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Thiol S-Conjugate Profiles: A Comparative Investigation on Dual Hop and Grape Must with Focus on Sulfanylalkyl Aldehydes and Acetates Adducts

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ABSTRACT

Cysteinylated and glutathionylated precursors of sulfanylalkyl alcohols have been extensively studied in hop and grape matrices, in contrast to those of sulfanylalkyl aldehydes and sulfanylalkyl acetates. Here, cysteinylated and glutathionylated adducts of 3-sulfanylpentanal (Cys-3SPal and G-3SPal), 3-sulfanylhexanal (Cys-3SHal and G-3SHal), 3-sulfanylpentyl acetate (only Cys-3SPA), and 3-sulfanylhexyl acetate (Cys-3SHA and G-3SHA) were first synthesized. Next, the occurrence of these compounds was studied by RP-HPLC-ESI(+)-MRM and apotryptophanase-GC-PFPD in a few dual hops and Belgian grape must samples. None of the Cys-adducts of 3-sulfanylalkyl aldehydes were detected in hop or grape extracts, while G-3SPal was identified for the first time in a natural matrix. Concentrations ranging from 3 to 15 mg/kg were found in several hop varieties, including Citra, Mandarina Bavaria, Mosaic, Nelson Sauvin, Polaris, and Saaz, whereas G-3SHal was detected (below the quantitation limit) only in the four latters. As for esters, Cys-3SPA was found in hop (4 mg/kg in Mosaic), while G-3SHA was detected only in grape extracts (Belgian Chardonnay and Johanniter). Besides the ubiquitous G-3SHol, G-3SPol was identified here for the first time in grape must (up to 7 mg/kg in Johanniter).

KEYWORDS
cysteinylated conjugates; glutathionylated conjugates; *Humulus lupulus L.*; polyfunctional thiols; *Vitis vinifera*

Introduction

Researchers, in recent years, have shown strong interest in polyfunctional thiols, a family of aromas with very low perception thresholds (ng/L level) and pleasant organoleptic impacts (fruity or floral odors for those with five or more carbon atoms).[1]

The free forms of these molecules have been investigated in wines and beers as well as in their raw materials: grapes and hops.[2–4] Significantly higher concentrations in the finished products than in the ingredients led to the hypothesis of hidden forms, precursors of these molecules.[5]

This hypothesis proved to be correct upon the discovery of cysteine (Cys-) and glutathione (G-) precursors in grapes and hops. These non-odorant molecules were believed to be cleaved by yeasts during fermentation in brewing and wine-making processes, thereby releasing their organoleptic potential. So far, the G- and Cys-precursors of only a few sulfanylalkyl alcohols, i.e., 3-sulfanyl-4-methylpentanol (3S4MPol), 3-sulfanylhexanol (3SHol) and 3-sulfanylpentanol (3SPol) and one sulfanylalkyl ketone, i.e., 4-sulfanyl-4-methyl-pentan-2-one (4S4M2Pone), have been identified and quantified.[6,7]

Table 1 summarizes their levels in grapes and hops, as available in the literature.[8–12] As a general trend, Cys-precursors are present at higher levels (up to 15.86 mg/kg) in grape, with a predominance of 3SHol adducts. In hop, higher concentrations of G-precursors are found (1.28-101 mg/kg G-3SHol for only 0.09-4.9 mg/kg of the cysteinylated counterpart). There are also differences in distribution within the plant: grape skins, for example, have much higher concentrations than musts.[10,12] Amarillo, Citra, Hallertau Blanc, and Polaris hops have proved to be the most interesting for the ubiquitous G-3SHol and G-3SPol, while Sauvignon Blanc and Semillon have emerged as the grapes with the highest levels of Cys-3SHol.

Besides grapes and hops, these precursors have been identified among others in peppers, passion fruits, and *Poncirus trifoliata* (a lemon species).[7,13,14] Their biosynthesis in plants is the result of defense mechanisms involving glutathione. This endogenous tripeptide (ɣ-Glu-Cys-Gly), practically omnipresent in organisms, is the most abundant non-protein thiol in eukaryotic cells. Essential to the proper development of cells and especially to their redox balance, glutathione synthesis requires the sequential action of gamma-glutamyl cysteine synthetase and glutathione synthetase, its failure leading *in fine* to death of the organism.[15]

Via this reaction sequence, glutathione can be added to *trans*-2-hexenal, a toxic messenger synthesized during environmental stress, whether biotic (*Botrytis cinerea*, *Agrobacterium tumefaciens*, mildew…) or abiotic (water stress, oxidation,

