Taste-guided isolation of sweet-tasting compounds from grape seeds, structural elucidation and identification in wines

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Abstract:

This work aimed at improving knowledge about sweetness in dry wines. Following on from the empirical observations of winegrowers, we assessed the contribution of grape seeds to wine sensory properties. An inductive fractionation method guided by gustatometry was used to isolate and characterize sweet-tasting compounds from grapes. Fractionation of grape seed macerates was achieved by liquid-liquid extraction, centrifugal partition chromatography (CPC) and preparative HPLC. Then, the structures of the purified compounds were elucidated by use of FTMS and NMR. Five compounds were identified: two new compounds, 2-hydroxy-3-methylpentanoic-2-*O*-*β*-glucopyranoside (H3MP-G) and 2-hydroxy-4-methylpentanoic-2-*O*-*β*-glucopyranoside acids (H4MP-G), along with gallic-4-*O*-*β*-glucopyranoside acid (AG-G), 3-indolyl-(2R)-*O*-*β*-D-glycoside lactic acid (ILA-G) and *epi*-DPA-3′-*O*-*β*-glucopyranoside acid (epi-DPA-G). These compounds exhibited various levels of sweetness in a hydro-ethanolic solution and in white and red wines. Additionally, H3MP-G, H4MP-G and epi-DPA-G were identified for the first time in grapes and wines, whereas AG-G has already been reported in white grapes but never in wine.

Keywords: Sweetness, Wine, Grape seed, Taste, *epi*-DPA-G

Highlights

Inductive fractionation of grape seed macerates guided by gustatometry.

Isolation and characterization of five sweet-tasting compounds in grape seeds.

Identification of two new compounds: H3MP-G and H4MP-G.

First identification of *epi*-DPA-G in grapes and wines.

First identification of AG-G in wines.

1. Introduction

Wine sensory quality is strongly dependent on grape composition. This composition is directly related to climate, cultivar and winegrowing conditions and is strongly modulated by the soil, these factors generally defining the terroir (van Leeuwen et al., 2004). Winemaking seeks to reveal the sensory characteristics associated with a given terroir and to produce a wine that is unique and typical (Dubourdieu, 2012). Thus, many enological studies investigated the chemical and biological mechanisms that occur during grape transformation and reveal aromas and tastes (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). To this end, knowledge of grape composition is a prerequisite to better understand the impact of grape compounds on wine sensory characteristics. While many volatile compounds responsible for varietal aromas have been identified in grapes under linked (Tominaga, Des Gachons, & Dubourdieu, 1998) or free (Roujou de Boubee et al., 2000, Siebert et al., 2008) forms, less is known about the non-volatile molecules involved in taste balance and in particular in the sweetness of dry wines (Noble & Bursick, 1984).

Sweet perception plays a major role in consumer preference (Sena-Esteves, Mota, & Malfeito-Ferreira, 2018) but the presence of residual sugars can imply microbiological instability and sanitary problems. For these reasons, most of the great red wines are dry and do not contain sugars above their detection threshold. Despite the absence of sugar, wines can display significant differences in sweet perception that can be enhanced by the winemaking process (Marchal, Pons, Lavigne, & Dubourdieu, 2013) but which are still only partially understood in molecular terms (Jones, Gawel, Francis, & Waters, 2008). Recently, the increase in sweetness at the end of alcoholic fermentation was attributed to the release of the Hsp12 protein during yeast autolysis (Marchal, Marullo, Moine, & Dubourdieu, 2011) with potential modulations due to environmental or genetic parameters (Marchal, Marullo, Durand, Moine, & Dubourdieu, 2015). Moreover, the empirical observations of winemakers and experimentations suggest the importance of post-fermentation maceration (PFM) in revealing sweetness in wines. Since wine is in contact with the solid parts of grapes during maceration, it would seem that skin and seeds contribute to sweetness. Furthermore, the contribution of seeds to the sensory characteristics of wine was already mentioned in the old winemaking manuals (Pacottet, 1908, Peynaud, 1983). Based on these observations, the present work aimed at studying the contribution of seeds to wine taste.

To investigate the gustatory impact of grape seeds and search for its molecular origins, taste-guided fractionation was performed. A grape seed extract was submitted to an inductive fractionation protocol guided by gustatometry using liquid-liquid extraction (L-L), centrifugal partition chromatography (CPC) and semi-preparative HPLC. At the end of each separation step, fractions were tasted and the most taste-active ones were submitted to the next step. Then, the isolated compounds were elucidated structurally by FTMS and NMR spectroscopy and their presence in commercial wines was assessed.

2. Materials and methods

2.1. Chemicals and commercial wines

Ultrapure water (Milli-Q purification system, Millipore, France) and HPLC grade solvent (acetonitrile, ethanol, ethyl acetate, *n*-heptane, methanol, propan-2-ol and butan-1-olfrom VWR International, Pessac, France) were used for sample preparation and compound purification. Acetonitrile and water used for mass spectrometry analysis were LC-MS grade and were purchased from Fisher Chemical (Illkirch, France).

The commercial wines used to assess the presence of the sweet-tasting compounds were a white Pessac-Leognan 2012 (80% Sauvignon blanc, 20% Semillon aged in oak barrels with 30% new oak) and a red Saint Emilion Grand Cru Classé 2003 (50% Merlot, 50% Cabernet franc, aged in 100% new oak barrels). These wines resulted from traditional winemaking practices.

2.2. Seed selection and extraction

Mature healthy grapes (from 21.5 to 22.7 Brix) were harvested in qualitative plots from various Bordeaux regions: Cadillac-Côtes de Bordeaux, Saint-Julien and Saint-Emilion. Grapes were from two vintages, 2014 and 2015, and from two varieties, Merlot and Cabernet Sauvignon. They were preserved at −20 °C after harvesting.

Seed extraction was carried out manually in order not to compromise their integrity. Seeds were removed from the frozen pulp and rinsed quickly with water to ensure the removal of sugars from the outer layer of the seeds. In this way, 70 g of seeds were removed from 1.3 kg of grapes (equivalent of 1L of wine). This step was repeated to obtain a total of 350 g of seeds.

