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Note

High-performance liquid chromatographic determination of the free *o*-quinones of *trans*-caffeoyltartaric acid, 2-S-glutathionylcaffeoyltartaric acid and catechin in grape must

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The enzymatic oxidation of hydroxyphenolic compounds to quinones, catalysed by polyphenoloxidases, is a ubiquitous phenomenon[1,2], responsible for many biologically and technologically important reactions, such as melanin pigmentation[3–5], insect cuticle sclerotization[6,7] and fruit and vegetable browning during processing and storage[2,8,9]. Numerous studies have dealt with the enzymatic substrates and/or the condensation products, but the quinone intermediates have not received much attention. The purpose of this work was to develop a method to measure free quinones in grape musts in order to establish the kinetics of phenolic oxidation and subsequent quinone reactions.

Hydroxycinnamic acids and especially *trans*-caffeoyltartaric (caftaric) and *p*-coumaroyltartaric (coutaric) acids are the major phenolic compounds of white grape musts[10–12]. When no special care is taken to avoid oxidation during crushing and pressing, they are rapidly oxidized by the grape polyphenol oxidase (PPO) to caftaric acid *o*-quinone. The latter reacts readily with the available glutathione to form 2-S-glutathionylcaffeoyltartaric acid, known as Grape Reaction Product (GRP)[13–15]. This compound is not a substrate for the grape PPO[15] but can be oxidized by the excess of caftaric acid quinones[16]. Similar coupled oxidations have been demonstrated with a number of flavans[17], which are minor components of white grape juice but can be extracted in larger amounts when pomace contact takes place during the wine-making process[18–21]. The resulting quinones are rapidly involved in polycondensation reactions, leading to the formation of brown polymers[17].

The high-performance liquid chromatographic (HPLC) separation of grape and must phenolics has been extensively studied[22–26]. On the other hand, no method is available for measuring the free quinones, probably because of their instability. In particular, they are readily reduced back to the corresponding hydroquinones by sulphur dioxide and/or ascorbic acid added to prevent sample oxidation when assaying for grape must phenolics. However, the instantaneous concentration of *o*-quinones, and especially that of caftaric acid quinones, might be fairly high in oxidizing musts.

The ability of benzenesulphinic acid to react with *o*-quinones has been known for some time[27–29]. The method reported here involves benzenesulphinic acid deriva-

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tization followed by reversed-phase HPLC separation for the determination of caftaric acid, GRP and catechin-free *o*-quinones in grape musts.

EXPERIMENTAL

Chemicals

(+)-Catechin and sodium benzenesulphinate were purchased from Fluka (Buchs, Switzerland), ascorbic acid and hydrogen peroxide (35% solution) from Merck (Darmstadt, F.R.G.) and horseradish peroxidase (E.C. 1.11.1.7) from Sigma (St. Louis, MO, U.S.A.). Crude grape PPO extract was prepared from grape juice as described previously[14].

Caftaric acid was extracted from grape juice following the procedure of Singleton *et al.*[12] and 2-S-glutathionylcaftaric acid prepared by aerating 2 m*M* caftaric acid and 10 m*M* reduced glutathione in the presence of 6 g/l crude grape PPO extract in 2.5 g/l aqueous potassium hydrogentartrate (pH 3.65). Both were purified by preparative HPLC. The preparative HPLC system was a Jobin-Yvon (Longjumeau, France) system, consisting of a Modulprep compression module, a Modulprep hydraulic module, a Modulprep pump, a manual injection system, an ISA-SM 25 UV detector set at 280 nm and a Linseis recorder. The column was an axial compression column (500 × 22 mm I.D.), filled with LiChrosorb RP-18 stationary phase (Merck, 15–25-μm packing). Isocratic elution was performed using 10% methanol in 3% acetic acid solution at a flow-rate of 20 ml/min.