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Table 1. Concentrations of thiol precursors in (A) grapes and (B) hops in mg/kg, found in literature.[8–12]

			Concentrations (mg/kg)						
	Matrix		Cys-3SPol	Cys-3SHol	Cys-4S4M2Pone	G-3SPol	G-3SHol	G-3S4MPol	G-4S4M2Pone
A. Grapes	Sauvignon blanc		N.A.	d-15.86	d-0.01	N.A.	d-0.64	N.A.	d
	Semillon		N.A.	d-0.69	N.A.	N.A.	N.A.	N.A.	N.A.
	Chardonnay		N.A.	d-0.04	N.A.	N.A.	0.11-0.52	N.A.	N.A.
	Riesling		N.A.	d-0.03	d	N.A.	d-0.27	N.A.	d
	Pinot Grigio		N.A.	d-0.03	N.A.	N.A.	0.34-0.47	N.A.	N.A.
	Melon B.		N.A.	d	d	N.A.	d	N.A.	nd
	Gewurztraminer		N.A.	0.05-0.06	d	N.A.	d	N.A.	d
	Petite Arvine		N.A.	d-0.08	N.A.	N.A.	N.A.	N.A.	N.A.
	Cabernet Sauvignon	Must	N.A.	d	N.A.	N.A.	N.A.	N.A.	N.A.
		Skins	N.A.	d	N.A.	N.A.	N.A.	N.A.	N.A.
	Merlot	Must	N.A.	d	N.A.	N.A.	N.A.	N.A.	N.A.
		Skins	N.A.	0.03	N.A.	N.A.	N.A.	N.A.	N.A.
	Koshu	Must	N.A.	d	N.A.	N.A.	d-0.04	N.A.	N.A.
		Skins	N.A.	d	N.A.	N.A.	d-0.06	N.A.	N.A.
		Leaves	N.A.	nd-0.61	N.A.	N.A.	d-1.22	N.A.	N.A.
B. Hops	Amarillo		d	2.10*	N.A.	7.50	101.00	nd	N.A.
	Citra		d	0.30*	N.A.	18.10	91.00	nd	N.A.
	Hallertau Blanc		d	1.30*	N.A.	3.00	77.10	0.30	N.A.
	Nelson Sauvin		d	0.20*	N.A.	1.40	20.10	d	N.A.
	Polaris		0.20	4.90*	N.A.	9,80	118.20	3.60	N.A.
	Saaz		d	0.20-0.40*	d	2.50	5.11-95.70	d	nd
	Chinook		N.A.	0.53	d	N.A.	4.48	N.A.	d
	Barbe Rouge		N.A.	1.14	d	N.A.	1.28	N.A.	nd
	Cascade		N.A.	0.43	d	N.A.	19.06	N.A.	nd
	Mistral		N.A.	0.22	nd	N.A.	2.84	N.A.	nd
	Aramis		N.A.	0.56	d	N.A.	2.36	N.A.	nd
	Strisselspalt		N.A.	0.48	0.05	N.A.	9.14	N.A.	nd
	Mandarina Bavaria		0.11	0.09-0.54	d	1.80-4.30	2.11-15.00	nd	nd

N.A.: not analyzed; nd: not detected (<0.01 mg/kg in grape; <0.02 mg/kg in hop); d: detected at trace levels (up to 3 times the detection limit); *Cys-3SHol + Cys-3S4MPol.

UV…).[16] Nucleophilic addition catalyzed by glutathione-S-transferase (GST) leads to glutathionylated 3-sulfanylhexanal (G-3SHal), which can be reduced to glutathionylated 3-sulfanylhexanol (G-3SHol) and possibly esterified. Probably all these adducts can be catabolized by γ-glutamyl transpeptidase (GGT) and carboxypeptidase, removing successively (in one way or another) the γ-glutamyl and glycine moieties so that finally the Cys-adduct is excreted from the cell.