2.3. Purification of taste-active compounds from grape seeds

2.3.1. Solid-liquid and liquid-liquid extractions

A quantity of seeds (70 g) was extracted with a hydro-alcoholic solution (15:85 ethanol/water, 1 L) at 30 °C for 10 days. After a 0.45 µm filtration and concentration *in vacuo* to remove ethanol, the aqueous solution was extracted three times with 500 mL of heptane (Hept), three times with 500 mL of ethyl acetate (EtOAc) and three times with 500 mL of butanol (BuOHsat) saturated with milli-Q water. The combined organic layers were evaporated to dryness, suspended in water and freeze-dried to obtain brownish powders of Hept (410 mg), EtOAc (640 mg), BuOHsat (1.44 g) and aqueous (1.34 g) prepurified extracts. This sequence of extractions was carried out five times to process all the seeds (350 g). The overall yield of the liquid-liquid extractions reached 92,3%.

2.3.2. Centrifugal partition chromatography

The CPC apparatus consisted of a Spot prep II LC system equipped with SCPC-100 + 1000 (Armen Instrument, Saint-Avé, France). Fractionation was performed on the 1 L rotor. The solvent was pumped into the column by a 4-way quaternary high-pressure gradient pump. The samples were introduced into the CPC column via an automatic high-pressure injection valve. The system was controlled by Armen Glider Prep V5.0 software. All the experiments were conducted at room temperature.

The choice of an appropriate biphasic system of solvents was based on the study of the partition of extract compounds in both phases according to the procedure described by Marchal, Waffo-Téguo, Génin, Mérillon, and Dubourdieu (2011). In optimal conditions in ascending mode, most of the compounds are partitioned equally between the two phases and separation is satisfactory. On this basis, various systems were tested and the ternary system (ethyl acetate/isopropanol/water 3:1:3 v/v) was selected to fractionate the aqueous prepurified extract. For each injection, 2 g of extract were solubilized in 30 mL of a mixture between the upper (10 mL) and lower (20 mL) phases of the system, and 0.45 µm-filtered. Three successive injections were necessary to process the entire aqueous prepurified extracts. Experiments were carried out in ascending mode at 1250 rpm with a flow rate of 25 mL/min for 120 min for the elution phase and 40 mL/min for 45 min for the extrusion. The Spot prep fraction collector was set to 25 mL/min. Every 10 CPC tubes, an aliquot (100 µL) was taken, evaporated, dissolved in 1 mL of H_2O/MeOH 95:5 and analyzed by LC-HRMS to obtain 12 fractions **F1** to **F12**. To constitute these fractions, CPC tubes with similar chromatographic profile were pooled, evaporated *in vacuo*, suspended in water and freeze-dried.

2.3.3. Semi-preparative liquid chromatography

Semi-preparative LC analyses were performed using a Waters Prep 150 LC including a 2545 Quaternary Gradient Module, a 2489 UV/Visible detector, a 2424 ELSD detector and a Fraction Collector III (Waters, Guyancourt, France). Separations were obtained using an XBridge C18 OBD column (19 × 250 mm, 5 µm, Waters, Guyancourt, France). The mobile phase was a mixture of ultrapure water containing 0.1% of formic acid (Eluent A) and acetonitrile with 0.1% of formic acid (Eluent B). The flow rate was set to 20 mL/min. Two gradients were developed and implemented depending on the injected fractions. Gradient I: 0 min, 5%; 3.3 min, 5%; 15 min, 13%; 29.5 min, 21%; 37 min, 30%, 53 min, 100%; 60 min, 100%; 61 min, 5%; 70 min, 5%. Gradient II: 0 min, 5%; 3.3 min, 5%; 29.5 min, 13%; 40 min, 21%; 47.5 min, 30%, 53 min, 100%; 60 min, 100%; 61 min, 5%; 70 min, 5%. Aliquots (20 mg) of CPC fractions were dissolved in methanol (200 µL), 0.45 µm-filtered and introduced manually into the system. UV detection was carried out at 254 and 280 nm and chromatographic peaks were collected manually just after the detector. Samples obtained after successive injections were pooled, evaporated in vacuo to remove acetonitrile and freeze-dried twice to obtain white amorphous powders. Three pure molecules were isolated from fractions **F3** and **F4** (compound **3**, 10.2 mg and **4**, 7.8 mg) and four other molecules from fractions **F8** and **F9** (compound **1**, 1.7 mg; **2**, 0.3 mg; **5**, 2 mg; and **6**, 2 mg).

2-hydroxy-3-methylpentanoic-2-*O*-*β*-glucopyranoside acid (**1**) (H3MP-G): white amorphous powder; $[\alpha]^{25}_{D}$ n.d.; ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz), see Table 2; HRMS *m/z* 293.1229 $[M-H]^-$($C_{12}H_{21}O_8^-$) (0.3 ppm).

2-hydroxy-4-methylpentanoic-2-*O*-*β*-glucopyranoside acid (**2**) (H4MP-G): white amorphous powder; $[\alpha]^{25}_{D}$ n.d.; ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz), see Table 2; HRMS *m/z* 293.1229 $[M-H]^-$ ($C_{12}H_{21}O_8^-$) (0.3 ppm).

Gallic-4-*O*-*β*-glucopyranoside acid (**3**) (AG-G): white amorphous powder; $[\alpha]^{25}_{D}$ -20 (*c* 0.05, H_2O); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz), see Table S1 (Supplementary data), in agreement with Pawlowska, De Leo, and Braca (2006); HRMS *m/z* 331.0670$[M-H]^-$ ($C_{13}H_{15}O_{10}^-$) (0.3 ppm).

3-indolyl-(2*R*)-*O*-*β*-D-glucopyranoside lactic acid (**4**) (ILA-G): white amorphous powder; $[\alpha]^{25}_{D}$ +2 (*c* 0.05, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz), see Table S2 (Supplementary data), in agreement with Fabre et al. (2014); HRMS *m/z* 366.1181 $[M-H]^-$ ($C_{17}H_{15}O_{10}^-$) (0.2 ppm).