Caftaric acid and catechin sulphones were synthesized by incubating 2 m*M* caftaric acid and 2 m*M* catechin, respectively, with 20 m*M* sodium benzene sulphinate and 10 g/l crude grape PPO extract in 2.5 g/l aqueous potassium hydrogentartrate. The GRP *o*-quinones were obtained by peroxidase oxidation of 2 m*M* GRP in the presence of a stoichiometric amount of hydrogen peroxide. After a few minutes, a red-brown colour characteristic of GRP quinones[16] developed and the sulphones were produced by addition of 20 m*M* sodium benzenesulphinate. Sulphur dioxide (0.5%) was added 30 s after the sodium benzenesulphinate to reduce the hydrogen peroxide remaining and inhibit peroxidase. The three sulphones were purified by preparative HPLC as described above but using 30% methanol in 3% acetic acid as the eluent.

Sample preparation

Model solutions were prepared by incubating the substrate(s) in the presence of crude grape PPO extract in 2.5 g/l aqueous potassium hydrogentartrate at 30°C and with air agitation on a magnetic stirrer. Oxidized must samples were obtained at the INRA experimental winery at Pech Rouge by crushing and pressing white grapes with regular winery equipment, unless specified otherwise.

Sodium benzenesulphinate crystals were added to all samples (approximately 2.5 mg/ml) and the mixture was stirred for 10–15 s, stabilized by addition of sulphur dioxide (0.2%) and filtered through 0.45-μm membrane filters prior to injection (injection volume 20 μl) on to the HPLC column.

Instrumentation

The HPLC apparatus was a Millipore-Waters (Milford, MA, U.S.A.) system including a 710B autoinjector, a 720 system controller and two M510 pumps

connected to a Spectromonitor 3100 (Milton Roy) variable-wavelength detector set at 280 nm and to an Enica 21 integrator (Delsi, France). The column was reversed-phase Spherisorb ODS-2 (5-μm packing) (250 × 4 mm I.D.) protected with a guard column of the same material (Knauer, F.R.G.). The elution conditions were as follows: flow-rate, 1 ml/min; solvent A, 2.5% acetic acid; solvent B, acetonitrile–solvent A (80:20, v/v); elution starting with 5% B, isocratic for 4 min, and continuing with a linear gradient from 5 to 20% B in 16 min and from 20 to 80% B in 10 min, followed by washing and reconditioning of the column.

The UV spectra were recorded from 250 to 400 nm using a Millipore-Waters photodiode-array detector under the same chromatographic conditions.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms for a combined standard of caftaric acid, GRP, catechin and their benzene sulphones and for an oxidizing white grape must sample. Each quinone gave a single derivative, as expected from the results of Piretti *et al.* [29] on catechin and Pierpoint [28] on caffeic and chlorogenic acids.

The mean retention times (± S.D.) for a series of twelve injections were 28.47 ± 0.04, 29.42 ± 0.05 and 30.84 ± 0.03 min for caftaric acid, GRP and catechin sulphones, respectively. It was checked that the quinone derivatives detected in grape

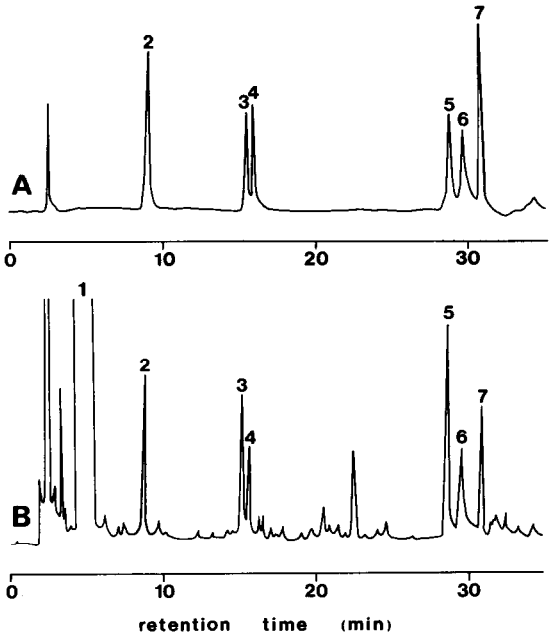

Fig. 1. HPLC traces of reduced (hydroquinones) and oxidized (*o*-quinones) grape juice phenolics in (A) 0.05 m*M* standard solution and (B) 0.1 m*M* catechin-treated Sauvignon must oxidized for 10 min. Peaks: (1) benzenesulphinic acid; (2) *trans*-caffeoyltartaric acid; (3) 2-S-glutathionylcaffeoyltartaric acid; (4) catechin; (5) *trans*-caffeoyltartaric acid *o*-quinone, (6) 2-S-glutathionylcaffeoyltartaric acid *o*-quinone; (7) catechin *o*-quinone.

musts were identical with the standards and did not coelute with other grape must components by co-injection of must samples with quinone derivative standards and comparison of the retention times and of the UV spectra recorded using a diode-array detector.