As mentioned above, G- and Cys-precursors of various sulfanylalkyl alcohols (and one ketone) have already been investigated in grapes and hops. On the other hand, only one sulfanylalkyl aldehyde (G-3SHal identified as precursor of 3SHol in Sauvignon Blanc)[17] and no ester *S*-conjugates have been investigated so far. Yet free 3-sulfanylheptanal (3SHptal) and 3-sulfanyl-3-methylbutanal (3S3MBal) have been found in Sauternes wines, while 3-sulfanylpentanal (3SPal) and 3-sulfanylhexanal (3SHal) have recently been evidenced in Chardonnay and Solaris Belgian wines.[18,19] In hops, 3SPal, 3SHal, 3SHptal, and 3-sulfanyloctanal (3SOal) have been identified.[20] In beer, furthermore, it has been demonstrated that bottle refermentation leads to the release of free 3SHal.[21]

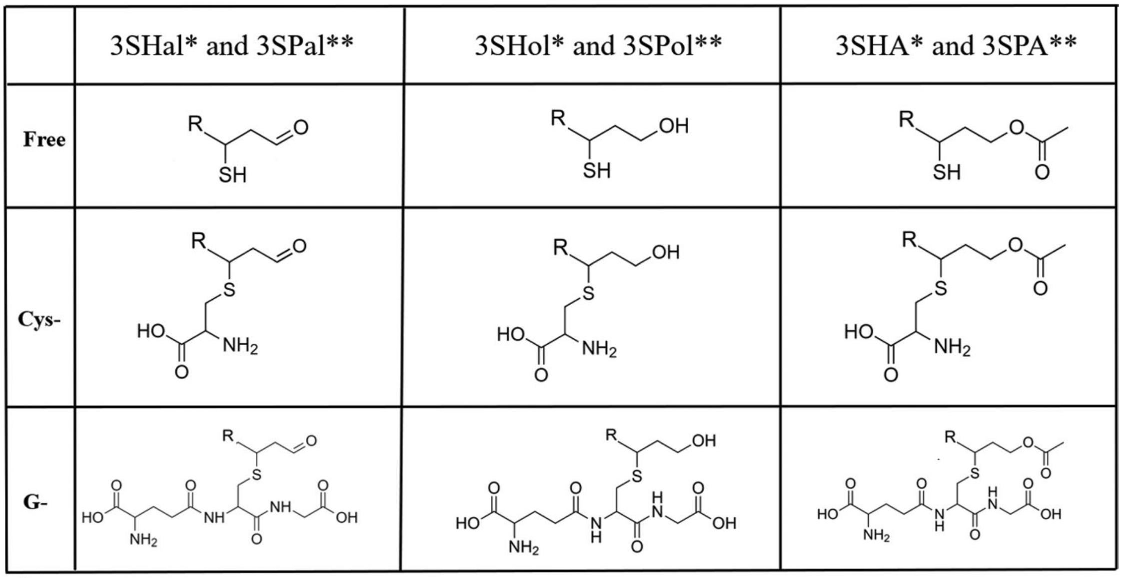
Likewise, several free sulfanylalkyl acetates including 3-sulfanylhexyl acetate (3SHA) and 3-sulfanylpropyl acetate (3SPrA) have been found in Belgian wines and beers.[19,22] It is known, however, that yeast can acetylate free sulfanylalkyl alcohols through fermentation, generating, for instance, organoleptically active 3SHA in both beers and wines.[5]

The aim of the present work was to investigate four new Cys-adducts with the aldehydes 3SPal and 3SHal and the esters 3SPA and 3SHA, along with three of their glutathionylated counterparts: G-3SPal, G-3SHal, and G-3SHA (Figure 1). After their chemical synthesis and after confirming their structures by nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS), we quantified them, using the direct HPLC-ESI(+)-MRM method and an indirect method (GC-PFPD analysis of apotryptophanase-released thiols) to quantify them in three varieties of grapes (one *Vitis vinifera* - Chardonnay, two interspecific grape varieties - Solaris and Johanniter), and ten hop cultivars (Amarillo, Citra, Columbus, Hallertau Blanc, Mandarina Bavaria, Mosaic, Nelson Sauvin, Polaris, Saaz, Sorachi Ace).

Experimental

Chemicals

Absolute ethanol, acetonitrile, Amberlite IR-120 resin, 28% ammonia, dichloromethane, diethylether, ethyl acetate, formic acid, 37% hydrochloric acid, magnesium sulfate, silica, glacial acetic acid, cyclohexane, and methanol were purchased from VWR (Leuven, Belgium). Anhydrous acetonitrile, cesium carbonate, deuterium oxide, >98% reduced L-glutathione, *trans*-2-pentenal, *trans*-2-hexenal, S-benzyl-L-cysteine, pyridine, and sodium borohydride were purchased from Sigma-Aldrich (Bornem, Belgium). Anhydrous sodium sulfate and sodium hydroxide were purchased from Acros Organics (Geel, Belgium). N-Boc-L-cysteine, monosodium phosphate, trifluoroacetic acid, and disodium phosphate were obtained from Merck (Darmstadt, Germany). Acetic anhydride was purchased from Alfa Aesar (Haverhill, Massachusetts, USA). Milli-Q water was used (Millipore, Bedford, MA, U.S.A.). The 10 g C18 Sep-Pak cartridges were purchased from Waters Millipore (Milford, MA, U.S.A.).



