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Disclosure of the nature of glycosylated varietal precursors of eugenol from the hybrid, tolerant grapevine variety Baco blanc: Methodology and quantification

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Abstract

A varietal origin of eugenol was previously demonstrated in Baco blanc, a major grapevine variety used to produce Armagnac wine spirits. Eugenol was found in high amount, both as the free and as unidentified glycosylated forms. To reveal their identity, a specific method was developed and applied to berry skin extracts. This EPIQ (Extraction-Purification-Identification-Quantification) procedure comprised HS–SPME GC–MS-guided LC fractionation, combined with specific enzymatic hydrolyses as well as LC–MS/MS and LC–HRMS analyses. Comparison with commercial standards allowed for the identification of geoside (eugenyl-6-*O*-α-L-arabinopyranosyl-b-D-glucopyranoside) as a major eugenol precursor. This is the first study to find geoside in grapevine. Three minor eugenol precursors were also putatively determined, including the monoglucoside, citrusin C. Two other diglycosides of eugenol were also hypothesised. LC–MS/MS quantifications confirmed presence of a larger amount of geoside in Baco blanc than in *Vitis vinifera* grapes. Geoside reach a maximum concentration in berry skins of Baco blanc at veraison.

Keywords: Geoside, Glycoconjugates, Grape berry, Citrusin C, Armagnac

Chemical compounds studied in this article

Eugenol (PubChem CID: 3314)

Geoside (eugenyl-6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside, PubChem CID: 25087713)

Citrusin C (PubChem CID: 3084296)

Eugenyl-6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside (PubChem CID: 15689810)

1. Introduction

Baco blanc is a hybrid grape variety (*Vitis labrusca* × *Vitis riparia* × *Vitis vinifera*) primarily grown in Bas Armagnac (Southwest of France) and used to produce Armagnac wine spirits. Indeed, Baco Blanc grapes were found to contain a higher amount of eugenol than Ugni Blanc and Folle Blanche grapes (two other *V. vinifera* varieties used to produce Armagnac wine spirits) originating from the same locations, regardless of the vineyard origin (Franc et al., 2023). It was shown that eugenol can subsequently be transferred to must, wine and wine spirits, before contact with oak wood (Franc et al., 2023). Contrarily, in wines and wine spirits from *V. vinifera*, the main source of eugenol is the contact with oak wood during ageing. In monovarietal Baco blanc wine spirits, without oak wood contact, the eugenol concentrations range from 31.0 to 174.7 $\mu g \cdot L^{-1}$ (Franc et al., 2023). These quantities are close to or even higher than those in wine spirits from Ugni blanc or Folle blanche aged in contact with oak wood.

Eugenol is an aroma compound that exhibits numerous interesting pharmacological and biological activities, such as anti-microbial, anti-fungal, anti-inflammatory, analgesic, anti-oxidant and anti-cancer properties (Kamatou et al., 2012; Pramod et al., 2010; Ulanowska & Olas, 2021). It is also described as an insect attractant (Dobson et al., 1999) or repellent (Born et al., 2012; Kamatou et al., 2012) as well as a strong acaricide and insecticide effective against a wide range of domestic arthropods (Chaieb et al., 2007; Ulanowska & Olas, 2021). In EU, eugenol has been an approved agro-chemical active substance since 2013, and its use is more related to its anti-fungal activity. Since 2017, eugenol in combination with thymol and geraniol has been approved in France for the treatment of grapevine aerial parts against grey mould or Botrytis bunch rot, caused by *Botrytis cinerea*. Indeed, eugenol has been shown to be highly effective against this major necrotrophic fungal pathogen (Amiri et al., 2008; Ben Arfa et al.,

2006; Olea et al., 2019; Wang et al., 2010), which drastically affects wine grapes in terms of quantity and quality (Hastoy et al., 2023; Ky et al., 2012). The first evidence of the anti-fungal activity of eugenol against *B. cinerea* was reported in 2000, with a complete inhibition of spore germination and a moderate effect on mycelial growth *in vitro* (Tsao & Zhou, 2000). Recently, we confirmed the *in vitro* efficiency of eugenol against two different isolates of *B. cinerea* (Hastoy et al., 2023).

The high content of eugenol found in Baco blanc may be one of the reasons why this variety is tolerant, thereby requiring fewer phytosanitary treatments overall compared with Ugni blanc or Folle blanche. According to vine practitioners, Baco blanc is relatively tolerant to fungal diseases, particularly black rot, grey rot and powdery mildew, and partially to downy mildew (Galet, 2015). The susceptibility of mature berries to *B. cinerea* confirmed the higher resistance of Baco blanc than Ugni blanc and Folle blanche (Hastoy et al., 2023). Moreover, within Baco blanc, the variations in clone susceptibility to *B. cinerea* could be related to the eugenol content in the berry skin, suggesting that eugenol is a good biochemical indicator of ontogenic resistance to the fungus in Baco blanc berries. Furthermore, (Hastoy et al., 2023) demonstrated the induction of eugenol following leaf removal in Baco blanc vines, consistent with the induction of many plant phenylpropanoid compounds in response to external stresses, such as pathogen attack, wounding or high ultraviolet light (Dixon & Paiva, 1995). Recently, (Wu et al., 2023) reported that *B. cinerea* leaf infection in two grape varieties, Victoria (*V. vinifera*) and Shine Muscat (*V. vinifera* × *V. labrusca*), induces the production of plant secondary metabolites with anti-fungal bioactivities, particularly eugenol, thereby enhancing plant resistance to diseases, such as grey mould.

In Baco blanc must, berry pulp, berry skin, vine leaf and vine shoot, a large part of eugenol is glycosidically bound, and the aglycone can be released via oenological enzymatic preparations containing α- and ß-glycosidase activities (Franc et al., 2023). It was recently found that Baco blanc berry skin contains higher concentrations of eugenol glycoconjugates than Ugni blanc or Folle blanche (Franc et al., 2023).

Glycosylated precursors of aroma compounds are widely found in the plant kingdom, including grapevines (Atkinson, 2018; Hjelmeland & Ebeler, 2015). According to (Hjelmeland & Ebeler, 2015), for all identified grape aroma glycoconjugates, the sugar moiety includes a direct linkage of the aroma compound to a β-D-glucose moiety. The monoglucosides are often esterified with a malonyl group (Hjelmeland & Ebeler, 2015). The diholosides and triholosides of aroma compounds are also described in grapes (Ferreira & Lopez, 2019). Apart from glucose, the three sugars most commonly found in these glycoconjugates are arabinose, apiose and rhamnose

(Ferreira & Lopez, 2019; Hjelmeland & Ebeler, 2015). However, although eugenol glycoconjugates have been found in grapes through enzymatic cleavage (Franc et al., 2023; García-Carpintero et al., 2011; Moio et al., 2004), their chemical structure has never been fully elucidated. Contrarily, several eugenol heterosides were already identified in other plants. Geoside (CAS # 585-90-0, eugenyl-6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside) was isolated from *Geum urbanum* (Hérissey & Cheymol, 1925), and sasanquin (CAS #18604-54-1, eugenyl-6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside) was extracted from *Camellia sasanqua* (Yamada et al., 1967). In 1988, citrusin C (CAS #18604-50-7, eugenyl-β-D-glucopyranoside) was isolated and identified from *Melissa officinalis* (Mulkens & Kapetanidis, 1988). In 1991, a eugenol heteroside, composed of a diholoside of apiose and glucose, was extracted and identified from the large panicle viburnum (*Viburnum dilatatum*) as eugenyl-6-*O*-β-D-apiosyl(1→6)-β-D-glucoside (CAS #136083-96-0) (Machida et al., 1991). The same year, eugenol rutinoside (CAS #138772-01-7), a diholoside of glucose and rhamnose, was extracted and identified from the bulbs of *Lilium mackliniae* (Sashida et al., 1991). Eugenyl-6-*O*-α-L-arabinofuranosyl-β-D-glucopyranoside (CAS #229333-98-6) was isolated from a rose extract (*Rosa damascena*) (Straubinger et al., 1999). Other potential eugenol heterosides were putatively identified from aspen (*Populus tremula*) (Koeduka et al., 2013), tomato (*Solanum lycopersicum*) (Tikunov et al., 2009) or oak wood (*Quercus petræa*) (Slaghenaufi, 2012) but without unambiguous identification.

Thus, in this context, the rationale of this study is to hypothesise that eugenol is stored in the form of precursors in the Baco blanc berry skin to be available and ready for release to defend the plant in the event of a pathogen attack. It cannot be ruled out that these precursors may also directly affect the pathogen. It is therefore of prime interest to understand the nature of these precursors to evaluate their potential as anti-fungal compounds. This knowledge may be a step forward in better understanding plant defence mechanisms.

The aims of this study are as follows: First is to unveil the nature of the glycosylated varietal precursors responsible for the high concentration of eugenol in Baco blanc, which may contribute to its greater tolerance to fungal diseases compared with other grapevine cultivars in Armagnac. For this purpose, an original method was specifically developed. This procedure involved LC fractionation followed by enzymatic hydrolysis and HS–SPME–GC–MS analysis to identify the fractions potentially containing eugenol glycosides, which could then be further characterised. Second is to quantify eugenol precursors in three *V. vinifera* grape varieties typical of the Armagnac region (Folle blanche, Ugni blanc and Plant de Graisse) to confirm the

varietal specificity for Baco blanc. Third is to quantify these precursors in Baco blanc grapes at various stages of maturity to assess temporal variations in their accumulation. To achieve a sensitive and accurate quantification, it was necessary to establish a specific LC–MS/MS quantification method. The entire procedure is summarised in the acronym EPIQ for: Extraction-Purification-Identification-Quantification.

2. Materials and methods

2.1 Samples

For the isolation of eugenol precursors and the following specific enzymatic hydrolyses, Baco blanc grape bunches were picked at veraison (8 August 2022, 971.4 growing degree days (GDDs), BBCH code 81) in an experimental plot at Château de Mons (Caussens, France). This experimental plot consisted of one row of Baco blanc divided into three blocks. Each block represented 15 vines with an average load of 18 bunches per vine. Six bunches from rank 1 and/or 2 (at shoot base) were picked by block. The bunches were de-stemmed in the laboratory, and all the berries were combined to obtain a representative sample. About 800–1,000 berries were required to conduct all the analyses presented below.

To compare the composition and amount of eugenol precursors in the *Vitis* genus, four typical grape varieties of the Armagnac region were investigated: Baco blanc (hybrid grape), Folle blanche, Ugni blanc and Plant de Graisse (all *V. vinifera* grapes). The grapes were sampled from Armagnac producers during the 2020 vintage. For each variety, two different origins (producers) were selected, except for the Baco blanc for which three different origins were sampled. All the bunches were sampled on the day of harvest by the growers. Therefore, they exhibited differences in Technology Maturity Index.