(1′*R*, 3′*S*, 5′*R*, 8′*S*)-dihydrophaseic-3′-*O*-*β*-glucopyranoside acid (**5**) (epi-DPA-G): white amorphous powder; $[\alpha]^{25}_{D}$ -60.9 (*c* 0.05, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz), see Table S3 (Supplementary data), in agreement with Del Refugio Ramos et al. (2004); HRMS *m/z* 443.1924 $[M-H]^-$ ($C_{21}H_{31}O_{10}^-$) (0.3 ppm).

Isolariciresinol-4′-*O*-*β*-glucopyranoside (**6**): white amorphous powder; $[\alpha]^{25}_{D}$ -50 (*c* 0.05, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz), see Table S4 (Supplementary data), in agreement with Marinos, Tate, and Williams (1992); HRMS *m/z* 521.2020 $[M-H]^-$ ($C_{26}H_{33}O_{11}^-$) (0.5 ppm).

2.3.4. LC-HRMS analysis

The LC-HRMS platform consisted of an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), an Accela U-HPLC system with quaternary pumps and an Exactive Orbitrap mass spectrometer equipped with a heated electrospray ionization (HESI I) probe (both from Thermo Fisher Scientific, Les Ulis, France). Liquid chromatography separation was performed on a C18 column (Hypersil Gold 2.1 mm × 100 mm, 1.9 µm particle size, Thermo Fisher Scientific) with water (A) and acetonitrile (B) as mobile phases. The flow rate was 600 µL/min and eluent B varied as follows: 0 min, 5%; 0.3 min, 5%; 2 min, 7%; 5.3 min, 17%; 6 min, 98%; 6.5 min, 98%; 6.6 min, 5%; 7 min, 5%. The injection volume was 5 µL. Mass acquisitions were performed in negative Fourier transform mass spectrometry (FTMS) ionization mode at a unit resolution of 10 000 (m/Δm, fwhm at 200 Th). The mass analyzer was calibrated each week using Pierce® ESI Negative Ion Calibration solution (Thermo Fisher Scientific). The sheath and auxiliary gas flows (both nitrogen) were optimized at 80 and 15 arbitrary units respectively. The HESI probe and capillary temperatures were 320 and 350 °C respectively. The electrospray voltage was set to −3.5 kV, the capillary voltage to −25 V, the tube lens voltage offset to −120 V and the skimmer voltage to −20 V. Mass spectra were recorded from 160 to 2000 Th, with an AGC value of 106. All data were processed using the Qualbrowser and Quanbrowser applications of Xcalibur version 2.1 (Thermo Fisher Scientific).

2.3.5. NMR analysis

All ^{1}D and ^{2}D NMR experiments were performed on a Bruker Avance 600 NMR spectrometer (^{1}H at 600 MHz and ^{13}C at 150 MHz) equipped with a 5-mm TXI probe. NMR spectra were acquired at 300 k in methanol-d_4 or D_2O. ^{1}H and ^{13}C chemical shifts were referenced to solvent signals. Data were processed using TOPSPIN 3.2 software (Bruker).

Molecule assignments were obtained by two-dimensional ^{1}H-^{1}H COSY, ^{1}H-^{1}H ROESY, ^{1}H-^{13}H HSQC and ^{1}H-^{13}H HMBC experiments.

2.3.6. Polarimetry

A JASCO P-2000 polarimeter with a sodium emission wavelength ($\lambda = 589$ nm) was used to determine the specific optical rotations of the isolated compounds in methanol or water at 20 °C.

2.4. Gustatory characterization

All the tasting sessions took place in a specific room equipped with individual booths and air-conditioned at 20 °C. INAO normalized glasses were used. Fractions or pure compounds were tasted by five experts in winetasting, in duplicate. They described the gustatory perception (bitterness, saltiness, sourness, sweetness, astringency) of each glass using the vocabulary of winetasting and were asked in particular to evaluate the sweetness intensity on a scale from 0 (not detectable) to 7 (strongly detectable).

After each fractionation step, prepurified extracts were evaporated in vacuo, suspended in water and freeze-dried. Fractions were tasted in 50 mL of a 12% vol. alc. hydro-alcoholic solution composed of pure and demineralized water (eau de source de Montagne, Laqueuille, France) and distilled ethanol. Fractions of prepurified seed extract were tasted at concentrations calculated in proportion to the quantities obtained for each of them and reduced to the equivalent of one liter of wine. Each fraction was tasted and compared to one control solution corresponding to a 12% vol. alc. hydro-alcoholic solution. The sweetness intensity of this solution was assigned to zero on a 0–7 scale.

After purification and identification, each compound was dissolved at 10 mg/L in a 12% vol. alc. hydro-ethanolic solution as well as in white (Bordeaux 2013) and red non-oaked wines (Bordeaux 2011).

3. Results and discussion

3.1. Extraction and purification of taste-active compounds from grape seeds

To reproduce the usual conditions of red wine post-fermentation maceration (PFM), a solid/liquid extraction of seeds was performed in a 15% vol. alc. hydro-ethanolic solution during. Then, a taste-guided fractionation protocol was undertaken to discover sweet-tasting

compounds. At the end of each separation step, a sensory assessment was carried out to select the fractions with the most intense perception of sweetness.

After ethanol removal, the first step consisted of successive partitions of the crude extract by sequential liquid/liquid extractions. Seed compounds were fractionated according to their affinity for the solvents, leading to four freeze-dried fractions: Hept, EtOAc, BuOHsat and aqueous prepurified extracts (Table S5, Supplementary data). The crude seed extract represented approximately 6% (4.15 g) of the total seed weight (70 g). This could suggest the potential importance of the contribution of seeds during PFM although these compounds diffuse better in model solutions than in real conditions. Indeed, during PFM in a wine cellar, most seeds were released and fell to the bottom of the vat while others remained inside the grape as marc. In general, compounds derived from seeds diffuse less than those from other grape components (Minana Castello, Cadot, Paravidino, Chevalier, & Moutounet, 2006).