*R=$CH_2CH_2CH_3$
**R= CH_2CH_3

Figure 1. Chemical structures of free, cysteinylated, and glutathionylated 3SHal, 3SPal, 3SHol, 3SPol, 3SHA, and 3SPA.

Hop samples

Vacuum-packed T90 hop pellets (7 – 9% moisture) from different varieties, harvest years, and countries were arbitrarily selected and stored at −20 °C until used. Saaz (Czech Republic, harvest 2019; α acids: 4.3%; oil content: 0.7%) and Mosaic (U.S.A., harvest 2019; α acids: 11.0%; oil content: 1.3%) were provided by Brouwland (Belgium); Hallertau Blanc (Germany, harvest 2015; α acids: 11.3%; oil content: 1.2%), Mandarina Bavaria (Germany, harvest 2019; α acids: 8.1%; oil content: 2.1%) and Polaris (Germany, harvest 2019; α acids: 19.1%; oil content: 3.6%) were provided by Hopsteiner (Germany); Nelson Sauvin (New Zealand, harvest 2018; α acids: 12.4%; oil content: 1.1%) was provided by Brouwerij Anders!; Amarillo (U.S.A., harvest 2015; α acids: 9.2%; oil content: 1.7%), Citra (U.S.A., harvest 2017; α acids: 12.3%; oil content: 2.3%), Columbus (U.S.A., harvest 2019; α acids: 17.3%; oil content: 2.7%), and Sorachi Ace (U.S.A., harvest 2015; α acids: 13.5%; oil content: 2.3%) were provided by Yakima Chief (Belgium).

Grape samples

Grapes of different varieties were sampled at the end of September 2020. The grape berries were crushed with a manual press and the resulting must was stored at −20 °C until used. Chardonnay was collected at the Domaine de la Ferme du Chapitre (Baulers, Belgium) while Solaris and Johanniter were collected at the Domaine du Chenoy (Émines, Belgium).

^{1}H NMR spectra of synthesized S-conjugates

To confirm the chemical structure of the synthesized products, they were analyzed by ^{1}H NMR. After dissolution of the product in deuterium oxide, ^{1}H NMR spectra were recorded on a Bruker Avance II 300 spectrometer operating at 300 MHz. After single-pulse NMR experiments, the resulting data were processed with Bruker TopSpin software (version 2.1). All chemical shifts (δ) are reported in parts per million relative to the reference [tetramethylsilane (TMS)]. The ^{1}H NMR spectra are detailed in the supplemental material online with this article.

HRMS of synthesized S-conjugates

To confirm the molecular formulas of the synthesized products, they were analyzed by HRMS. Mass spectrum determinations were performed with a Q-Exactive Orbitrap Exactive mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ion source operating in positive mode (ESI+). The ESI inlet conditions were as follows: source voltage, 4.5 kV; capillary temperature, 320 °C; sheath gas (nitrogen), 20 psi; and auxiliary gas (nitrogen) flow rate, 5 mL/min. Synthesized compounds were solubilized in water and directly introduced into the mass spectrometer controlled with Xcalibur software, version 2.0.7 (Thermo Fisher Scientific). The measured and calculated masses are given in daltons at the end of each synthesis section.

Synthesis of cysteinylated sulfanylalkyl aldehydes

Michael addition of N-Boc-L-cysteine (500 mg, 2.26 mmol, 0.9 equiv) to *trans*-2-pentenal or *trans*-2-hexenal (2.51 mmol, 1 equiv) was performed overnight (16 hours) under anhydrous conditions with anhydrous acetonitrile as solvent (7 mL) and in the presence of cesium carbonate (350 mg, 1.13 mmol, 0.45 equiv). The solvent was then evaporated under reduced pressure. The solid residue was dissolved in 10 mL water. The aqueous solution was extracted three times with 25 mL ethyl acetate. The combined organic phases were washed with 25 mL water, dried over sodium sulfate, and concentrated under reduced pressure. Deprotection of amines was achieved by reaction with HCl 4 M (5 ml) in 10 mL water (deprotection with TFA having proved to be too aggressive for the aldehyde function). After evaporation of the solvent under reduced pressure, the final product was obtained.