To monitor eugenol precursors during Baco blanc berry development, another set of samples was analysed. Bunches of Baco blanc were harvested from the experimental plot at Château de Mons (Caussens, France), as described above, at four phenological stages previously determined by the number of GDDs since mid-flowering with a vine threshold temperature of 10°C and using the BBCH scale (Hack et al., 1992; Lorenz et al., 1995): cluster closure (11 July 2022, 548 GDD, BBCH code 77), veraison (8 August 2022, 971.4 GDD, BBCH code 81), harvest (6 September 2022, 1381.8 GDD, BBCH code 89) and post-harvest (26 September 2022, 1573.2 GDD, BBCH code 91). The last stage has been defined as over-ripening of the grapes.

All samples were stored at −20°C.

2.2 Chemicals

Geoside (eugenyl-6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside, CAS RN [585-90-0], 90% purity) was purchased from Glentham Life Sciences Ltd. (Corsham, UK). Eugenyl-6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside (CAS RN [136083-96-0], 85% purity) was purchased from Aurora Fine Chemicals LLC (San Diego, USA). Citrusin C (eugenyl β-D-glucopyranoside, CAS RN: [18604-50-7], >95% purity) was obtained from MuseChem (Fairfield, New Jersey, USA). Eugenol-d_3 (CAS RN: [1335401-17-6], 95% purity, 99.7% isotopic purity), used as the GC–MS internal standard, was supplied by Santa Cruz Biotechnology Inc. (Dallas, USA). Eugenol (CAS RN: [97-53-0], 98% purity), sodium phosphate dibasic (CAS RN: [7558-79-4], >98.5% purity), citric acid (CAS RN: [77-92-9], >99.5% purity) and ammonium acetate (CAS RN: [631-61-8], 98% purity) were purchased from Sigma-Aldrich Chimie (Saint-Quentin-Fallavier, France). Phosphoric acid (CAS RN: [7664-38-2], 85% purity), sulphuric acid (CAS RN: [7664-93-9], 98% purity), sodium chloride (CAS RN: [7647-14-5], 99% purity) and ammonium formate (CAS RN: [540-69-2], 98.1% purity) were supplied by VWR-Prolabo (Fontenay-sous-Bois, France). Dichloromethane (CAS: [75-09-2], HPLC grade, 99.8% purity) and acetonitrile (CAS: [75-05-8], HPLC grade and Optima® LC–MS grade, 100% purity) were obtained from Fisher Scientific SAS (Illkirch, France). The following solvents (HPLC grade) were obtained from VWR-Prolabo (Fontenay-sous-Bois, France): methanol (CAS RN: [67-56-1], 99.9% purity), absolute ethanol (CAS RN: [64-17-5], >99.7% purity) and acetone (CAS RN: [67-64-1], 100% purity). Formic acid (CAS RN: [64-18-6], high purity grade, 100%) was obtained from Amresco Inc. (Solon, Ohio, USA). Ultrapure water (18.2 MΩ/cm) was obtained from purified water (Prima System; ELGA LabWater, Veolia Water STI, Anthony, France) filtered using a USF Maxima system (ELGA LabWater, Veolia Water STI, Anthony, France). Rapidase® Revelation Aroma (Oenobrands SAS, Montferrier-sur-Lez, France) was used for enzymatic hydrolysis of eugenol glycoconjugates before HS–SPME–GC–MS analysis of eugenol. Pure enzymes, α-rhamnosidase (prokaryote, 1500-U/mL suspension in 3.2 M ammonium sulfate, CAS RN: [37288-35-0]), α-L-arabinofuranosidase (*Aspergillus niger*, 300-U/mL suspension in 3.2 M ammonium sulfate, CAS RN: [9067-74-7]) and β-glucosidase (*Aspergillus niger*, 40 U/mL suspension in 3.2 M ammonium sulfate, CAS RN: [9001-22-3]), were obtained from Megazyme Ltd. (Bray, Ireland).

A citrate–phosphate buffer solution (500 mL, 0.1 M; pH 5) was prepared using 5.322 g of sodium phosphate dibasic and 2.401 g of citric acid in ultrapure water and adjusted to pH 5 with a dilute phosphoric acid solution (threefold dilution from the commercial solution). By

dissolving 97.8 mg of ammonium acetate in 50-mL ultrapure water, a 24.9-mM buffer solution of ammonium acetate (pH 6.7) was obtained.

Stock solutions of analytical standards were prepared as follows: 5 mg of geoside was dissolved in 5-mL ethanol, and 5 mg of eugenyl-6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside was dissolved in 10 mL of an ethanol/water (1:1) solution. Citrusin C was prepared at 89 $mg \cdot L^{-1}$ in ethanol 100%, eugenol at 68.8 $mg \cdot L^{-1}$ in ethanol 50% and eugenol-d_3 at 50 $mg \cdot L^{-1}$ in ethanol 100%.

2.3 Analytical strategy for isolating eugenol glycoconjugates from Baco blanc grape berry skin

2.3.1 Solid–liquid extraction from berry skin

Frozen Baco blanc grape berries were peeled, and the skins, which were dried on adsorbent paper, were finely ground with liquid nitrogen using a MM400 ball mill (Retsch France, Verder S.A.R.L., Eragny sur Oise, France). The ground and homogenised powder was kept in liquid nitrogen until approximately 12 g was weighed into each of eight 50-mL Falcon® tubes (8 replicates for a total of 97.94 g of berry skin powder). After the introduction of a ceramic homogeniser (reference 5982-9313, Agilent Technologies, Massy, France), three successive solid–liquid extractions (SLE) were performed using 20, 15 and 15 mL of acetone/water (1:1) for 1 H each with shaking on an IKA HS 501 digital reciprocating shaker (VWR International SAS, Fontenay-sous-Bois, France) at 250 rpm. Each SLE was followed by centrifugation at 5000 g for 10 min at 20°C (High-Speed Refrigerated Centrifuge Himac CR22N, Hitachi Life-Sci, Tokyo, Japan) to separate the berry skin residues from the solvent extract. The combined supernatant was concentrated under reduced pressure using a rotary evaporator (Laborota 4010 digital, Heidolph Instruments, Schwabach, Germany, combined with a vacuum controller and a cryobath) and centrifuged again (5000 g for 10 min at 5°C) to obtain 134 mL of solid–liquid (S/L) extract.

To check for the presence of eugenol glycoconjugates, 500 µL of S/L extract (approximately equivalent to 365 mg of skin extract) was enzymatically hydrolysed (as described in Section 2.3.5) and further analysed via HS–SPME–GC–MS. HS–SPME–GC–MS analyses of the samples were conducted as described by Franc et al. (2023) using the same instrument and conditions.

2.3.2 Solid-phase extraction of berry skin extract

For the solid-phase extraction (SPE), a Visiprep 12-port vacuum manifold (Sigma-Aldrich Chimie, St Quentin Fallavier, France) was used. The first SPE on the C_{18} phase was performed on 1-g/20-mL Strata cartridges (Phenomenex, Le Pecq, France). The S/L extract obtained as described in Section 2.3.1 was divided into four portions of approximately 33.5 mL each, and four SPEs were performed in parallel. The cartridges were first conditioned and equilibrated with 20 mL of methanol and then with 20 mL of water. The four aliquots of the S/L extract were then loaded onto the cartridges. After complete elution of the extract, the cartridges were washed with 12 mL of water to remove polar compounds, such as sugars. Subsequently, 12 mL of dichloromethane was eluted to remove apolar compounds and aglycones, such as eugenol. An elution step with 12 mL of 30% acetonitrile in water was performed to recover glycosylated precursors, particularly those of eugenol. The acetonitrile eluates were then combined and concentrated under reduced pressure to yield approximately 34 mL of C_{18}–solid-phase extract. To check for the presence of eugenol glycoconjugates, 200 µL of the solid-phase extract (roughly equivalent to 574-mg skin extract) was enzymatically hydrolysed and further analysed via HS–SPME–GC–MS.

2.3.3 Anion-exchange SPE of berry skin extract

To further purify the C_{18}–solid-phase extract, a strong anion-exchange SPE was achieved with 500-mg/6-mL Polyclean 30HAX cartridges (Interchim SAS, Montluçon, France). The C_{18}–solid-phase extract obtained in Section 2.3.2 was divided into three portions of approximately 11 mL, and three anion-exchange SPEs were performed in parallel. The cartridges were first conditioned and equilibrated with 10 mL of methanol and then with 10 mL of water. Subsequently, the C_{18}–solid-phase extract was loaded onto the cartridges, which were subsequently washed with 12 mL of a 25-mM buffer solution of ammonium acetate to remove unretained chemical species. The first elution step was performed with 12 mL of pure methanol to recover basic and neutral chemical species, followed by the second elution step with 12 mL of a 5% formic acid solution in methanol to recover acidic compounds. Eugenol glycosylated precursors were recovered in the pure methanol fraction consistently with their neutral state at this pH. The combined methanol eluate yielded approximately 35.5 mL of anion-exchange solid-phase extract, from which 200 µL (approximately equivalent to 546 mg of skin extract) was withdrawn for enzymatic hydrolysis, followed by HS–SPME–GC–MS analysis to confirm the presence of eugenol glycoconjugates. The anion-exchange solid-phase extract was then evaporated to dryness under reduced pressure. The remaining solid was dissolved in 1 mL of methanol, of which 500 µL was withdrawn for enzymatic hydrolysis to provide the hydrolysed

sample for comparison. For further LC–MS/MS and LC–HRMS analyses, LC fractionation and acidic chemical hydrolysis, the remaining 500 µL was diluted with 500 µL of water. Both hydrolysed and non-hydrolysed samples were roughly equivalent to 48 g of skin extract. After enzymatic hydrolysis (Section 2.3.5), the hydrolysed sample was freeze-dried overnight (freeze-dryer Christ Alpha 2-4 LSCbasic, Grosseron SAS, Couëron, France), and the resulting solid was dissolved in 1 mL of methanol using an ultrasonic bath (Branson 2800, Emerson Electric, Saint-Louis, Missouri, USA).

The solution was then centrifuged to remove the hydrolysis salts, and finally, the supernatant was diluted by half with water.