Tasting of the prepurified extracts revealed a clear fractionation of the taste. The bitterness and astringency perceived in Hept, EtOAc and BuOHsat extracts were rather expected owing to the presence of various phenolic compounds in seeds (Di Lecce et al., 2014, Gambuti et al., 2009, Ky and Teissedre, 2015), many of which have been described as bitter and astringent (Arnold and Noble, 1978, Brossaud et al., 2001, Hufnagel and Hofmann, 2008, Peleg et al., 1999, Soares et al., 2013). The intense sweet taste of the aqueous extract was established for the first time (Table S5, Supplementary data) and suggested the presence of sweet-tasting compounds in seeds. Glucose and fructose were not responsible for this sweetness since they were present only at trace levels (< 0.1 g/L), well below their detection threshold (< 2 g/L). Therefore, the aqueous extract was submitted to further fractionation. This first liquid–liquid extraction step led to the elimination of more than 62% of the total seed extract.

The chemical complexity of the aqueous prepurified extract suggested a fractionation by CPC. Preliminary tests showed that the ternary solvent system ethyl acetate/isopropanol/water 3/1/3 (v/v) allowed the best partition of the aqueous extract between the two phases. Therefore, we eluted it in ascending mode. Since many tubes (192) were collected, fractions were constituted by grouping tubes together on the basis of their LC-MS profiles. After solvent evaporation and freeze-drying, 12 fractions were obtained as powder in variable quantities. The largest quantities were obtained in the most polar fractions (**F11** and **F12**), which was consistent with the aqueous origin of this prepurified extract. The qualitative and quantitative taste evaluations of the fractions were recorded in a gustatogram (Table 1). Four of the fractions exhibited a sweet taste, **F3**, **F4**, **F8** and **F9**, with an intensity of 4–6 on a 0–7 scale. This demonstrated the good fractionation of the prepurified aqueous extract and

confirmed the presence of sweet compounds in the fractions that were not the most polar. The yield of this separation step was quite high, since the freeze-dried CPC fractions had a total weight representing 92% of the initial sample mass.

The four sweet-tasting fractions were then submitted to semi-preparative HPLC with UV detection. For each fraction, a preliminary injection of 1 mg showed that the chromatograms (Fig. S1, Supplementary data) presented a refined profile with only a few peaks detected both in ELSD and in UV at 280 nm. Thus, appropriate gradients were chosen to isolate the main compounds and only the UV detector was used to avoid losses due to ELSD. In this way, peaks were well separated and collected just after the detector. After acetonitrile removal and freeze-drying, six compounds were obtained as white amorphous powders.

3.2. Structural characterization of compounds isolated from grape seeds

Compounds **1** and **2** have the same mass spectrum with a quasi-molecular ion $[M-H]^-$ *m/z* 293.1229. Given the isotopic ratio (around 13%), the empirical formula $C_{12}H_{21}O_8^-$ was attributed to the deprotonated molecule, with a deviation of 0.3 ppm between experimental and theoretical values. A fragment ion was observed at *m/z* 131.0705 ($C_6H_{11}O_3^-$), corresponding to the neutral loss of 162.0524 ($C_6H_{10}O_5$). A second fragmentation with a neutral loss of 46.0057 (CH_2O_2) suggested by comparison with the literature that these two compounds possessed a glycosyl moiety fixed on an α-hydroxycarboxylic acid (Von Saint Paul et al., 2011) (Fig. S2, Supplementary data). Moreover, the elementary composition of isomers **1** and **2** established the presence of two insaturations, which supported this hypothesis. The presence of such compounds in some plant metabolites has been suggested, but their structure has never been elucidated (Von Saint Paul et al., 2011). No correspondence was found between experimental NMR data and the literature.

^1H and ^{13}C NMR signals of compound **1** were assigned (Table 2) by analyzing 1D and 2D NMR data (Fig. S3, Supplementary data). Among the 12 carbons, six were assigned to a glucosyl unit and the other six to a genin. The ^1H NMR spectrum of compound **1** showed the presence of characteristic resonances in two distinct regions. The former signals were characterized by a triplet of methyl protons at δ_H 0.77 (3H, *t*, CH_3-5), a doublet of methyl protons at δ_H 0.8 (3H, *d*, CH_3-6), two multiplets at δ_H 1.15 (1H, *m*) and 1.34 (1H, *m*) of H-4 methylene protons, a multiplet of H-3 methine proton at δ_H 1.68 (1H, *m*), and a doublet at δ_H 3.85 (1H, d) corresponding to H-2 oxy-methine. The HMBC NMR spectrum showed the presence of a carbon at δ_C 178.2 corresponding to the C-1, which is characteristic of a carboxylic

group. Interpretation of the ^{1}H and ^{13}C NMR signals established the genin of compound **1** to be 2-hydroxy-3-methylpentanoic acid.

The second set showed six characteristic signals of a glycosyl unit associated with the following signals: an anomeric proton signal at δ_H 4.29 (d, H-1′, J = 7.4 Hz) indicative of a *β*-configuration for the glycosyl bond and six other signals between δ_H 3.22 and 3.67. The interpretation of the ^{1}H-^{1}H COSY spectrum confirmed the presence of a seven-spin system characteristic of a hexose unit allowing the identification of the following signals: a multiplet at δ_H 3.22 (1H, *m*, H-2′), a triplet at δ_H3.28 (1H, *t*, H-3′, J = 9.4 Hz), another triplet at δ_H3.32 (1H, *t*, H-4′, J = 8 Hz), a multiplet at δ_H 3.22 (1H, *m*, H-5′) and two doublet of doublets at δ_H 3.55 (1H, *dd*, H-6′, J = 12.6 ; 5.3 Hz) and δ_H 3.67 (1H, *dd*, H-6′, J = 12.3 ; 2.1 Hz). The coupling constants of the glycosyl protons are all trans-diaxial orientations, which suggests a *β*-glucopyranosyl unit. Moreover, ^{13}C NMR shifts were also typical of a *β*-glucopyranose unit. Therefore, compound **1** could be a *β*-glucopyranosyl-derivative of the 2-hydroxy-3-methylpentanoic acid. The position of the glucosyl unit in **1** was determined by HMBC, which showed a long-range correlation between the carbonyl carbon at δ_C 82.8 (C-2) and the anomeric proton H-1′ (δ_H 4.29) of the glucosyl unit (Fig. S3, Supplementary data). Accordingly, compound **1** was established to be 2-hydroxy-3-methylpentanoic-2-*O*-*β*-glucopyranoside acid (H3MP-G) (Fig. 1).