For Cys-3SHal, the final product is a brown, sticky solid; yield: 33%. ^{1}H NMR (300 MHz, deuterium oxide) (supplemental material 4): δ 0.89 (t, 3H, –CHCH$_2$CH$_2$**CH**$_3$), 1.40 (m, 2H, –CHCH$_2$**CH**$_2$CH$_3$), 1.61 (m, 2H, –CHC**H**$_2$CH$_2$CH$_3$), 2.23 (m, 2H, –**CH**$_2$CHO), 2.89 (m, 1H, -C**H**S-), 3.17 (m, 2H, –**CH**$_2$S–), 4.20 (m, 1H, α **CH** in Cys), 9.66 (t, $J = 2$ Hz, 1H, –CH$_2$C**H**O); MS(ESI+) m/z $[M+H]^+$ 220; HRMS(ESI+) m/z calculated for $C_9H_{17}O_3NS$, 220.10019 Da; found, 220.10019 Da.

For Cys-3SPal, the final product is a brown, sticky solid; yield: 29%. ^{1}H NMR (300 MHz, deuterium oxide) (supplemental material 5): δ 0.89 (t, 3H, –CHCH$_2$**CH**$_3$) 1.47-1.77 (m, 2H, –CHC**H**$_2$CH$_3$), 2.25 (m, 2H, –**CH**$_2$CHO), 2.87 (m, 1H, -C**H**S-), 3.14 (m, 2H, –**CH**$_2$S–), 4.21 (m, 1H, α **CH** in Cys), 9.58 (t, $J = 2$ Hz, 1H, –CH$_2$C**H**O); MS(ESI+) m/z $[M+H]^+$ 206; HRMS(ESI+) m/z calculated for $C_8H_{15}O_3NS$, 206.08461 Da; found, 206.08454 Da.

Synthesis of cysteinylated sulfanylalkyl acetates

This synthesis was adapted from the method described by Starkenmann et al.[14] It was performed starting from the Boc-cysteine sulfanylalkyl alcohol (Boc-Cys-3SHol or Boc-Cys-3SPol obtained by the method described by Gros et al. and Chenot et al.) and diluted in 5 mL pyridine.[8,11] Esterification was carried out with 1 mL acetic anhydride under stirring at room temperature for 4 h. After this time, 25 mL toluene was added and the reaction medium was concentrated under reduced pressure. Deprotection of amines was achieved by reaction with TFA (3.5 ml) in 10 mL dichloromethane for 1 h. After deprotection, 25 mL toluene was added for azeotropic distillation and the reaction medium was concentrated under reduced pressure. For purification, a 10-g Sep-Pak C18 cartridge was used. After conditioning with 200 mL acetonitrile and 300 mL water, the sample diluted in 5 mL water was loaded on the cartridge. The cartridge was rinsed with 100 mL water before elution with 20-mL water-methanol solutions of increasing methanol concentration (10-20-30-40-50%). The extract was concentrated under reduced pressure to obtain the final product.

For Cys-3SHA, the final product is a translucent, oily solid; yield: 11%. ^{1}H NMR (300 MHz, deuterium oxide) (supplemental material 6): δ 0.89 (t, 3H, –CHCH$_2$CH$_2$**CH**$_3$), 1.42 and 1.57 (m, 4H, –CHCH$_2$**CH**$_2$CH$_3$ and – CHC**H**$_2$CH$_2$CH$_3$) 1.81-2.02 (m, 2H, –**CH**$_2$CH$_2$OCOCH$_3$), 2.08 (s, 3H, –CH$_2$CH$_2$OCOC**H**$_3$), 2.89 (m, 1H, -C**H**S-), 3.09 (m, 2H, -C**H**$_2$S-), 3.96 (m, 2H, –CH$_2$**CH**$_2$OCOCH$_3$), 4.24 (m, 1H, α **CH** in Cys); MS(ESI+) m/z $[M+H]^+$ 264; HRMS(ESI+) m/z calculated for $C_{11}H_{21}O_4NS$, 264.12632 Da; found, 264.12641 Da.