Known dilutions of each compound in water and in a Chardonnay wine must prepared at the INRA experimental winery were used to determine the response factors (concentration/unit peak area) at 280 and 313 nm. The calibration graphs for all compounds were linear over a concentration range of 0–2 m*M* (0–40 nmol injected). The coefficients of variation over the range 0.01–2m*M* were 1.46, 4.8 and 3.9% ($n=8$) for caftaric acid, GRP and catechin quinones, respectively. The detection limits for caftaric acid, GRP and catechin benzene sulphones were 10, 20 and 13 ng, respectively, in grape must.

The derivatization rates were studied on a solution containing 1 m*M* caftaric acid, 0.5 m*M* GRP and 0.5 m*M* catechin incubated for 10 min with 1 g/l PPO. Derivatization appeared to be very rapid for all three compounds, as immediate discoloration of the samples was observed following addition of benzenesulphinate and no further increase in the amount of catechin and GRP sulphones was obtained when benzenesulphinate was allowed to react longer with the quinones before addition of sulphur dioxide (Fig. 2). In addition, the amount of caftaric acid quinone derivative increased slightly, indicating that the enzymatic oxidation but not the coupled oxidations continues in the presence of excess of benzenesulphinate until sulphur dioxide is added. It was therefore concluded that the optimum delay between benzenesulphinic acid and sulphur dioxide addition was 5–10 s.

The quinone derivatives were stable over a period of several weeks when the samples to which sulphur dioxide had been added were kept in the dark at 4°C.

Reproducibility studies were performed on model solutions containing caftaric acid and GRP or caftaric acid and catechin (each 0.2 m*M*) with the crude grape PPO extract and on a white must prepared by crushing Sauvignon grapes under vacuum, adding 0.1 m*M* catechin and oxidizing by stirring in air at 30°C. Samples were taken in

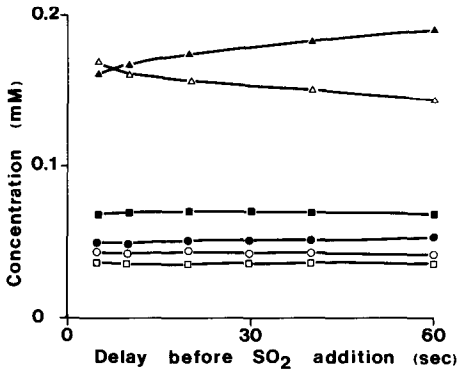

Fig. 2. Influence of the delay between sodium benzenesulphinate and sulphur dioxide addition on the concentration of hydroquinones (open symbols) and *o*-quinones (full symbols) in samples taken after 10-min oxidation from a solution containing initially (△,▲) 1 m*M* caffeoyltartaric acid, (□,■) 0.5 m*M* 2-S-glutathionylcaffeoyltartaric acid, (○,●) 0.5 m*M* catechin and 1 mg/ml crude grape polyphenol oxidase extract.

five replicates from each solution after oxidation for 10 min. The coefficients of variation for the determination of quinones in the model solution and grape must were 2.4, 3 and 2.3% for caftaric acid, GRP and catechin, respectively, in model solutions, and 2.4, 4 and 2.5%, respectively, in Sauvignon must.

The method described is simple, fast and of adequate sensitivity for the measurement of free caftaric acid, GRP and catechin *o*-quinones in oxidizing grape must. Further work on its application to the determination of other *o*-quinones potentially present in grape musts, in particular epicatechin and procyanidin *o*-quinones, is in progress. One of the advantages of the method is that it is suitable for the simultaneous determination of hydroquinones and *o*-quinones. It should also be adaptable to other phenolic substances such as DOPA and its derivatives and, consequently, offer a very useful approach to the study of phenolic oxidation and condensation mechanisms in various research fields.

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