2.3.4 GC–MS-guided LC fractionation

LC fractionation was achieved on a 1200 series liquid chromatograph (Agilent Technologies, Massy, France) consisting of 1260 binary pump, 1260 high-performance degasser, 1290 thermostat for the 1260 autosampler and 1290 thermostatted column compartment, which was set to 40°C. A Kinetex Polar C18 column (150 × 2.1 mm, length × I.D.; 2.6-µm particle size) from Phenomenex (Le Pecq, France) was installed, and pure water (solvent A) and pure acetonitrile (solvent B) were used. The flow rate was 0.3 mL/min, and the gradient was 0–2 min, 2% B; 5–30 min, 5% B; 60 min, 20% B; 70 min, 40% B; 80–90 min, 100% B; 95 min, 2% B; equilibration 6 min, for a total run duration of 101 min. For each one of the three attempts detailed below, 10 µL of the non-hydrolysed sample from Section 2.3.3 was injected in the liquid chromatograph. Each of the collected fractions was enzymatically hydrolysed (Section 2.3.5) to narrow down the search area at each attempt and ultimately identify the fraction that released the largest amount of eugenol. For comparison, 10 µL of the non-hydrolysed sample from Section 2.3.3 (roughly equivalent to 479 mg of skin extract) was also enzymatically hydrolysed. On the first HPLC fractionation, fractions were collected every 15 min from 0 to 90 min, yielding 6 fractions of 4.5 mL each. Enzymatic hydrolysis was performed on 1 mL of each fraction, whereas 100 µL of each fraction was used for LC–MS analysis. The only fraction that enzymatically released a quantifiable amount of eugenol (8.8 $\mu g \cdot L^{-1}$) was the one collected between 45 and 60 min. In the second HPLC fractionation, fractions were collected every 3.5 min from 40 to 64.5 min, yielding 7 fractions of 1.05 mL each. Enzymatic hydrolysis was performed on 950 µL of each fraction, whereas 100 µL was used for LC–MS analysis. The fraction that enzymatically released the highest amount of eugenol (29.9 $\mu g \cdot L^{-1}$) was the one collected between 50.5 and 54 min. In the third HPLC fractionation, fractions were collected every minute from 49.5 to 56.5 min, yielding 7 fractions of 0.3 mL each. Enzymatic hydrolysis

was performed on 240 µL of each fraction, whereas 60 µL was used for LC–MS analysis. The fraction that mainly released eugenol (53.0 $\mu g \cdot L^{-1}$) via enzymatic hydrolysis was the one collected between 51.5 and 52.5 min. However, traces of released eugenol were also detected in fractions from 49.5 to 50.5 min (3.6 µg/L) and 52.5 to 53.5 min (3.5 $\mu g \cdot L^{-1}$). GC overlaid chromatograms of released eugenol by enzymatic hydrolysis are available in Figure S1 (Supplementary material).

2.3.5 Enzymatic cleavage of eugenol precursors

At each stage, the withdrawn sample (10 µL to 1 mL) was enzymatically hydrolysed to confirm the presence of eugenol glycosylated precursors by eugenol release from the various samples, as previously described (Franc et al., 2023). Briefly, a sample was prepared pouring in a 15 mL hydrolysis tube: a sufficient quantity of the citrate–phosphate buffer solution (0.15 M, pH 5) to dilute the sample to 10 mL, plus 300 µL of glycosidase-rich enzyme solution, Rapidase® Revelation Aroma (oenological enzymatic preparation), prepared in ultrapure water (120 $g \cdot L^{-1}$) and finally 20 µL of the internal standard solution (eugenol-d_3, 50 $mg \cdot L^{-1}$ in ethanol). The sealed tube was incubated for 24 h at 40°C and 150 rpm in a SW22 water bath (Julabo GmbH, Seelbach, Germany). After centrifugation (Thermo Scientific™ Sorvall™ ST 8 Small Benchtop Centrifuge, Fisher Scientific SAS, Illkirch, France) at 3000 g for 5 min, the supernatant was poured into a 20-mL vial containing 3.5 g of sodium chloride. The vial was sealed with a PTFE-lined cap (Chromoptic, Courtaboeuf, France) and vortexed before analysis via HS–SPME–GC–MS, as previously described (Franc et al., 2023).

2.4 Acidic chemical hydrolysis of eugenol precursors

Acidic hydrolysis was performed in triplicate on 10 µL of a twofold diluted solution of the non-hydrolysed sample obtained in Section 2.3.3 (roughly equivalent to 240 mg of skin extract) and compared with enzymatic hydrolysis, which was performed in triplicate on the same sample (10 µL), as described in Section 2.3.5. For the acidic chemical hydrolysis of eugenol precursors, 10 µL of the sample was diluted with 10 mL of acidified water at pH 1 (adjusted with H_2SO_4 98%) and poured in a 20-mL glass vial sealed with a PTFE-lined cap. The three vials were then placed in a heated oven at 100°C for 1 h. The hydrolysed samples were subsequently analysed via HS–SPME–GC–MS.

2.5 Enzymatic hydrolysis of eugenol precursors with specific enzymes

To elucidate the composition of the sugar moieties of the eugenol precursors, three pure enzymes (β-D-glucosidase, α-L-arabinofuranosidase and α-L-rhamnosidase) and the enzymatic preparation Rapidase® Revelation Aroma were tested on three commercial eugenol precursors (citrusin C **(1)**, geoside **(2)**, and eugenyl-6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside **(3)**, Figure 1) and on a solid-liquid extract of Baco blanc berry skin powder (cf. section 2.3.1). Briefly, 1 mL of a solution of one of the commercial precursors (whose concentrations were [citrusin C] = 445 $\mu g \cdot L^{-1}$; [geoside] = 630 $\mu g \cdot L^{-1}$; [eugenyl-6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside] = 595 $\mu g \cdot L^{-1}$) or 1 mL of Baco blanc S/L extract, 9 mL of a citrate–phosphate buffer solution (0.15 M, pH 5) and 50 µL of a suspension of pure enzyme or 300 µL of Rapidase® Revelation Aroma at 120 $g \cdot L^{-1}$ were successively added in a 15-mL hydrolysis tube. Each experiment was prepared in triplicate. As a control, samples without enzyme addition were prepared. The sealed tubes were incubated for 72 h at 40°C and 150 rpm in a SW22 water bath (Julabo GmbH, Seelbach, Germany). After enzymatic reaction, 1 mL from each tube was filtered over PTFE in a 2-mL glass vial sealed with a PTFE-lined cap and analysed via LC–MS/MS in the multiple reaction monitoring (MRM) mode, as described in Section 2.8.2**.** For the quantification of eugenol precursors, calibration lines were obtained in citrate–phosphate buffer solutions (0.15 M, pH 5).

2.6 LC–MS analyses of samples

LC–MS analyses were conducted using a 1200 series liquid chromatograph described in Section 2.3.4 coupled with a 6430 triple quadrupole mass spectrometer from Agilent Technologies (Massy, France). The mass spectrometer was equipped with an electrospray ionisation (ESI) source. Nitrogen was produced by NiGen LC–MS 40-1 from Gengaz (Wasquehal, France) and used as drying, nebulising and collision gas. The ESI interface simultaneously operated in the positive and negative modes with the capillary voltage set to 3500 V, the nebuliser pressure to 40 psi, the drying gas flow to 11 $L \cdot min^{-1}$ and the gas temperature to 325°C. The system operates with the MassHunter Workstation software version B.05.00.

Separation was performed on a Kinetex Polar C18 column (150 × 2.1 mm, length × I.D.; 2.6 µm particle size) from Phenomenex (Le Pecq, France) or a Poroshell 120 EC C18 column (150 × 2.1 mm, 2.7 µm) from Agilent Technologies (Massy, France). The mobile phase flow was 0.3 $mL \cdot min^{-1}$, and the injection volume was 10 µL.

Two mobile phase systems were used. In the first column, mobile phase A consisted of 0.1% (v/v) formic acid in ultrapure water, whereas mobile phase B consisted of 0.1% (v/v) formic

acid in acetonitrile (LC–MS quality grade). In the second column, mobile phase A consisted of 0.05% (w/v) ammonium formate and 0.01% (v/v) formic acid in ultrapure water, whereas mobile phase B consisted of 0.05% (w/v) ammonium formate, 5% (v/v) ultrapure water and 0.01% (v/v) formic acid in acetonitrile (LC–MS quality grade).

Two solvent gradients were used for the separation. The first started with 98% A, ramped to 100% B linearly over 15 min and held for an additional 5 min at 100% B. The solvent ratio was then returned to 98:2 (A/B) within 5 min and held for 6 min for column equilibration before the next injection for a run duration of 31 min. The second started with 98% A held for 5 min, ramped to 100% B linearly over 40 min and held for an additional 10 min at 100% B. The solvent ratio was then returned to 98:2 (A/B) within 5 min and held for 6 min for column equilibration before the next injection for a run duration of 66 min.

Detection was performed in the scan mode (*m/z* range 50–2000, scan frequency 200 ms) with ESI^+/ESI^- switching. The fragmentor voltage was 82 V, and the cell accelerator voltage was 5 V.

2.7 LC–HRMS analyses of samples

HRMS analyses were conducted on a QExactive+ instrument (Thermo) based on the Orbitrap technology. The system was equipped with a HESI source, and the ionisation parameters were set as follows: negative mode, spray voltage: 3000V, sheath gas: 45 au, Aux gas: 15, capillary temperature: 320°C, probe heater temperature: 250°C, S-lens RF level: 100 V. MS data were acquired in full-scan data-dependent MS/MS (dd-MS^2) analysis with the following acquisition parameters: for full MS, resolution: 70000, AGC target: 3e6, maximum IT: 100 ms, scan range: 70–750 m/z; for dd-MS^2: resolution: 17500, AGC target: 1e5, maximum IT: 50 ms, isolation window: 2 m/z, number of precursor ions (top N): 5, normalised collision energy: 30 V.

The instrument was coupled to a Vanquish UHPLC (Thermo). Separation was performed on a Phenomenex Luna Omega Polar C18 column (50 × 2.1 mm, 1.6 µm) at 40°C using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) at a flow rate of 0.5 $mL \cdot min^{-1}$; the gradient was 0 min, 1% B; 11.5 min, 40% B; 12.5–14 min, 95% B; 15.5–16 min; 1% B.

2.8 Quantitative LC–MS/MS analysis

2.8.1 Sample preparation

Samples were prepared in triplicates. Finely ground grape berry skin (250 mg, RETSCH MM400 ball mill) was weighed in 2-mL Eppendorf® tubes. Then, SLE was performed using

800, 400 and 400 µL of acetone/water (1:1) with 1-h shaking each on a vortex mixer (Vortex-Genie 2, Scientific Industries Inc., Bohemia, NY, USA) equipped with a vortex adapter (MO BIO Laboratories Inc., Carlsbad, CA, USA). Each SLE step was followed by 10-min centrifugation at 3000 g (Thermo Scientific™ Sorvall™ ST 8 Small Benchtop Centrifuge equipped with a MicroClick® (30 × 2 mL) fixed-angle micro-tube rotor, Fisher Scientific SAS, Illkirch, France). The three combined extracts were again centrifuged in a 2-mL Eppendorf® tube for 10 min at 3000 g to separate the berry skin residues from the solvent extract. The supernatant (1.6 mL) was diluted to 5 mL with Milli-Q water and then vortexed. Subsequently, the diluted sample (16% acetone in water) was injected in LC–MS/MS.

2.8.2 Quantitative LC–MS/MS conditions

Separation was performed on a Kinetex Polar C18 column (150 × 2.1 mm, 2.6 µm) from Phenomenex (Le Pecq, France); the mobile phase flow was 0.3 $mL \cdot min^{-1}$, and the injection volume was 10 µL.

Mobile phase A consisted of 0.05% (w/v) ammonium formate and 0.01% (v/v) formic acid in ultrapure water, whereas mobile phase B consisted of 0.05% (w/v) ammonium formate, 5% (v/v) ultrapure water and 0.01% (v/v) formic acid in acetonitrile (LC–MS quality grade).

The solvent gradient was as follows: starting with A:B (90:10) maintained for 1 min, then ramped to A:B (85:15) linearly over 16 min and held for 1 min, then ramped to 100% B linearly over 5 min, and finally held for an additional 5 min. The solvent ratio was returned to 90:10 (A:B) within 5 min and held for 6 min for column equilibration before the next injection for a run duration of 39 min.