For Cys-3SPA, the final product obtained is a yellow oily solid; yield 13%. ^{1}H NMR (300 MHz, deuterium oxide) (supplemental material 7): δ 0.97 (t, 3H, –CHCH$_2$**CH**$_3$), 1.64 (m, 2H, –CHC**H**$_2$CH$_3$), 1.81 (m, 2H, –**CH**$_2$CH$_2$OCOCH$_3$), 2.09 (s, 3H, –CH$_2$CH$_2$OCOC**H**$_3$), 2.93 (m, 1H, -C**H**S-), 3.12 (m, 2H, -C**H**$_2$S-), 3.78 (m, 2H, –CH$_2$**CH**$_2$OCOCH$_3$), 4.23 (m, 1H, α **CH** in Cys); MS(ESI+) m/z $[M+H]^+$ 250; HRMS(ESI+) m/z calculated for $C_{10}H_{19}O_4NS$, 250.11070 Da; found, 250.11076 Da.

Synthesis of glutathionylated sulfanylalkyl aldehydes

The *trans*-2-pentenal or *trans*-2-hexenal was added in three portions (0.33 equiv at 3-h intervals) to a solution of glutathione (500 mg, 1.63 mmol, 1 equiv) in phosphate buffer (monosodium phosphate/disodium phosphate, 1 M, pH 8, 10 mL). The reaction mixture was stirred for 10 h (after the first addition) at room temperature. The reaction medium was then concentrated under reduced pressure. For purification, the solid residue dissolved in 5 mL water was filtered on a silica layer. The extract was then dried under reduced pressure to obtain the final product.

For G-3SHal, the final product is a yellow solid; yield: 41%. ^{1}H NMR (300 MHz, deuterium oxide) (supplemental material 8): δ 0.87 (t, 3H, –CHCH$_2$CH$_2$**CH**$_3$), 1.27-1.65 (m, 4H, –CHCH$_2$**CH**$_2$CH$_3$ and – CHC**H**$_2$CH$_2$CH$_3$), 1.75 (m, 2H, –**CH**$_2$CHO), 2.01 (m, 2H, β **CH**$_2$ in Glu), 2.48 (m, 2H, γ **CH**$_2$ in Glu), 2.62-3.15 (m, 3H, -C**H**$_2$S- and -C**H**S-), 3.72 (m, 3H, α **CH**$_2$ in Gly and α **CH** in Glu), 4.49 (m, 1H, α **CH** in Cys), 9.27 (t, 1H, –CH$_2$C**H**O). MS(ESI+) m/z $[M+H]^+$ 406; HRMS(ESI+) m/z calculated for $C_{16}H_{27}O_7N_3S$, 406.16409 Da; found, 406.16425 Da.

For G-3SPal, the final product is an orange solid; yield: 53%. ^{1}H NMR (300 MHz, deuterium oxide) (supplemental material 9): δ 0.92 (t, 3H, –CHCH$_2$**CH**$_3$), 1.43-1.91 (m, 4H – CHC**H**$_2$CH$_3$ and – **CH**$_2$CHO), 2.11 (m, 2H, β **CH**$_2$ in Glu), 2.48 (m, 2H, γ **CH**$_2$ in Glu), 2.88 (m, 3H, -C**H**$_2$S- and -C**H**S-), 3.70 (m, 3H, α **CH** in Glu and α **CH**$_2$ in Gly), 4.49 (m, 1H, α **CH** in Cys), 9.25 (t, 1H, –CH$_2$C**H**O). MS(ESI+) m/z $[M+H]^+$ 392; HRMS(ESI+) m/z calculated for $C_{15}H_{25}O_7N_3S$, 392.14834 Da; found, 392.14860 Da.

Synthesis of glutathionylated sulfanylalkyl acetates

The synthesis was performed starting from glutathionylated sulfanylalkyl alcohol (G-3SHol obtained by the method described by Kankolongo Cibaka et al.) and diluted in 5 mL acetonitrile.[23] Reflux Fischer esterification was performed at 80 °C for 4 h with glacial acetic acid (3 equiv) and with a drop of 37% HCl for acid catalysis. The reaction medium was then concentrated under reduced pressure. The residual acetic acid was removed via azeotropic distillation with 25 mL cyclohexane to obtain the final product. Two other methods were tested: a Steglich esterification and an esterification with acetic anhydride in pyridine, with protection of the amino group of glutathione by Boc_2O. As these two techniques failed to yield the desired products, they are not detailed here.

