilised (Leybold-Heraeus GT2) for 20 h. After lyophilisation, the homogeneous sample of each tissue was pulverised with a chilled mortar and pestle. The powders were kept at −20 °C until analyses.

Table 1
Open-field treatments applied on grapevine parcels.

Treatment
CHT (0.03% chitosan)
CHT/Cu (0.03% chitosan + 150 g h L^{-1} copper hydroxide)
BTH (0.3 mM benzothiadiazole)
Fungicides (penconazole + methyldinocap, positive control)
No treatment (negative control)

Experimental Groppello wines were produced, by standard microvinification techniques, in the Centro Vitivinicolo Provinciale di Brescia (Italy) and stored at 4 °C until analysis.

2.2. Sterol extraction from berry and seed tissues

In all plant tissues, phytosterols occur in the form of free sterols, steryl esters, steryl glycosides and acylated steryl glycosides. Therefore, the optimal sample preparation procedure for total sterol determination should consider sterols from all possible conjugates. Acid hydrolysis prior to alkaline hydrolysis has been used to release sterols in their free and conjugated forms, including glycosidic sterols. Cholesterol was added to each sample (1 g), as internal standard (0.5 mL of a solution 1:1 w/v in ethanol), together with 2 mL of ethanol and 10 mL of 6 N HCl. The suspension was stirred at 80 °C for 60 min. After cooling, 10 mL of ethanol was added, and the mixture was stirred for a further 5 min. Finally, 50 mL of hexane/diethyl ether (1:1 v/v) was added and, after 10 min of stirring, 35 mL of the organic layer was removed and evaporated under vacuum by a Büchi Rotavapor R-114 (temperature not greater than 40 °C). The residue was treated with ethanolic pyrogallol (10 mL of a solution 2% w/v) and KOH (6 mL of a solution 25% w/v). After 30 min at 80 °C, the solution was cooled, and 20 mL of water and 40 mL of cyclohexane were added therein. The mixture was centrifuged at 6500 rpm for 5 min at 4 °C. Part of the organic layer (30 mL) was removed, and the solvent was evaporated by rotavapor. Dry samples were stored at −20 °C.

2.3. Sterol extraction from experimental wines

The extraction process consisted in transferring 50 mL of experimental wine to a separatory funnel and adding 0.5 mL of a solution of 1 mg/mL cholesterol in ethanol as internal standard. Sterols were extracted by shaking, with three successive 50 mL portions of $CHCl_3$, in a separatory funnel. In order to break down the water–chloroform

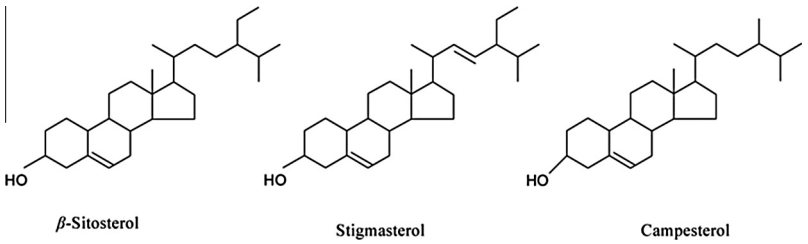

Fig. 1. Chemical structures of the main phytosterols.