FTMS analysis of compound **2** exhibited a quasi-molecular peak at *m/z* $[M-H]^-$ 293.1229 in negative mode, in agreement with the molecular formula $C_{12}H_{22}O_8$. ^{1}H and ^{13}C NMR data (Table 2) of **2** were very comparable to those of compound **1** except for some inversion in NMR assignments of the genin protons. Interpretation of the ^{1}H and ^{13}C NMR data of the glucosyl unit indicated the presence of a *β*-glucopyranosyl unit, as for compound **1**. The ^{1}H NMR spectrum showed five other signals that were characterized by a doublet of methyl protons at δ_H 0.78 (6H, *d*, CH_3-5 and CH_3-6), two multiplets at δ_H 1.36 (1H, *m*) and 1.56 (1H, *m*) of H-3 methylene protons, a multiplet of H-4 methine proton at δ_H 1.62 (1H, *m*) and a doublet of doublets at δ_H 3.9 (1H, *dd*) corresponding to H-2 oxy-methine. This genin may be considered to be 2-hydroxy-4-methylpentanoic acid, a regioisomer of compound **1**. However, no correlation was observed on the HMBC spectrum indicating the presence of a carboxylic group, probably owing to the low quantities (0.3 mg) of compound **2** used for the NMR experimentation. Nevertheless, the chemical shift of carbon C-2 at δ_C 79.4 suggested a similar chemical environment to carbon C-2 of compound **1** and by extension the presence of a carboxylic group, as supported by MS data. Finally, the position of the glucosyl unit in **2** was determined by interpreting HMBC, which showed a long-range correlation between the

carbonyl carbon at δ_C 79.4 (C-2) and the anomeric proton H-1′ (δ_H 4.3) of the glucosyl unit (Fig. S4, Supplementary data). Therefore, compound **2** may be considered to be 2-hydroxy-4-methylpentanoic-2-*O*-*β*-glucopyranoside acid (H4MP-G) (Fig. 1). Thus, compounds **1** and **2** were regioisomers that had never been identified in natural products.

The HRMS spectrum of compound **3** exhibited a quasi-molecular $[M-H]^-$ ion at *m/z* 331.0670. Considering the isotopic ratio (around 15%) and the experimental mass (+ 0.3 ppm) of the deprotonated ion, the empirical formula $C_{13}H_{16}O_{10}$ was assigned to compound **3**. The spectrum also exhibited an ion at *m/z* 169.0134 ($C_7H_5O_5^-$) corresponding to a neutral loss of 162.0536 ($C_6H_{10}O_5$) (Fig. S5, Supplementary data). These MS data suggested that compound **3** might be a glycosyl derivative of gallic acid with 6 insaturations. The interpretation of 2D NMR spectra and comparison with literature ^1H and ^{13}C data confirmed that compound **3** was a mono-galloyl glucose (Pawlowska et al., 2006, Santos et al., 2013). The position of the glycosyl unit was determined by HMBC, which showed a long-range correlation between C-4 of the galloyl group and the anomer proton at δ_H 4.9 (H-1′) of the *β*-glucospyranoside. Thus, compound **3** was established to be gallic-4-*O*-*β*-glucopyranoside acid (Fig. 1), already identified by Pawlowska et al. (2006) in strawberries. Gallic acid and its derivatives are widespread in the vegetal kingdom and constitute a large family of secondary metabolites. Among the monoglycosyl derivatives of the gallic acid, esters have been studied the most and are widely used in food, cosmetics and the pharmaceutic industry for their anti-oxidative, biological and diverse pharmaceutical properties (Abe et al., 2000, Kanai and Okano, 1998).

The HRMS spectrum of compound **4** exhibited a quasi-molecular $[M-H]^-$ ion at *m/z* 366.1181. Given the isotopic ratio (around 18%) and the experimental mass (+ 0.2 ppm) of the deprotonated ion, the empirical formula $C_{17}H_{21}O_8N$ was attributed to compound **4**. The spectrum also exhibited three fragment ions at *m/z* 204.0657 ($C_{11}H_{10}NO_3^-$), *m/z* 186.0551 ($C_{11}H_8NO_2^-$) and *m/z* 142.0651 ($C_{10}H_8N^-$) (Fig. S6, Supplementary data). The first fragment corresponded to a neutral loss of 162.0526 ($C_6H_{10}O_5$) and suggested that the molecule might contain a glycosyl group and a nitrogenated genin with 7 insaturations. Interpretation of NMR spectra and comparison with literature data confirmed this hypothesis and established compound **4** to be 3-indolyl-(2*R*)-*O*-*β*-D-glucopyranoside lactic acid (Fig. 1). This molecule had already been described in wine by Fabre et al. (2014). Indoles mostly originate from tryptophan catabolism and their biosynthesis is supposedly catalyzed by wine micro-organisms (Arevalo-Villena, Bartowsky, Capone, & Sefton, 2010). They are mainly known to present plastic or animal off-odors (Hoenicke et al., 2002) but the sensory properties of compound **4** had never been studied.

Compound **5** HRMS spectrum exhibited a quasi-molecular $[M-H]^-$ ion at *m/z* 443.1924. Given the isotopic ratio (around 23%) and the experimental mass (+0.3 ppm) of the deprotonated ion, the empirical formula $C_{21}H_{32}O_{10}$ was assigned to compound **5**. With a 30 eV collision energy in the HCD cell, one fragment ion was observed at *m/z* 281.1396 ($C_{15}H_{21}O_5^-$), corresponding to the neutral loss of 162.0528 ($C_6H_{10}O_5$) (Fig. S7, Supplementary data). These data suggested that the molecule might contain a glucosyl group and a genin with 5 insaturations. Interpretation of NMR spectra and comparison with literature data confirmed this hypothesis and established compound **5** to be *epi*-dihydrophaseic-*β*-D-glucopyranoside acid, which was identified by Del Refugio Ramos et al. (2004) (Fig. 1).

