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Quantification of stilbenoids in grapevine canes and grape cluster stems with a focus on long-term storage effects on stilbenoid concentration in grapevine canes

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ABSTRACT

On the one hand there is an enormous amount of underutilized side-streams rich in bioactives during wine-production, while on the other hand there is a growing demand for promising phytochemicals for e.g. dietary and pharmaceutical purposes. In the present study we analyzed the stilbenoid contents of grape canes and grape cluster stems collected in Germany. Likewise we investigated the effect of long-term post-harvest storage on stilbenoid levels in grape canes and also the variability of stilbenoids in grape cluster stems during growth cycle. The predominant stilbenoids in either canes or stems were *trans*-resveratrol and *trans*-ε-viniferin. The contents in canes ranged from 441 to 7532 and 1218 to 5341 mg/kg DW for *trans*-resveratrol and *trans*-ε-viniferin, respectively, depending on variety, vintage and storage time. Within storage of 6 months the content in canes increased by up to a factor of fourteen. Stilbenoid contents in grapevine cluster stems varied from 16 to 289 and 23 to 253 mg/kg DW for *trans*-resveratrol and *trans*-ε-viniferin, respectively, depending on vintage and wine-growing area.

1. Introduction

The wine making process is known to generate substantial quantities of organic waste, so called side-streams or by-products. This can inter alia be derived from the fact, that an amount as high as 20% of the grape does not find its way into the wine. By-products accumulate in each production step, most of which accumulate during vinification but considerable amounts of side-streams are also generated during viticulture. Noteworthy side-streams are especially canes, stems, pomace (consisting of skins, pulp and seeds) and lees (Ye, Harrison, Cheng, & Bekhit, 2015). Grape canes and grapevine cluster stems are woody residues of wine-making and can also be described as lignified side-streams. These side-streams represent a so far underutilized source of bioactive stilbenoids whose economic potential has been conclusively evaluated, for especially grape canes, by Rayne, Karacabey, & Mazza, 2008.

Grape canes are formed during annual pruning which is carried out during vegetation rest in winter and is necessary for an adequate cultivation of the grapevine. Each year 1 to 3 t/ha of grape canes are generated. Nowadays emission protection regulations mostly prohibit the burning of grape canes which was the traditional way of disposal of these woody residues. The usage as fertilizer is also questionable, as it enables wood destructive fungi, e.g. esca-disease to persist in the vineyard (Bauer, Regner, & Schildberger, 2015).

Grapevine cluster stems accumulate during the destemming process. Destemming is essential since cluster stems introduce green tannins and herbaceuos and vegetabile aromas to the wine, furthermore they are rich in water and poor in sugar hence reducing potential alcohol in the must if not removed (Christmann & Freund, 2010).

Lignified tissues of grapevine are known to contain huge amounts of stilbenoids, i.e. *trans*-resveratrol and its derivatives and seem to be a promising source for the recovery of these substances. Indeed, there is still a need for alternative sources of resveratrol, as can be seen by the recent permission of resveratrol as a novel food ingredient in the European Union (Commission Implementing Decision (EU) 2016/1190).

Resveratrol and even more its oligomers are phytochemicals with promising biological effects, at least in vitro (Empl, Albers, Wang, & Steinberg, 2015; Empl et al., 2014; Macke, Jerz, Empl, Steinberg, & Winterhalter, 2012; Willenberg et al., 2015). A good overview about attributed health effect to *trans*-resveratrol is given by Baur and Sinclair (2006).

Several studies deal with stilbenoid contents in grapevine canes (Guerrero et al., 2016; Lambert et al., 2013; Pawlus et al., 2013;

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Vergara et al., 2012; Zhang et al., 2011) as well as grapevine cluster stems (Anastasiadi, Pratsinis, Kletsas, Skaltsounis, & Haroutounian, 2012; Piñeiro, Guerrero, Fernández-Marin, Cantos-Villar, & Palma, 2013). But so far little is known about post-harvest accumulation of stilbenoids in grapevine canes. To the best of our knowledge there are only two studies explicitly dealing with post-harvest accumulation of stilbenoids in grape canes down to the present day, both of which investigating short-term storage effects on stilbenoid levels (Gorena et al., 2014; Houillé et al., 2015). Surprisingly, up to date there is also no data available concerning stilbenoid content in woody residues of winery from German cultivation areas. To the best of our knowledge, the present study is the first to deal with long term storage effects on stilbenoid levels of canes from the Palatinate, which is the second largest German wine growing area by size. In addition, stilbenoid levels in grapevine cluster stems from two German cultivation areas are investigated with a focus on variability of stilbenoids in grape cluster stems during growth cycle.

2. Materials and methods

2.1. Chemicals

Ethanol absolute, analytical reagent grade, methanol, HPLC-grade and LC-MS-grade and acetic acid, LC-MS-grade were purchased from Fisher Chemical (Loughborough, United Kingdom). Acetic acid, HPLC-grade was purchased from Applichem (Darmstadt, Germany). Deionized water (Nanopure, Werner, Leverkusen, Germany) was used. *trans*-Resveratrol was purchased from Sigma-Aldrich (Steinheim, Germany), *trans*-ε-viniferin was purchased from Phytolab (Vestenbergsgreuth, Germany).