Detection was performed in the MRM mode. The fragmentor voltage was optimised for each parent ion, and the collision energy was optimised for each MRM transition. The cell accelerator voltage was set to 5 V and the dwell time to 20 ms for all transitions. Data are given in Table S1 (Supplementary material) together with parent and daughter ions monitored. For quantification, transition 344→165 was used for **(1)**, 476→295 for **(2)** and 476→133 for **(3)**.

2.8.3 Performance of the quantitative method

Linearity was evaluated by injecting five times 10 calibration levels prepared in a 16% solution of acetone in water, from 0.9 to 900 $\mu g \cdot L^{-1}$ (equivalent to 0.018 to 18 $mg \cdot kg^{-1}$ of berry skin) for geoside, from 4.3 to 4250 $\mu g \cdot L^{-1}$ (equivalent to 0.085 to 85 $mg \cdot kg^{-1}$ of berry skin) for eugenyl-6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside and from 0.89 to 890 $\mu g \cdot L^{-1}$ (equivalent to 0.018 to 17.8 $mg \cdot kg^{-1}$ of berry skin) for citrusin C. The calibration levels were then injected

on a regular basis (every other day). The limits of detection (LODs) and limits of quantification (LOQs) were determined using the regression line method in $\mu g \cdot L^{-1}$ and converted to $mg \cdot kg^{-1}$ of berry skin. Data are available in Table 1.

Intra-day precision was evaluated by injecting the same sample of berry skin (from the 4 grape varieties studied in this work) 10 times as is or spiked with a mix of the three analytical standards at two concentration levels, 36 and 180 $\mu g \cdot L^{-1}$ (equivalent to 0.72 and 3.6 $mg \cdot kg^{-1}$ of berry skin) for **(2)**, 17 and 85 $\mu g \cdot L^{-1}$ (equivalent to 0.34 and 1.7 $mg \cdot kg^{-1}$ of berry skin) for **(3)** and 35.6 and 178 $\mu g \cdot L^{-1}$ (equivalent to 0.71 and 3.6 $mg \cdot kg^{-1}$ of berry skin) for **(1)**. In addition, intra-day precision, including SLE, was evaluated on sample triplicates without spiking, taking into account weighing.

Inter-day precision was evaluated by injecting four times over 7 days the same sample of berry skin as is or spiked with a mix of the three analytical standards at the same two concentration levels used for intra-day precision for the four grape varieties under study.

Recoveries were evaluated using the intra- and inter-day samples spiked at the two aforementioned concentration levels. All data are available in Table S2 (Supplementary material).

2.9 Data statistics

Statistical analyses, including the Shapiro–Wilk normality test, Levene homoscedasticity test, two-way ANOVA, Kruskal–Wallis test and Newman–Keuls post-hoc test and a part of plots, were performed using the RStudio software (version 2021.09.2). Specific use of adapted tests is indicated in the figure captions.

3. Results and discussion

3.1 Analytical strategy for isolating eugenol glycoconjugates from Baco blanc grape berry skin

To isolate eugenol glycosylated precursors from Baco blanc berry skin, the GC–MS-guided LC–MS analytical strategy was developed. Berry skin was chosen as it was found to contain the highest concentrations of eugenol, particularly at the veraison stage, either free or released through enzymatic cleavage (Franc et al., 2023). Using this strategy, the samples obtained at each step were analysed via LC–MS in the full-scan mode and checked via GC–MS analysis following enzymatic hydrolysis to ensure the presence of eugenol glycosylated precursors. A

large amount of Baco blanc berry skin (97.94 g) was processed to obtain a sufficient amount of precursors to be used.

In the first step, to extract polar and less-polar components from the grape berry skin powder, SLE was performed using a 1:1 mixture of acetone and water, as described for oak wood (Slaghenaufi et al., 2013).

In the second step, SPE using a C_{18} phase was performed to simplify the matrix while preserving all the eugenol precursors. The extraction protocol was adapted from (Genovese et al., 2013). The final elution step was optimised by comparing elution with pure acetonitrile and gradual elution with increasing percentage of acetonitrile in water (Hastoy, 2023). LC–MS analysis in the scan mode revealed that a cleaner extract was obtained via elution with 30% acetonitrile in water than with pure acetonitrile while producing the same quantity of enzymatically released eugenol, indicating no loss of eugenol glycoconjugates.

In the third step, an ion-exchange SPE was performed to further purify the Baco blanc berry skin extract. As many compounds from berry skin can be ionised under acidic or basic conditions, we tested both cationic and anionic SPEs. The cation-exchange SPE tested according to (Cebrián-Tarancón et al., 2021) proved to be ineffective (results not shown). LC–MS analysis in the scan mode revealed that a cleaner extract was obtained with the anion-exchange SPE than with the cation-exchange SPE, with no loss of eugenol glycoconjugates, as indicated by the GC–MS analysis of the enzymatically hydrolysed eluates.

Following the anion-exchange SPE, LC–MS was performed to compare the eluate with the hydrolysed eluate to highlight the disappearance of glycoconjugates. However, this disappearance was difficult to determine by comparing the chromatograms owing to the complexity of the extracts and the multiple glycoconjugates of other aroma compounds potentially present in Baco blanc grape skin extracts. To address this drawback, we implemented a step-by-step semi-preparative LC fractionation coupled with GC–MS analysis of the enzymatically hydrolysed fractions to identify the richest fractions in eugenol glycosylated precursors. Three successive tests were conducted to narrow down the search area, as described in Section 2.3.4, and ultimately identify the fraction that released the largest amount of eugenol for further analyses.

3.2 Evidence on the nature of eugenol glycosylated precursors

3.2.1 Predominance of glycosylated precursors in the extracts

Enzymatic hydrolysis was compared with acidic chemical hydrolysis, which is known to be versatile and effective in the hydrolysis of all types of precursors, not just glycosides (Ibarz et

al., 2006). On the extract obtained after anion-exchange SPE, the amount of released eugenol, determined via HS–SPME–GC-MS analysis, was 53.9 ± 0.4 $\mu g \cdot L^{-1}$ for enzymatic hydrolysis and 46.4 ± 3.8 $\mu g \cdot L^{-1}$ for acidic chemical hydrolysis. No significant difference was observed between the two hydrolysis methods (Student's *t*-test, *p*-value < 0.05). The results indicate that the majority of eugenol precursors are glycosylated. Their glycone moieties were indeed cleaved by the complex mixture of enzymes present in the commercial enzymatic preparation.

3.2.2 Molecular formula of the main eugenol precursor

After the third HPLC fractionation, LC–MS analysis of the fraction releasing the highest amount of eugenol (51.5–52.5 min as described in Section 2.3.4) revealed the presence of ions of *m/z* 481 in ESI^+ and 503 in ESI^- using mobile phases added with formic acid (Figure S2), as well as ion of *m/z* 476 in ESI^+ when using mobile phases added with ammonium formate. These ions were interpreted as the sodium adduct (+23) and the ammonium adduct (+18) of a compound with a molecular weight of 458 in ESI^+ and its formate adduct (+45) in ESI^-. Thus, it was hypothesised that this glycosylated precursor of eugenol contains eugenol (164), a hexose (162) and a pentose (132). This hypothesis was validated by LC–HRMS analysis in ESI^- providing the exact mass of 503.1781 and the molecular formula of $C_{22}H_{31}O_{13}$ consistent with the formate adduct of a diglycoside of eugenol containing a pentose and a hexose. Two commercial eugenol glycoconjugates with this molecular formula were purchased: geoside **(2)** and compound **(3)**. As a reminder, their structures are depicted in Figure 1. Both only differ in the five-carbon sugar conformation: arabinose for **(2)** and apiose for **(3)**.

3.2.3 Specific enzymatic hydrolysis of eugenol precursors

The main precursor of eugenol is hydrolysed via commercial enzymatic preparation, as indicated by the disappearance of the peaks of *m/z* 503 (ESI^-) and 476 (ESI^+) in the chromatogram of the hydrolysed sample compared with the non-hydrolysed one (data not shown). The oenological enzymatic preparation is described with glucosidase, rhamnosidase, arabinofuranosidase and apiosidase activities. Arabinose and apiose (pentoses) have exactly the same molecular formula ($C_5H_{10}O_5$). Furthermore, they are both compatible with the hypothesis of a diholoside of eugenol containing a hexose, probably glucose directly linked to the eugenol aglycone (as generally described in literature including (Hjelmeland & Ebeler, 2015)), and a pentose (as arabinose or apiose) linked to this glucose unit. Meanwhile, rhamnose with the molecular formula $C_6H_{12}O_5$ is not compatible with our hypothesis. To elucidate the glycone moiety of the main eugenol precursor, the enzymatic activities were tested separately using

available specific enzymes (glucosidase, rhamnosidase, arabinofuranosidase) on grape skin extract. To address the unavailability of apiosidase, **(3)** was purchased. Compound **(4)** (Figure 1) was not commercially available to test the arabinofuranosidase activity. Moreover, **(2)** which is commercially available should be hydrolysed by arabinopyranosidase but not by arabinofuranosidase. However, arabinopyranosidase was not available. Compound **(1)** was also purchased to monitor the effect of the enzymes by measuring the release of citrusin C from the eugenol precursors. The experiments were monitored by LC-MS/MS using the optimised separation method described in section 2.8.2 in order to reduce analysis time.

For control, the pure eugenol precursor reference standard solutions of **(1)**, **(2)** and **(3)** were tested separately against three specific enzymes (i.e. β-glucosidase, α-rhamnosidase and α-arabinofuranosidase) and against the commercial enzymatic preparation, to ensure proper hydrolysis with the available commercial enzymes (Figure 2). Concerning **(3)**, the purity was low, showing two peaks in LC–MS at 16.8 and 17.6 min in a ratio of 3:1 (*m/z* 481). We assumed that the first one corresponded to **3** and the second peak (**Unk 3b**) was a synthesis secondary product. With the α-rhamnosidase or the α-arabinofuranosidase, all three commercial eugenol precursors were kept intact, including **Unk 3b**. Moreover, no citrusin C was released by enzymatic hydrolysis from the three commercial precursors using α-rhamnosidase and α-arabinofuranosidase. This was expected as none of the precursors possessed terminal rhamnose or arabinofuranose. As expected, citrusin C was totally removed using a pure β-glucosidase suspension, whereas precursors **(2)** and **(3)**, including **Unk 3b**, were not affected. Finally, the commercial enzymatic preparation was the only one to completely hydrolyse precursors **(1)**, **(2)** and **(3),** including **Unk 3b**, even if its arabinopyranosidase activity was not described (Figure 2).

Enzymatic hydrolyses were then performed on a raw Baco blanc grape skin extract rather than on the purified fractions so as not to be limited by the amount of precursor. Notably, in the raw S/L extract, apart from the main precursor detected at RT = 15.8 min, a small amount of citrusin C at 16.3 min and three other small peaks were present (Figure 3a). These three other small peaks at RT = 14.6, 16.8 and 17.6 min presented the same *m/z* 481 in ESI^+ and 503 in ESI^-.