The position of the glucosyl unit in **5** was determined by HMBC NMR, which showed a long-range correlation between the genin carbon C-3′ and the proton H-1″ of the hemiacetal glucose. The relative configuration of the stereogenic carbon C-3′ was established by ROESY NMR. The presence of NOE between the equatorial protons H-2′, H-4′ and H-3′ indicated that the latter is also in an equatorial position. Moreover, the sign of the optical rotation value was negative, as for the compound published by Del Refugio Ramos et al. (2004). Accordingly, **5** was established to be (1′*R*, 3′*R*, 5′*R*, 8′*S*)-*epi*-dihydrophaseic-3′-*O*-*β*-D-glucopyranoside acid (epi-DPA-G) (Fig. 2).

Compound **5** has already been established in a few plants and fruits such as avocado (Del Refugio Ramos et al., 2004) and cherry (Setha, Kondo, Hirai, & Ohigashi, 2005), although it had never been identified in grapes or wine. The diastereoisomer of compound **5**, (1′*R*, 3′*S*, 5′*R*, 8′*S*)-dihydrophaseic-3′-*O*-*β*-glucopyranoside acid (DPA-G), has been observed in various plants and fruits such as avocado, grape (Hirai & Koshimizu, 1983) and sunflower (Barthe, Hogge, Abrams, & Le Page-Degivry, 1993). PA-G has been established to be one of the major metabolites of abscisic acid (ABA) via phaseic acid (PA) and dihydrophaseic acid (DPA) in avocado and tomato (Hirai and Koshimizu, 1983, Milborrow and Vaughan, 1982). It has been suggested that DPA-G might intervene in germ growth (Sannohe et al., 2011). Many authors have also suggested the role of ABA in grape maturation (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006). In general, *epi*-DPA-G has been studied less than its isomer. Recently, Hirai, Kondo, and Ohigashi (2003) showed that *epi*-DPA, and not DPA, is the main metabolite of ABA in avocado. The reaction of the ABA metabolism might be stereoselective (Del Refugio Ramos et al., 2004). These studies suggest that *epi*-DPA-G might be involved in ABA metabolism in grapes and that its content might be linked with grape berry maturity and, thus, with seed maturity.

The HRMS spectrum of compound **6** exhibited a quasi-molecular $[M-H]^-$ ion at *m/z* 521.2020. Given the isotopic ratio (around 27%) and the experimental mass (+0.5 ppm) of the deprotonated ion, the empirical formula $C_{26}H_{34}O_{11}$ was assigned to compound **6**. The spectrum also exhibited a fragment ion at *m/z* 359.1496 ($C_{20}H_{23}O_6^-$), corresponding to a neutral loss of 162.0528 ($C_6H_{10}O_5$) (Fig. S8, Supplementary data) and suggesting that the molecule might contain a glucosyl group and a genin with 9 insaturations. Interpretation of NMR spectra and comparison with data in the literature confirmed this hypothesis and established compound **6** to be a glucosyl derivatives of the lignan isolariciresinol. The position of the glucosyl unit in **6** was determined by HMBC NMR, which showed a long-range correlation between the C-4′ carbon of the genin and the H-1″ proton of the glucose hemiacetal. Accordingly, **6** was established to be the lignan isolariciresinol-4′-*O*-*β*-glucopyranoside (Fig. 1). This molecule has already been described in wine (Marinos et al., 1992) but never in grapes.

3.3. Gustatory properties of isolated compounds

This study assessed for the first time the gustatory properties of the isolated compounds **1**–**6**. Compounds were dissolved in a hydro-alcoholic solution, in a white wine and in a red wine. For each compound, five wine experts characterized the taste and evaluated the perceived intensities on a 0–7 scale in comparison with the control solution or the control wines as a reference. Five compounds presented sweetness with various intensities whereas one had no particular taste (no difference was perceived between the control and the spiked solutions). Results are presented in Table 3.

Isomers H3MP-G (**1**) and H4MP-G (**2**) were not tasted separately owing to their low quantities. Only their mixture was tasted by the panel in two matrixes, in hydro-alcoholic solution and in white wine. Its sensory attribute was evaluated as mild sweet. ILA-G (**4**) exhibited only a light sweetness, while AG-G (**3**) was evaluated as having a mild sweetness. It is noteworthy to mention that AG-G sweetness is perceived at 10 mg/L, which is a content 200 times lower than the glucose detection threshold. The sweetest isolated compound was *epi*-DPA-G (**5**) which modified the red wine taste balance distinctly and to a lesser extent the white wine taste balance. The lignan **6** did not presented any taste at a concentration of 10 mg/L, so it may not have any gustatory impact on wine.

3.4. Assessment of sweet-tasting compounds in wines by LC-HRMS

Compounds **1**–**6** were isolated from grape seed extract so we wondered whether they are present in commercial white and red wines. Thanks to its mass measurement accuracy, LC-

HRMS allowed samples to be screened by targeting *m/z* ions characteristic of specific empirical formulas. Fig. 3 presents extracted ion chromatograms (XIC) obtained in a grape-seed maceration (a), in a red wine (b) and in a white wine (c) for *m/z* ratios specific to the isolated compounds **1**–**5**.

Fig. 3 shows that similar signals were detected in all three matrices. Moreover, analysis in HCD fragmentation mode revealed the same main fragment ions in the three matrices. Specificity of mass measurement (<5 ppm) and retention time similarity (<0.04 min) demonstrated that compounds **1**, **2**, **3**, **4** and **6** were present in wines. Whereas the most abundant compound of each XIC was purified, minority isomers also seemed to be present for some $[M-H]^-$ ions. Therefore, this study demonstrates for the first time the presence of H3MP-G (**1**) and H4MP-G (**2**) and the presence of *epi*-DPA-G (**6**) in grapes and in wines. ILA-G (**4**) has already been described in grapes and wines, while Fig. 3 shows the first evidence of AG-G (**3**) in wines. Comparison of the signal intensity of the various compounds suggested that ILA-G (**4**) and *epi*-DPA-G (**5**) might be the most abundant of the isolated compounds in wines. Robust quantitative studies now need to be performed to confirm this hypothesis.