The final product (G-3SHA) is a translucent solid; yield: 8%. ^{1}H NMR (300 MHz, deuterium oxide) (supplemental material 10): δ 0.88 (t, 3H, $-CHCH_2CH_2C\mathbf{H}_3$), 1.22 (m, 2H, $-CHCH_2C\mathbf{H}_2CH_3$), 1.54 (m, 2H, $-CHC\mathbf{H}_2CH_2CH_3$), 1.90 (m, 2H, $-C\mathbf{H}_2CH_2OCOCH_3$), 2.00 (s, 3H, $-CH_2CH_2OCOC\mathbf{H}_3$), 2.10 (m, 2H, β C$\mathbf{H}_2$ in Glu), 2.5 (m, 2H, γ C$\mathbf{H}_2$ in Glu), 2.76 (m, 1H, -C**H**S-), 3.02 (m, 2H, -C$\mathbf{H}_2$S-), 3.9 (s, 2H, α C$\mathbf{H}_2$ in Gly), 4.16 (m, 2H, $-CH_2C\mathbf{H}_2OCOCH_3$), 4.35 (m, 1H, α C**H** in Glu), 4.56 (m, 1H, α C**H** in Cys); MS(ESI+) m/z $[M+H]^+$ 450; HRMS(ESI+) m/z calculated for $C_{18}H_{31}O_8N_3S$, 450.19057 Da; found, 450.19046 Da.

Extraction of cysteine and glutathione S-conjugates from hop pellet samples

The compound S-benzyl-L-cysteine was used as the internal standard (IST) at 8 mg/kg hop. The extraction method is identical to that referred to by Chenot et al[8,24] (solid/liquid extraction with ethanol 20% v/v, centrifugation, cation-exchange resin, elution with ammonia solutions of increasing concentrations, concentration under vacuum). The obtained extract was dissolved in 2 mL of 0.1% v/v formic acid aqueous solution.

Extraction of cysteine and glutathione S-conjugates from grape must samples

The compound S-benzyl-L-cysteine was again used as the internal standard (IST), at 4 mg/kg for HPLC and 0.5 mg/kg for GC analysis. One kilogram of grapes was pressed to obtain at least 200 mL must. Two hundred milliliters of must was accurately measured and formic acid was added to 1% v/v final concentration along with the IST. This solution was loaded onto the column of IR-120 cation exchange resin. The next steps are identical to those used for the hop samples.

Reversed-phase high-performance liquid chromatography – positive electrospray ionization [RP-HPLC – ESI(+)-MRM] on a Hypersil GOLD aQ column and on astec cyclobond I 2000 RSP

Analyses were performed on a 100 × 2.1 mm, 3 µm Hypersil GOLD aQ column, a polar end-capped C18 phase offering superior retention of polar compounds (Thermo Fisher Scientific). The elution solvents were water (solvent A) and acetonitrile (solvent B), both containing 0.1% v/v formic acid.

The previously published gradient was used for elution of most Cys- and G-adducts: 100% solvent A for 10 min, from 100 to 98.6% in 15 min, maintained for 5 min, from 98.6 to 85% in 20 min, decrease to 10% in 1 min, 10 min of washing, and back to the original conditions in 5 min for 15 min (called Program A).[8] Yet as Cys- and G-sulfanylalkyl esters were eluted during the washing step of this elution program, programs B and C (see below) were used, respectively, for these compounds (both with a higher concentration of acetonitrile).

Program B: 99% solvent A for 5 min, from 99% to 92% in 10 min, maintained for 10 min, from 92% to 10% in 11 min, 10 min of washing, and back to the original conditions in 4 min for 15 min; program C: 92% solvent A for 30 min, from 92% to 10% in 1 min, 10 min of washing, and back to the original conditions in 4 min for 15 min.

The flow rate was set at 0.35 mL/min for all the elution programs. A total of 10 µL sample was injected into the column at 50 °C. A system equipped with an autosampler and a quaternary pump (Agilent Technologies, 1200 Series) was used. The system was controlled with Agilent ChemStation software. The parameters of the mass spectrometer are identical to those detailed by Chenot et al.[8]

To distinguish G-3SHol from G-3S4MPol (present in a few hop varieties), the Astec Cyclobond I 2000 RSP chiral column was used with the parameters described in a previous work.[8]

Direct HPLC quantification of cysteine and glutathione S-conjugates in hop and grape extracts

Calibration curves of conjugates relative to IST were determined for compounds detected in quantifiable quantities. Water with 0.1% formic acid, 5 – 10 – 15 – 20 – 25 mg/kg of synthesized conjugate, and 25 mg/kg IST were used to plot the linear curves (area ratio versus concentration ratio). The obtained slopes gave the conjugate-to-IST response coefficient ratio ($R^2 > 0.97$). The following equation was used for each conjugate quantitation: concentration of the conjugate in the extract (mg/kg) = concentration of IST in the extract (mg/kg) × (conjugate peak area/IST peak area) × (response coefficient of IST/response coefficient of conjugate). All extractions were performed in duplicate.