Concerning the various enzymatic hydrolyses performed, different observations were noticed. First, all the five peaks, previously cited, disappeared after hydrolysis by the commercial enzymatic preparation.

Second, citrusin C was not released with the use of α-rhamnosidase, and all the other peaks with *m/z* 481 (ESI^+), 503 (ESI^-), remained stable. This proves the absence of a diholosidic

precursor of eugenol, which is composed of one unit of glucose and one unit of rhamnose. This observation also indicates the absence of the triholosidic precursors of eugenol with a terminal unit of rhamnose. Triholosidic precursors that would have been able to release the precursors of eugenol detected with *m/z* 481 (ESI^+) and 503 (ESI^-).

Next, hydrolysis with α-arabinofuranosidase strongly increased the concentration of citrusin C from 50.4 µg· to 227.3 µg·L^{-1}. This had no effect on the main peak at 15.8 min nor on the small peaks at 16.8 and 17.6 min, but it totally hydrolysed the small peak at 14.6 min. This further supports the hypothesis of a minor diholosidic precursor of eugenol at 14.6 min. A minor diholosidic precursors presumably composed of a hexose, more precisely a glucose and a pentose assimilated here to an arabinofuranose.

Finally, the use of the pure suspension of β-glucosidase slightly reduced citrusin C concentration from 50.4 to 31.2 µg·L^{-1}. This reduction of 38.0% reached 99.7% when the enzymatic hydrolysis with β-glucosidase was performed on the commercial standard of citrusin C. This could be explained by the presence of numerous glycosylated compounds in the Baco blanc berry skin extract competing with citrusin C for enzymatic hydrolysis. However, this enzyme exerted no effect on the main precursor at 15.8 min.

All these specific enzymatic hydrolyses clearly indicate that concerning the main precursor of eugenol, i) glucose is not the terminal unit and ii) pentose is not an arabinose in the form of a five-membered ring.

3.2.4 Comparison with genuine standards

Next, the main precursor isolated through LC fractionation was compared in LC–MS and LC–HRMS with the two commercial standards available, (**2)** and (**3)**. The comparison was performed on two different instruments, with three different LC columns, two different mobile phase systems and three different gradients. Whatever the system used, compound (**2**) and the main Baco blanc eugenol precursor were co-eluted and showed: a close exact mass and similar fragmentation patterns (Figure 4). Meanwhile, **(3)** and **Unk 3b** were only slightly detected in the Baco blanc fractions. On the LC–HRMS instrument using a Luna Polar C18 column, both the geoside standard and the purified fraction exhibited a peak at 6.45 min (Figure 4a). At this retention time, the full-scan spectra in ESI^- showed the presence of an ion with *m/z* = 503.1777 (Figure 4b), which was assigned to the molecular formula of $C_{22}H_{31}O_{13}$ consistent with the hypothesis of a formate coating of a eugenol diholoside (Δm=3.6 ppm). Furthermore, an ion with *m/z* = 457.1722 was detected with both MS instruments and assigned with the non-adduct form $C_{21}H_{29}O_{11}$ (Δm = 4.0 ppm). This precursor ion was also fragmented in the data-dependent

MS/MS experiment using a normalised collision energy of 30 V. The fragmentation unequivocally showed (Figure 4b) the formation of a fragment with *m/z* = 163.0758, which is coherent with the eugenol aglycone form $C_{10}H_{11}O_2$ (Δm = 2.8 ppm), after losing the sugar moieties.

Based on all this evidences, we can formally conclude that the main eugenol precursor in Baco blanc is geoside, which consists of a direct linkage between eugenol and a glucose molecule attached to an arabinose in the form of a six-membered ring. This hypothesis could be confirmed if arabinopyranosidases were available. This is the first identification of geoside in grapes.

Apart from geoside at RT = 15.8 min, other minor eugenol precursors were detected in the S/L extracts of Baco blanc berry skin, namely, i) a compound hypothesised as eugenyl–arabinofuranosyl–glucopyranoside (**Unk 4**) at RT = 14.6 min, ii) compound **(1)** at RT = 16.3 min, iii) compound **(3)** at RT 16.8 min and iv) an unknown compound (**Unk 3b**) at RT = 17.6 min, which is also apparently present in analytical standard **(3)**.

3.3 Method development for the quantitation of eugenol glycoconjugates

A quantitative MRM-LC–MS method was next developed for the three commercially available eugenol glycoconjugates, i.e. **(1)**, **(2)** and **(3)**. First, these analytical standards exhibited better abundances when ammonium formate together with formic acid were used as mobile phase additives than when using formic acid alone. The use of ammonium formate favours ammonium adducts in ESI^+. Indeed, ions with *m/z* 476 and 344 were the main ones observed for the diglycosides and the monoglucoside of eugenol, respectively. In ESI^-, the main ions corresponded to formate adducts at *m/z* 503 and 371, respectively. Manual optimisation of MRM transitions had to be performed. As matter of fact these adducts proved difficult to break in the collision cell. The same problem appeared with sodium adducts when no ammonium formate was used. Compound **(1)** ammonium adduct was fragmented to $[\text{eugenol} + H]^+$ of *m/z* 165 and its sodium adduct to $[\text{glucosyl} + Na]^+$ of *m/z* 185. The ammonium adducts of diglycosides were fragmented to $[\text{eugenol} + H]^+$ of *m/z* 165, $[\text{glucosyl} + H]^+$ of *m/z* 163, $[\text{pentosyl} + H]^+$ of *m/z* 133 and $[\text{glucosyl-pentosyl} + H]^+$ of *m/z* 295. Their formate adducts were fragmented to $[\text{glucosyl-pentosyl-H}]^-$ of *m/z* 293 and $[\text{eugenol-glucosyl-pentosyl-H}]^-$ of *m/z* 457. Second, LC separation was optimised to avoid co-elutions of the eugenol glycoconjugates, in particular those with the same molecular weight. This optimisation consisted in a reduced run time and a better separation as illustrated in Figure 3b.

As regards the compound hypothesised as eugenyl–arabinofuranosyl–glucopyranoside (**Unk 4**) at RT 14.6 min, as no standard was available, semi-quantitation was performed using the geoside calibration curve.

For the sample preparation, to provide an extremely simple and repeatable procedure, only the first step was applied: a SLE using a mixture of acetone/water (1:1) on the berry skin powder sample. The anion-exchange SPE employed for fractionation was not needed as the methodology showed sufficient sensitivity. To process 250 mg sample of berry skin powder, the solid/solvent ratio had to be slightly modified to obtain a sufficient amount of liquid to soak the solid. As eugenol glycoconjugates are abundant in Baco blanc berry skin, acetone evaporation was not performed; instead, the sample was diluted to reduce the amount of acetone at the injection. The tests conducted using 25%, 17% and 10% of acetone in water showed that 17% was compatible for maintaining the peak shape and width. A standardised sample preparation was finally adopted, extracting the sample in 16% acetone (see Section 2.4.1). Next, calibration curves were obtained with sample extracted with a mixture of 16% acetone in water.

3.4 Performance of the quantitative method for eugenol glycoconjugate analysis

Calibration curves were plotted for **(1)**, **(2)** and **(3)**. For compound **(3)**, two peaks were present in the full-scan chromatogram of the standard, and the given purity was 85%. Then the concentration of the peak at 16.8 min was estimated as 85% of the total amount. As regards the linearity of each compound in the method, the calibration lines were stable over time with coefficients of determination greater than 0.998. The calibration parameters, LOQ and LOD, are given in Table 1.

Intra-day precision showed good method repeatability as the relative standard deviations (RSDs) for 10 replicates were extremely low. The RSDs were lower than 4.3% for citrusin C, except for the Baco blanc sample without spiking (13.5%) for which the concentration value was close to LOQ. For geoside, the RSDs were lower than 7.2%, and for compound **3**, they were lower than 7.9%**.** As for the sample analysis including extraction, the intra-day precision of triplicates (n = 7) ranged from 4.5% to 16.9% for citrusin C, from 1.4% to 14.0% for geoside and from 0.6% to 10.5% for the unknown compound **4** (**Unk 4**). For the inter-day precision, the RSDs were slightly higher than those of the intra-day precision but still correct, ranging from 7.6% to 12.3% for citrusin C, except for the Baco blanc sample without spiking (38.2%) for

which the concentration value was close to LOQ, from 7.3% to 14.5% for geoside and from 7.1% to 11.9% for compound **(3).** Finally, the recoveries exhibited extremely good accuracy: from 82.8% to 98.2% for citrusin C, from 84.6% to 99.6% for geoside and from 81.2% to 105.0% for eugenyl-6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside. All these data are available in the Table S2 (Supplementary material).

3.5 Quantification of eugenol glycoconjugates in grape berry skin samples

3.5.1 Quantification of eugenol glycoconjugates in *Vitis* genus

To demonstrate the specific origin of geoside, the main eugenol precursor identified in Baco blanc grapes, different grape varieties were also analysed. The other eugenol precursors were also monitored in these samples. In particular, we analysed and compared berry skin samples from Baco blanc (3 different origins), Ugni blanc (2 different origins), Folle blanche (2 different origins) and Plant de Graisse (2 different origins) samples. The quantification results (Table2) are expressed in mass unit of compound (mg) per mass unit of matrix (kg of grape skin) and then converted to equivalent of eugenol in mg per kg of grape skin. Compound **(2)** was significantly more abundant in Baco blanc grape skin (4.1 ± 1.5 $mg \cdot kg^{-1}$, which is equivalent to 1.5 ± 0.6 mg of eugenol/kg of grape skin on average) than in the other varieties. In Ugni blanc and Plant de Graisse, **(2)** concentration was below LOD, whereas in Folle blanche, an extremely small amount of geoside, below LOQ (i.e. <0.098 $mg \cdot kg^{-1}$), was detected. These results confirm the specificity of geoside for Baco blanc grapes. Compound **Unk 4** (eugenyl–arabinofuranosyl–glucopyranoside) followed the same trend as **(2):** it was highly abundant in Baco blanc berry skin but not detected in Ugni blanc and Plant de Graisse, and finally slightly detected in Folle blanche (close to LOD). As no standard was available for its absolute quantitation, semi-quantitation using geoside provided a ratio of approximately 80:20 in favour of geoside. Compound **(1)** was detected only in Baco blanc samples and close to LOQ (0.028 ± 0.008 $mg \cdot kg^{-1}$ equivalent to 0.014 ± 0.004 mg of $eugenol \cdot kg^{-1}$of grape skin on average) and not in the three other grape varieties. In these samples, compound **(1)** represented less than 1% of the eugenol equivalent out of the three eugenol glycoconjugates detected. This confirms the minor presence of citrusin C in Baco blanc berry skin compared with geoside. Compound **(3)** was not detected in these samples (cf. Table 1, LOD = 0.141 $mg \cdot kg^{-1}$, which is equivalent to 0.050 mg of $eugenol \cdot kg^{-1}$ of grape skin).