4. Conclusion

These findings demonstrate the presence of sweet-tasting molecules released from grape seeds into wine, thereby confirming the empirical observations of the old winemaking manuals. An inductive approach guided by gustatometry was used to fractionate a grape-seed macerate. This protocol led to the isolation and identification of five sweet-tasting compounds. Indeed, their addition to a hydro-alcoholic solution and to a white or red wine clearly impacted the taste balance by increasing the sweetness. Fractionations of the other prepurified extracts (data not shown) did not demonstrate any other sweet-tasting molecules in seeds. Nevertheless, the presence of such molecules cannot be ruled out owing to perceptual interactions that could mask their sensory properties.

Among the isolated sweet-tasting molecules, two are described for the first time: H3MP-G and H4MP-G. Additionally, *epi*-DPA-G is reported for the first time in grapes and in wines, while AG-Glc has already been reported in white grapes but never in wine. The establishment of their detection threshold and quantification studies would allow their sensory impact on wine taste to be determined. Moreover, this research opens promising perspectives. A study of the levels of these new markers during ripening would throw light on the conditions modulating

their accumulation in grapes. Furthermore, the evolution of their concentrations during winemaking and ageing would provide useful data about their extraction and stability. Beyond the gain in fundamental knowledge, such studies could have practical consequences and lead to recommendations for winemakers.

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Figures

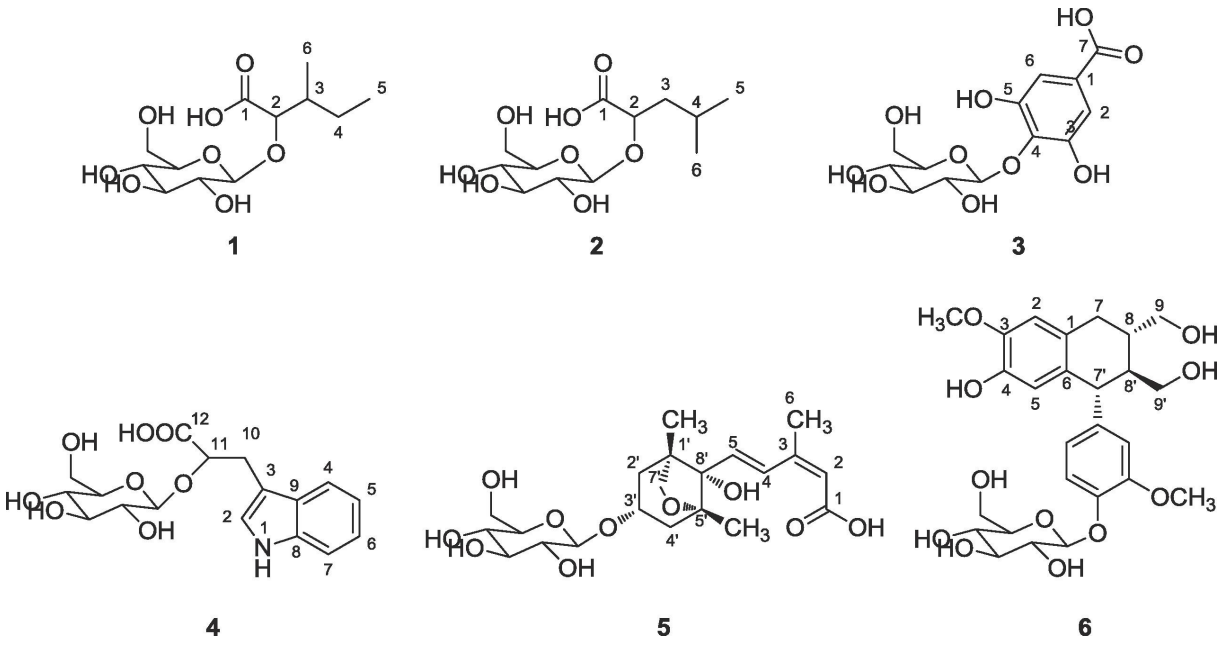

Fig. 1. Chemical structures of isolated compounds (**1**–**6**).

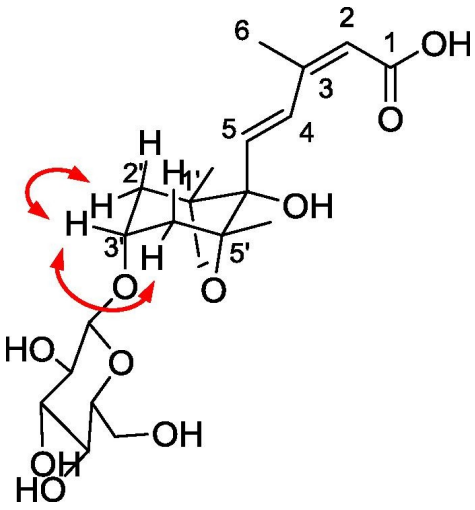

Fig. 2. NOE correlations observed on ROESY NMR spectrum of compound **5**.