2.2. Samples

2.2.1. Grape canes

The canes of 15 samples were analyzed, all of which were *Vitis vinifera* varieties, in particular “Cabernet sauvignon”, “Merlot”, “Regent”, “Riesling”, “Pinot gris”, “Sauvignon blanc”, “Pinot noir”, “Pinot blanc”. The grape canes were collected druing the annual pruning seasons in the viticultural region of Palatinate (Neustadt a. d. W) in 2013 and 2014. They were chopped to pieces (10–20 cm) and stored under well-aerated conditions in the dark, at ambient temperature, for up to 30 months. The canes harvested during 2013 (8 samples) were analyzed approximately 6 month after pruning. From the canes harvested in 2014 (7 samples) a representative amount of each sample (approx. 100–200 g) was analyzed directly after harvest and furthermore every 6 months during storage.

2.2.2. Grape cluster stems

Grape cluster stems of *Vitis vinifera* varieties, “Müller-Thurgau”, “Pinot blanc”, “Rieslaner” and “Riesling” from the viticultural region of Palatinate (Neustadt a. d. W) were directly harvested from the grape vine in 2015 without being involved in the destemming and vinification process. The grape cluster stems from Palatinate region were picked at four different dates. Harvest dates in 2015 were the July 24, August 17, September 2 and September 9 for each variety, with the last date indicating full ripeness of the grapes.

Grape cluster stems of *Vitis vinifera* varieties from the viticultural region Saale-Unstrut were harvested in 2014 as described above. Harvest dates were September 30 and October 1 to 3 (“Riesling”/“Pinot blanc”/“Gewürztraminer”/“Gutedel”) at full ripeness of the individual grapes.

2.3. Sample preparation and extraction

Sample preparation was done according to the study of Vergara et al. (2012) but with several modifications. The plant material was lyophilized until a constant weight was reached (freeze dryer Christ Alpha 2–4, Osterode, Germany). The lyophilized samples were ground by a cutting mill equipped with a parallel section rotor and a bottom sieve with trapezoid holes of 1.5 mm (Retsch SM 1, Haan, Germany).

An amount of 2 g of the ground sample were extracted four consecutive times with 20 mL of an ethanol/water mixture (80/20; v/v) for 5 min, using an ultrasonic homogenizer equipped with a 1/8″ ultrasonic horn in order to transmit ultrasonic energy into the sample solution (Branson Ultrasonics Sonifier S450A, Danbury, USA). The sonifications were carried out with the output control set to 20%. Each individual extraction step was followed up by a 5 min centrifugation step at 8000 rpm (Hettich Universal 30F, Lauenau, Germany). The extracts were combined and made up to 100 mL. The processed extracts were either analyzed directly or stored in the dark at − 18 °C prior to analysis. For the analysis of the grapevine cluster stems a concentration step was included. The extracts were dried under nitrogen and afterwards reconstituted with ethanol/water mixture (80/20; v/v), resulting in 10 fold concentrated solutions. Each sample of grape cane was extracted in triplicate while extraction grapevine cluster stems was performed at least in duplicate.

2.4. HPLC analysis

To quantify stilbenoids, an HPLC system from Jasco (Gross-Umstadt, Germany), with a PU-2080 plus pump combined with a degasser (DG-2080-53), ternary gradient unit (LG-2080-02), and a photodiode array detector (MD-2010 plus) was used. HPLC separation was achieved on a Kromasil 100-5-C18 column (250 mm × 4.6 mm, 5 µm, Eka Chemicals AB, Bohus, Sweden) protected with a guard column of the same material (10 mm × 4 mm). The mobile phases consisted of 1% aqueous acetic acid (v/v) (A) and methanol (B). The separation was carried out at 25 °C with a flow rate of 0.8 mL/min, under the following conditions: 0 min (20% B), 5 min (30% B), 15 min (30% B), 18 min (37% B), 29 min (37% B), 35 min (50% B), 57 min (50% B), 58 min (100% B), 71 min (100% B), 72 min (20% B), and 75 min (20% B). *trans*-Resveratrol and *trans*-ε-viniferin were quantified at 306 and 324 nm, respectively.

2.5. HPLC-ESI-MS

For qualification and peak identification purposes an HPLC-ESI-MS system consisting of a binary HPLC Pump (1100 series) and an autosampler (1200 series) from Agilent Technologies (Waldbronn, Germany) equipped with an HCT Ultra ETD II LC-ESI-MS/MS ion-trap system from Bruker Daltonics (Bremen, Germany) was used. Mass spectra were recorded in the negative ionization mode, with the capillary voltage set at 3500 V, the end plate at − 500 V, and the capillary exit at − 115.0 V. Drying gas was nitrogen at 330 °C, and the flow rate was 10.0 L/min. The nebulizer pressure was set to 50 psi, the target mass at *m/z* 400, and the scan range from *m/z* 100 to 3000. Compass Hystar Software (Bruker Daltonics) was used for analysis and data collection. HPLC separation was carried out on a Luna 3u; C18; 100 A; 3 µm (150 mm × 2.0 mm i.d.) from Phenomenex (Aschaffenburg, Germany). The mobile phase was the same as described in Section 2.4. LC conditions for ESI-MS analysis were 0 min (20% B), 5 min (35% B), 15 min (35% B), 18 min (45% B), 29 min (45% B), 35 min (60% B), 57 min (60% B), 58 min (100% B), 71 min (100% B), 72 min (20% B), and 75 min (20% B).

3. Results and discussion

3.1. Analytical validation

The extraction parameters were extensively evaluated by Vergara et al. (2012), nevertheless extraction cycle number, solid-liquid ratio and extraction methodology (i.e. stirred maceration, maceration in