Thus, geoside **(2)** appeared to be a varietal eugenol precursor of Baco blanc. It was either not found or found at extremely low concentrations in *V. vinifera* skin extracts. Moreover, in Baco blanc, geoside was the most abundant eugenol precursor compared with citrusin C **(1)**, which represented a minor fraction of bound eugenol in this variety. Indeed in the samples analysed in these experiments the ratio between geoside and citrusin C was 99:1 in molar concentration or in eugenol equivalent content. In addition, a ratio of approximately 80:20 was found between geoside and the compound hypothesised as eugenyl–arabinofuranosyl–glucopyranoside (**Unk 4**). The latter was also not detected in the three other grape varieties.

Free and bound eugenol were also found in some other grapevine hybrid varieties - in particular with V. riparia or V. cinerea ancestor (Sun, Gates, et al., 2011) as well as in the wild spine grapes *V. davidii* "Foëx" (Lan et al., 2020). Thus, it would be interesting to determine whether geoside, or any of the other compounds identified in our research are also main precursors in these varieties.

3.5.2 Time progress of eugenol glycoconjugates during the Baco blanc berry development

The last section of this study was aimed at monitoring eugenol glycoconjugates and, in particular, **(2), (1)** and **Unk 4** during the Baco blanc berry development. Recently, (Hastoy et al., 2023) showed that bound eugenol, which represented eugenol precursors, reached a maximum at veraison stage and decreased during grape maturation.

To confirm this observation, the concentrations expressed in mg equivalent of eugenol per kg of grape skin $mg \cdot kg^{-1}$ for **(2), (1)** and **Unk 4** were compared with the concentrations of enzymatically released eugenol (bound eugenol, measured via HS–SPME–GC–MS) in berry skin (Figure 5). Geoside and **Unk 4** showed the same concentration profile as bound eugenol: 131% increase from cluster closure to veraison for **(2)**, 167% for **Unk 4** and 98% for bound eugenol. From veraison to harvest, the contents decreased by 43% for **(2),** 33% for **Unk 4** and 31% for bound eugenol. Finally, from the harvest to the post-harvest stage, the concentrations slightly decreased: 3% for **(2)**, 20% for **Unk 4** and 25% for bound eugenol. As regards these three eugenol precursors, the variations from one stage to the next were statistically different, proving the time-progress profile of these eugenol precursors.

Contrarily, the concentration of **(1)** statistically decreased continuously from cluster closure to the post-harvest stage: 62% from cluster closure to veraison, 31% from veraison to harvest and 25% from harvest to post-harvest.

The general concentration profile of geoside **(2)** and **Unk 4** in berry skin might correspond to the defence mechanism of the plant against some pathogens, i.e. an accumulation during the

berry development and a slow decline during the berry maturation (Deytieux-Belleau et al., 2009; Pañitrur-De la Fuente et al., 2020).

Conclusion

An EPIQ strategy was employed to isolate, identify and quantify the eugenol glycosylated precursors of Baco blanc from a raw extract of berry skin. While searching for multiple glycosylated precursors, it has been found that a particular compound was predominant. This is how geoside (eugenyl-6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside) was identified following an original research approach combining HRMS data, selective enzymatic hydrolyses and comparison with the genuine commercial molecule. This is the first evidence of the presence of geoside in grapes (*Vitis* genus). We determined that geoside accounts for approximately 80% of the eugenol released from Baco blanc berry skin extracts. An other eugenol glycosylated precursor was quite abundant in Baco blanc samples. It is hypothesised as a diholoside containing a glucose and an arabinofuranose unit. The ratio between geoside and this compound (**Unk 4**) reached approximately 80:20. Citrusin C, the monoglucoside of eugenol, is a very minor compound in Baco blanc berry skin, accounting for less than 1% of all glycoconjugates. Eugenyl-6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside was not quantifiable in our samples and was only detected in strongly concentrated extracts used for structure elucidation.

Moreover, compared with Baco blanc, the quantification of geoside in three *Vitis vinifera* grape varieties showed high specificity or even exclusivity of this compound. During maturity of Baco blanc grapes, the geoside concentration increased until veraison and then decreased. Geoside production could result from the glycosylation of eugenol. This eugenol would then be produced as a biofungicide and preserved in glycosylated form, soluble and non-toxic to the plant, in the event of a fungal attack.

It would be interesting to determine whether geoside is present in other grapevine hybrid varieties and whether it improves tolerance to *Botrytis cinerea* in these varieties. Now that geoside is acknowledged as the major precursor of eugenol in Baco blanc, its capacity to inhibit *Botrytis cinerea* needs further investigation. Furthermore, its properties should be compared with the already known major anti-fungal properties of eugenol. In addition, considering that eugenol could be an odour active compound, mainly depending on its concentration in the free form, its sensory role in Armagnac wine spirits need to be clarified. Consequently, studying the fate of the precursor forms of eugenol throughout the vinification process of Baco blanc wines,

as well as during distillation, and examining the variables influencing the release of eugenol would be of prime importance.

Acknowledgments

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FIGURES AND TABLES

Table 1: Calibration parameters, limits of quantification and limits of detection.

Compound	Calibration range		slope	R^2	LOD		LOQ	
	µg·L^{-1}	equiv. mg·kg^{-1} of berry skin			µg·L^{-1}	equiv. mg·kg^{-1} of berry skin	µg·L^{-1}	equiv. mg·kg^{-1} of berry skin
(**1**)	0.89-890	0.018-17.8	468.60	0.9988	0.3	0.007	1.1	0.023
(**2**)	0.9-900	0.018-18.0	110.57	0.9989	1.5	0.029	4.9	0.098
(**3**)	4.3-4250	0.085-85.0	58.57	0.9991	7.1	0,141	23.5	0.470
Unk **4** †	4.5-4500	0.090-90.0	1609.58	0.9991	3.3	0.067	11.1	0.222

†Calibration and LOD/LOQ are relative to geoside reference standard **(2)**

Table 2: Quantification of eugenol precursors in Vitis genus

Compound	citrusin C (1)			geoside (2)			Unk **4** * (eugenyl-arabinofuranosyl-glucopyranoside)			Sum glycosides	ratio geoside:Unk 4	% citrusin C	% geoside	% Unk 4
	mg·kg^{-1} of berry skin	**eq. eugenol mg·kg^{-1} of berry skin**	**RSD (%)**	**mg·kg^{-1} of berry skin**	**eq. eugenol mg·kg^{-1} of berry skin**	**RSD (%)**	**mg·kg^{-1} of berry skin**	**eq. eugenol mg·kg^{-1} of berry skin**	**RSD (%)**	**eq. eugenol mg·kg^{-1} of berry skin**				
Baco blanc A	0.037 ± 0.05	0.019 ± 0.002	12.2%	5.870 ± 0.211	2.102 ± 0.075	3.6%	1.162 ± 0.056	0.416 ± 0.020	4.8%	2.537	83:17	0.7	82.9	16.4
Baco blanc B	0.023 ± 0.02	0.012 ± 0.001	10.7%	3.079 ± 0.066	1.102 ± 0.024	2.2%	0.672 ± 0.018	0.241 ± 0.007	2.7%	1.355	82:18	0.9	81.3	17.8
Baco blanc C	0.025 ± 0.03	0.012 ± 0.001	10.5%	3.335 ± 0.045	1.194 ± 0.016	1.4%	0.799 ± 0.005	0.286 ± 0.002	0.6%	1.492	81:19	0.8	80.0	19.2
Ugni blanc A	< LOD	< LOD		< LOD	< LOD		< LOD	< LOD						
Ugni blanc B	< LOD	< LOD		< LOD	< LOD		< LOD	< LOD						
Folle blanche A	< LOD	< LOD		< LOQ	< LOQ		< LOD	< LOD						
Folle blanche B	< LOD	< LOD		< LOQ	< LOQ		< LOD	< LOD						
Graisse A	< LOD	< LOD		< LOD	< LOD		< LOD	< LOD						
Graisse B	< LOD	< LOD		< LOD	< LOD		< LOD	< LOD						

concentration given as average ± SD (n = 3)

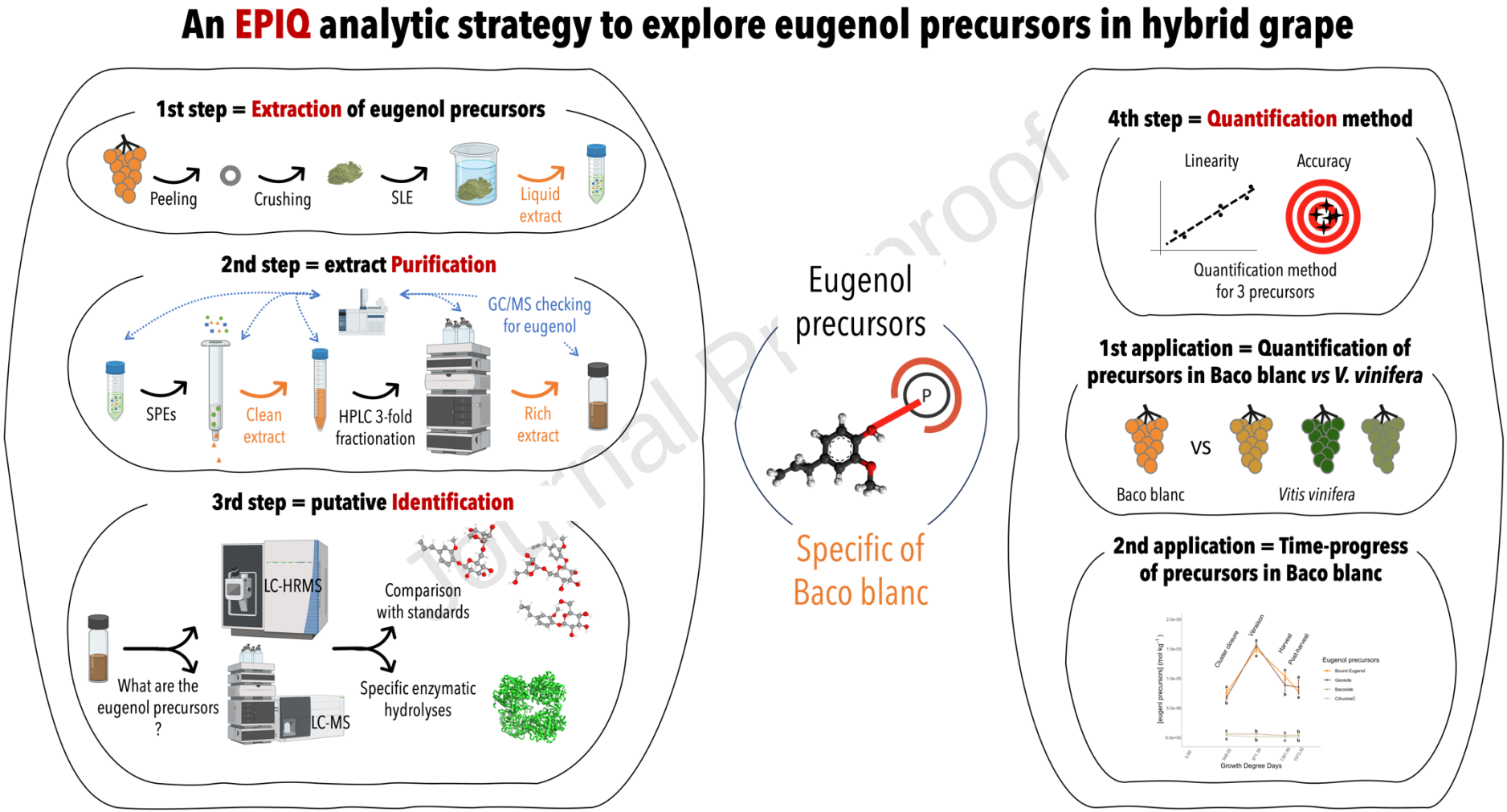
* quantification relative to geoside standard