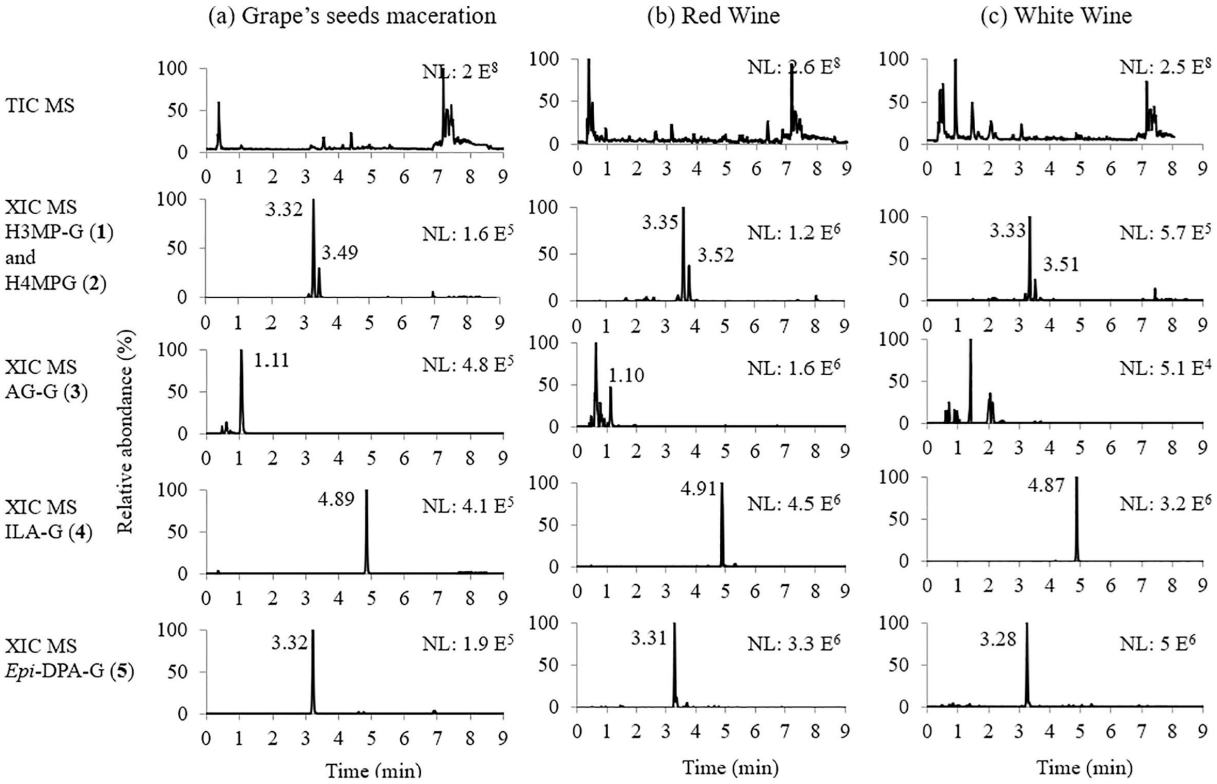

Fig. 3. Negative LC-ESI-FTMS XIC of a grape-seed maceration (a), a red wine (b) and a white wine (c) corresponding to $[M-H]^-$ ions of isolated compounds **1**–**6** (from top to bottom).

Tables

Table 1. Gustatogram of aqueous pre-purified extract after CPC fractionation.

Fraction	Tubes	Taste*	Aspect	Obtained masses (mg)
F1	21–32	Salty 0.5/7	Beige powder	17.4
F2	33–47	Bitter 1/7	Brown powder	132.1
F3	**48–62**	**Sweet 5/7**	**Beige powder**	**101.1**
F4	**63–67**	**Sweet 4/7**	**Beige powder**	**20**
F5	68–79	–	Beige powder	33.8
F6	80–87	–	Beige powder	17.6
F7	88–97	–	Pink powder	15.3
F8	**98–108**	**Sweet 6/7**	**Beige powder**	**17.6**
F9	**109–152**	**Sweet 5/7**	**Beige powder**	**56.9**
F10	153–159	–	Brown viscous solid	281.9
F11	160–169	Astringent 7/7	Brown powder	4827.6
F12	170–180	Sweet 1/7	Brown powder	75.5

–: Fractions with no taste compared to control solution.

Fractions in bold characters have been submitted to semi-preparative liquide chromatography.

* Taste and its intensity rated on a 0–7 scale.

Table 2. ^{1}H and ^{13}C NMR data of compounds **1** and **2**.

	Compound 1		**Compound 2**	
Atom number	**δ ^{1}H (ppm) multiplicity (J)**	**δ ^{13}C (ppm)**	**δ ^{1}H (ppm) multiplicity (J)**	**δ ^{13}C (ppm)**
1		178.2		n.d.
2	3.85 *d* (5 Hz)	82.8	3.9 *dd* (8.5; 4.7 Hz)	79.4
3	1.68 *m*	37.1	1.36 *m* 1.52 *m*	41
4	1.15 *m* 1.34 *m*	24.1	1.62 *m*	23.8
5-CH_3	0.77 *t* (7; 7 Hz)	10	0.78 *d* (6.5 Hz)	22
6-CH_3	0.8 *d* (7 Hz)	13.4		
1′	4.29 *d* (7.4 Hz)	103.2	4.3 *d* (7.9 Hz)	102.9
2′	3.24 *m*	72.8	3.22 *m*	72.8
3′	3.32 *t* (8.8; 8.8 Hz)	75.2	3.32 *t* (9; 9.3 Hz)	75.2
4′	3.28 *t* (9.1; 9.4 Hz)	68.8	3.29 *t* (9.3; 9.3 Hz)	68.8
5′	3.22 *m*	75.3	3.22 *m*	75.3
6′	3.55 *dd* (12.6; 5.3 Hz) 3.67 *dd* (12.3; 2.1 Hz)	60	3.57 *dd* (12.7; 5.3 Hz) 3.67 *dd* (12.4; 2.2 Hz)	60

n.d.: not determined.

Table 3. Gustatory characteristics of isolated compounds.

Compounds		Taste* in the hydro-alcoholic solution		Taste* in the White wine		Taste* in the Red wine	
1 and **2** mix	H3MP-G and H4MP-G mix	Sweet	3/7	Sweet	3/7	n.d.	
3	AG-G	Sweet	3/7	Sweet	3/7	Sweet	3/7
4	ILA-G	Sweet	1/7	Sweet	1.5/7	Sweet	1.5/7
5	*epi*-DPA-G	Sweet	3/7	Sweet	4/7	Sweet	6/7
6	Isolariciresinol-glucopyranoside	–	–	–	–	–	–

n.d.: not determined.

–: no taste.

* Taste and its intensity rated on a 0–7 scale.