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

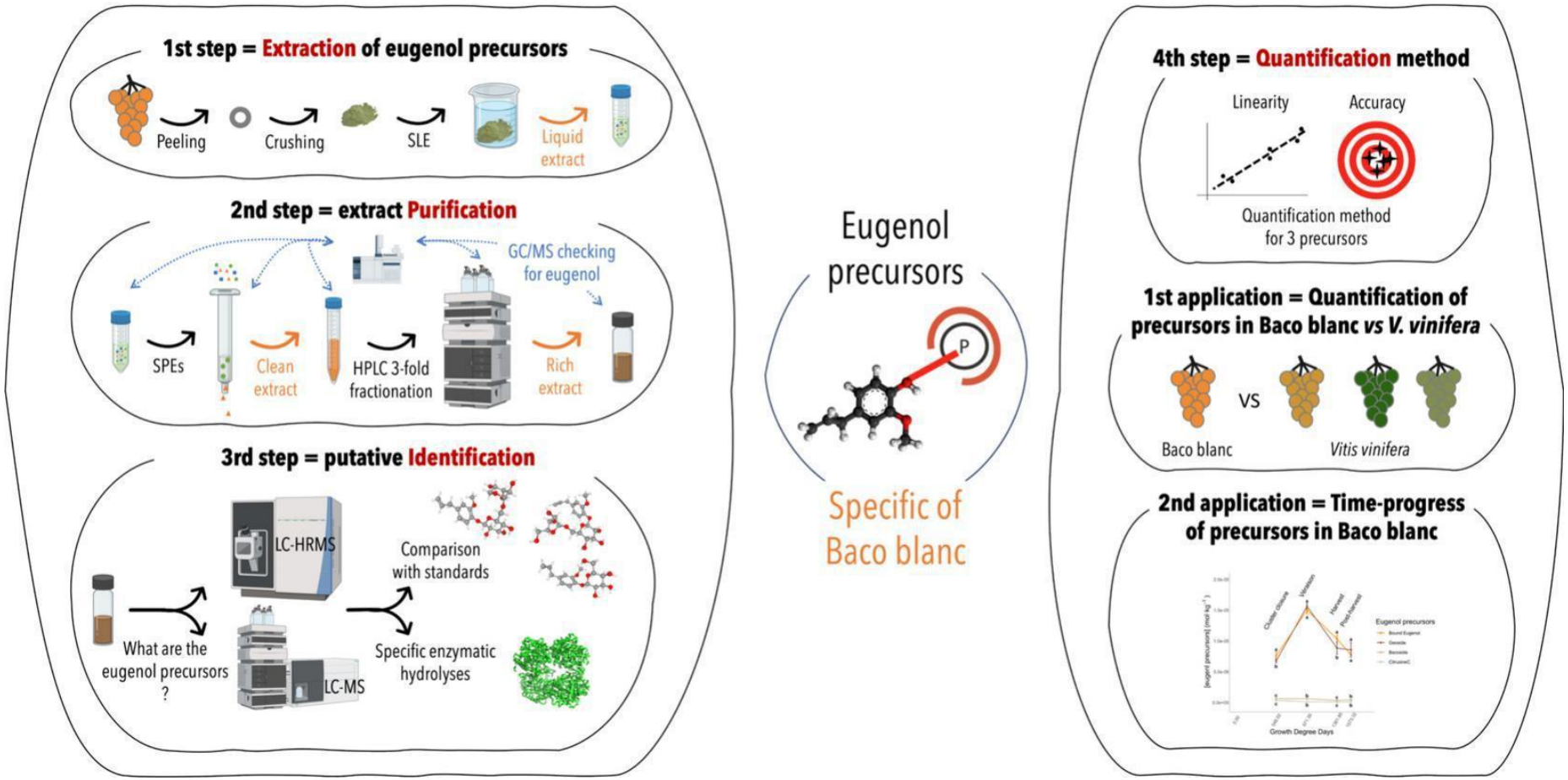
☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract



Highlights

- GC–MS-guided LC fractionation led us to isolate glycosidic precursors of eugenol
- Geoside is the main eugenol precursor in Baco blanc grape variety
- Geoside is almost exclusively found in Baco blanc compared with *V. vinifera* fruit
- The geoside content in Baco blanc berry skin is maximum at the veraison fruit stage



Graphics Abstract

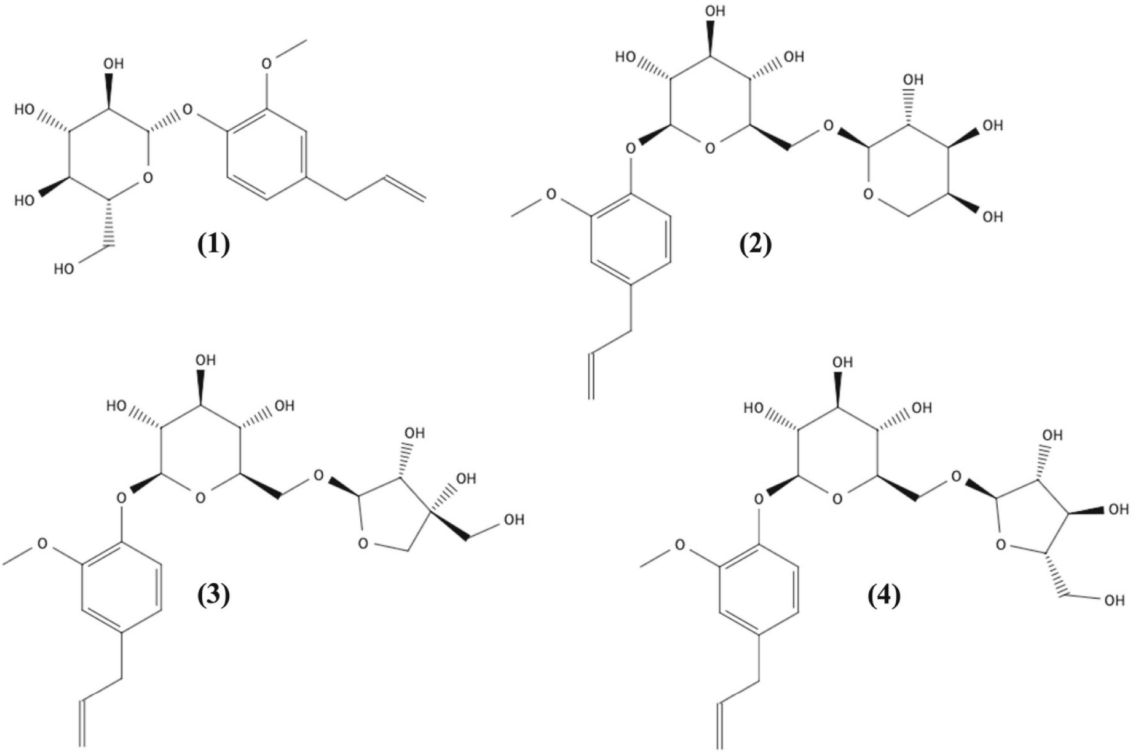


***Figure 1**: Molecular structures of potential eugenol glycoconjugates. **(1)** citrusin C (eugenyl β-D-glucopyranoside), **(2)** geoside (eugenyl-6-O-α-L-arabinopyranosyl-β-D-glucopyranoside), **(3)** eugenyl-6-O-β-D-apiofuranosyl-β-D-glucopyranoside, and **(4)** eugenyl-6-O-α-L-arabinofuranosyl-β-D-glucopyranoside. It should be noted that **(4)** was not commercially available, unlike the other three*

Figure 1

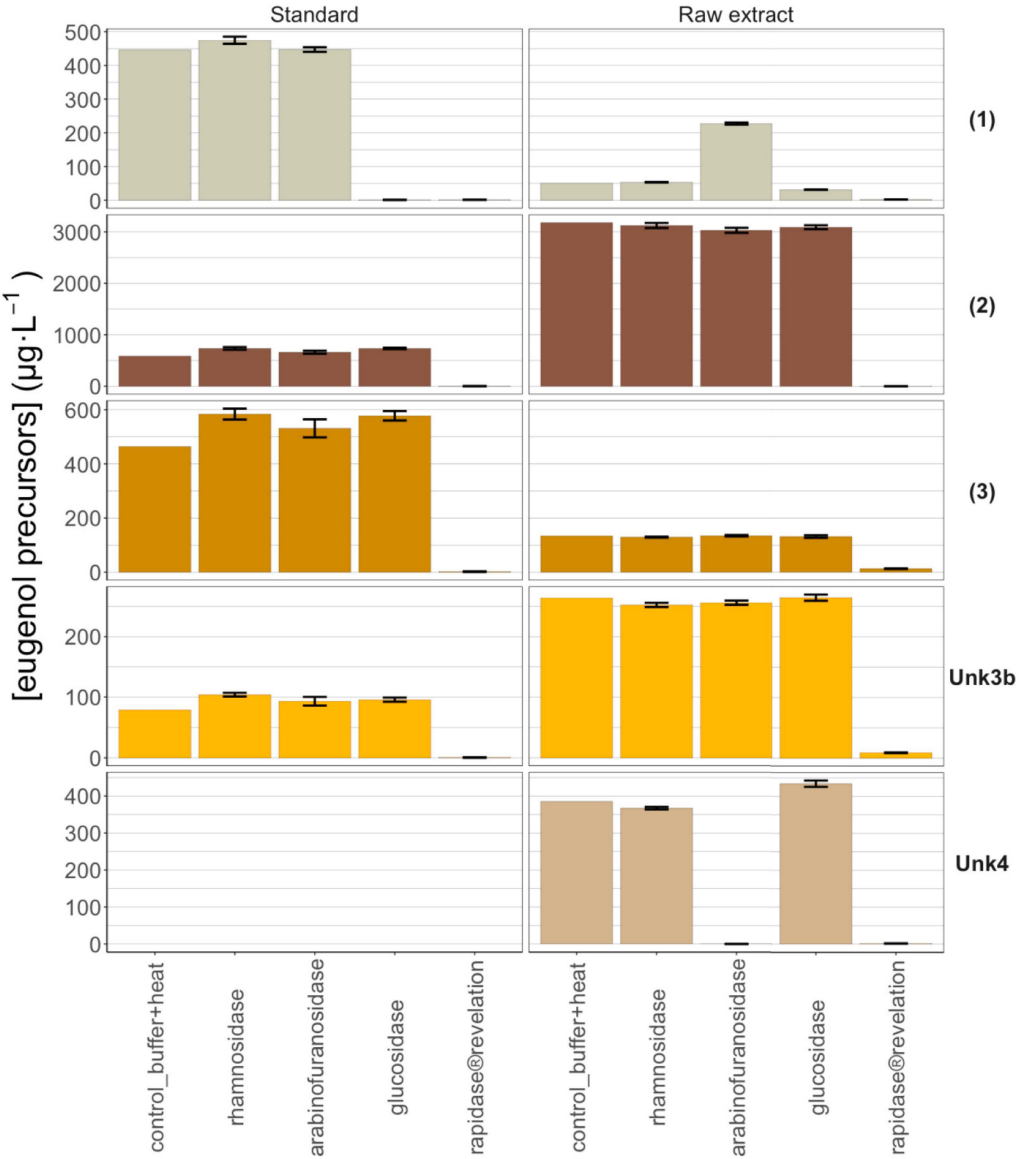


Figure 2: *Effect of specific enzymes on eugenol precursors in standard solutions (left) and in raw S/L berry skin extract (right)*

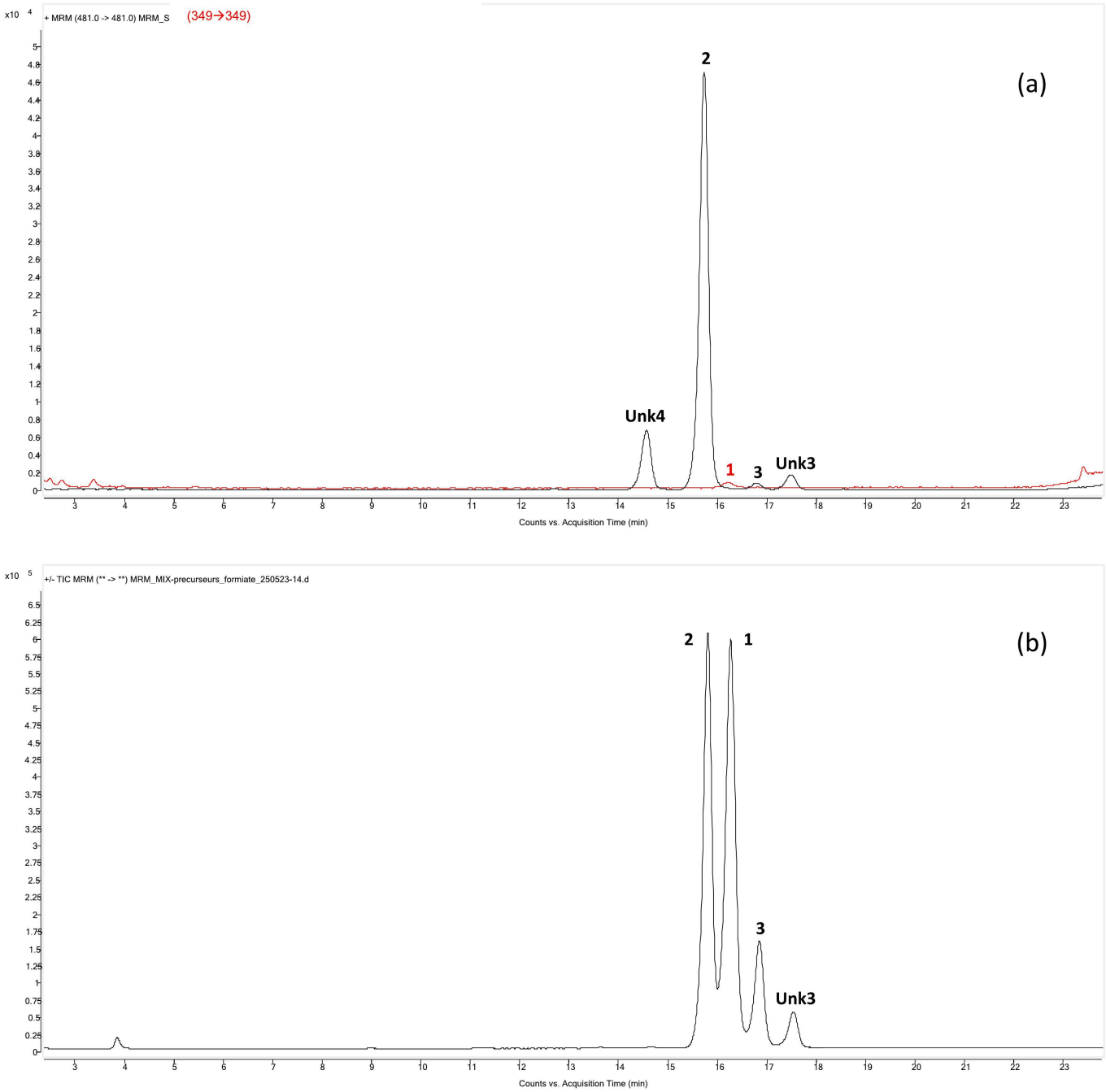


Figure 3: *LC-MS chromatograms in MRM mode for (a) a raw S/L extract of Baco blanc grape skin (black line m/z 481 (eugenol diglycosides), red line m/z 349 (citrusin C) and (b) a solution of commercial standards* ***(1)****,* ***(2)*** *and* ***(3)*** *at approximately 3 mg/L each*

Figure 3

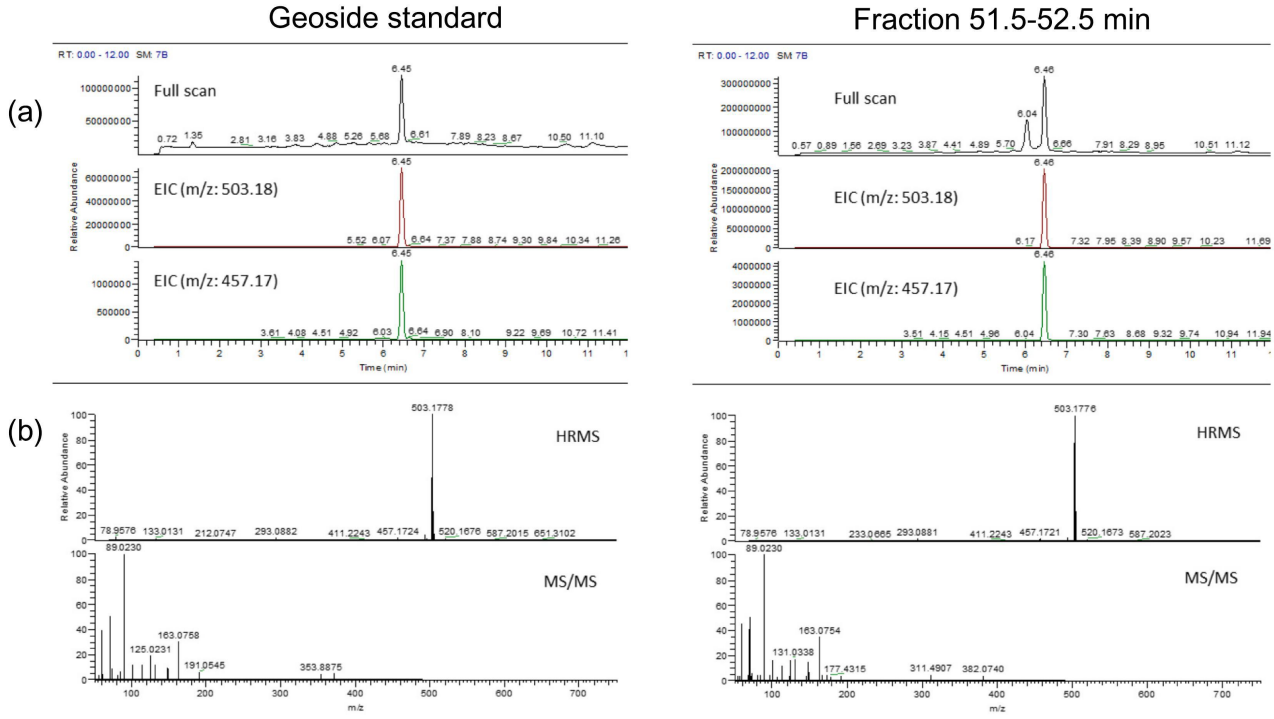


Figure 4: LC-HRMS analysis of the geoside standard (left) and the purified fraction (right). (a) Full scan chromatograms and extracted ion chromatograms (EIC) for m/z = 503.18 and m/z = 457.17 (ESI^-), showing a common peak at RT = 6.45 min. (b) HRMS (ESI^-) for this peak (m/z = 503.1777, consistent with the formate adduct) and MS/MS fragmentation of the ion 457.17 coincident for both the standard and the purified fraction

Figure 4

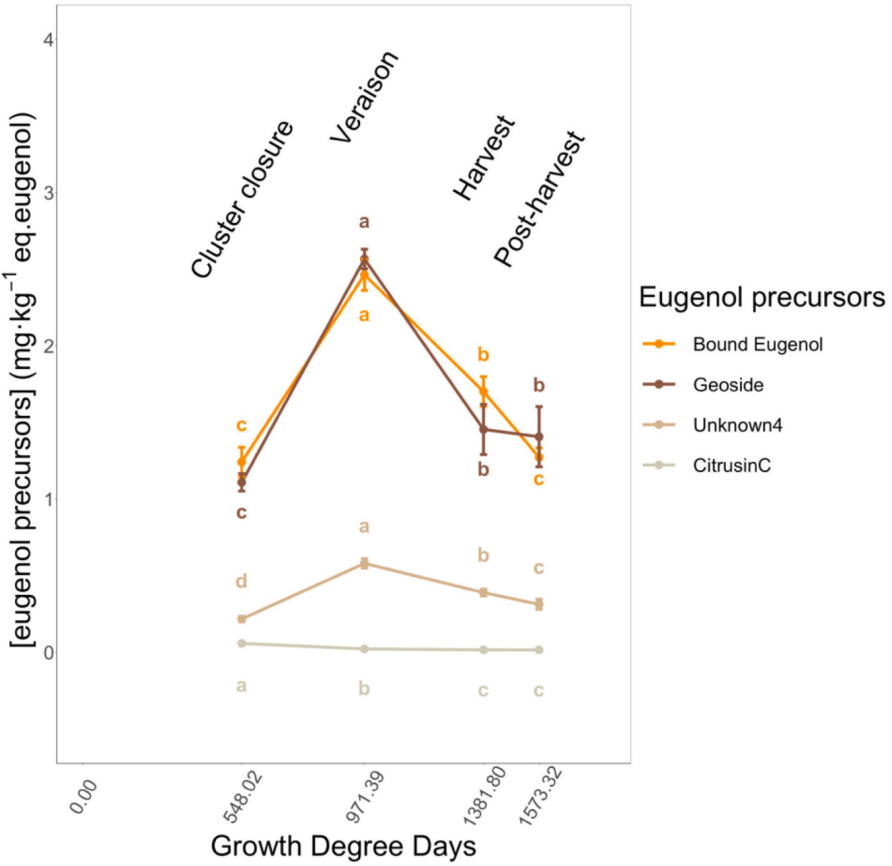


Figure 5: Time-progress of eugenol precursors. Newman-Keuls tests were performed by molecule to observe differences between phenological stages. Different letters indicate a significant difference ($\alpha = 0.05$)

Figure 5