Indirect GC-PFPD analysis after hydrolysis with apotryptophanase

Apotryptophanase treatment was applied as described by Chenot et al. [24] One microliter of extract was analyzed with an Agilent 6890 N gas chromatograph equipped with a splitless injector maintained at 250 °C. Analyses were carried out on a wall-coated tubular (WCOT) apolar CP-Sil5-CB capillary column (50 m x 0.32 mm i.d., 1.2 µm film thickness). The carrier gas was helium, and the pressure was set at 90 kPa. The oven temperature was programmed to rise from 36 to 85 °C at 25 °C per min, then to 145 °C at 1 °C per min, and finally to 220 °C at 3 °C per min, held for 30 min. The column was connected to the OI Analytical PFPD detector (model 5380) with the following parameters: temperature, 250 °C; voltage, 600 V; gate width, 18 ms; gate delay, 6 ms; trigger level, 400 mV; pulse frequency, 3.33 Hz. PFPD chromatograms were recorded throughout elution; ChemStation software was used to process the resulting data.

For quantification of bound X, the following equation was used: concentration of X (µg/L) = concentration of IST (µg/L) × (free X peak area/free IST peak area) × (bound X molecular weight/bound IST molecular weight) × (free IST molar response coefficient/free X molar response coefficient) × (IST recovery factor/X recovery factor).[19]

Results and discussion

Synthesis of sulfanylalkyl aldehyde S-conjugates

Cys-3SHal

As detailed in Figure 2A, the pseudo-molecular ion (m/z 220), the ion corresponding to loss of NH_3 (m/z 203), the pseudo-molecular ion of cysteine (m/z 122) and the carbocation of the volatile thiol (m/z 99) were observed in the MS/MS-ESI(+) spectrum. For fragments containing the aldehyde moiety, peak at m/z −1 is better observed, e.g., m/z 219 and 202. The m/z 202 fragment was used for RP-HPLC-ESI(+)-MRM quantitation in hop and in grape. A double peak (corresponding to the mixture of the two diastereomers) was observed at 9-13 min after injection onto the Hypersil GOLD aQ column and elution according to Program A.

Cys-3SPal

The mass spectrum is shown in supplemental material 1 online. The fragmentation pathway was similar to that of Cys-3SHal, with an m/z difference of 14 (one CH_2 less) for the fragments containing the thiol moiety (fragments corresponding to the amino acid moiety fragmentation are identical). The m/z 188 fragment was used for RP-HPLC-ESI(+)-MRM quantitation. A peak was observed at 4.5-6.5 min after injection onto the Hypersil GOLD aQ column (Program A).

G-3SHal

As shown in Figure 2B, the MS/MS-ESI(+) mass spectrum was characterized by the pseudo-molecular ion (m/z 406), the ion corresponding to glycine loss (m/z 331), the ion corresponding to glutamyl and NH_3 loss (m/z 260), the dipeptide cysteinylglycine ion (m/z 162) and the carbocation of the volatile thiol (m/z 99). Similarly to its cysteinylated counterpart, for fragments containing the aldehyde moiety, a higher peak at m/z −1 is observed (m/z 405, 330 and 259). The m/z 259 fragment was used for RP-HPLC-ESI(+)-MRM quantitation. A double peak was observed at 35-38 min after injection onto the Hypersil GOLD aQ column (Program A).

G-3SPal

The mass spectrum is shown in supplemental material 3 online. The fragmentation pathway was similar to that of G-3SHal, with an m/z difference of 14 (one CH_2 less) for the fragments containing the thiol moiety (fragments corresponding to the peptide moiety fragmentation were identical). The m/z 246 fragment was used for RP-HPLC-ESI(+)-MRM quantitation. A peak was observed at 10.5-14.5 min after injection onto the Hypersil GOLD aQ column (Program A).

Synthesis of sulfanylalkyl acetate S-conjugates

Cys-3SHA

In the MS/MS-ESI(+) fragmentation detailed in Figure 2C, were found the pseudo-molecular ion (m/z 264), the ion corresponding to loss of NH_3 (m/z 247), the carbocation of the volatile thiol (m/z 143) and the pseudo-molecular ion of cysteine (m/z 122). The m/z 247 fragment was used

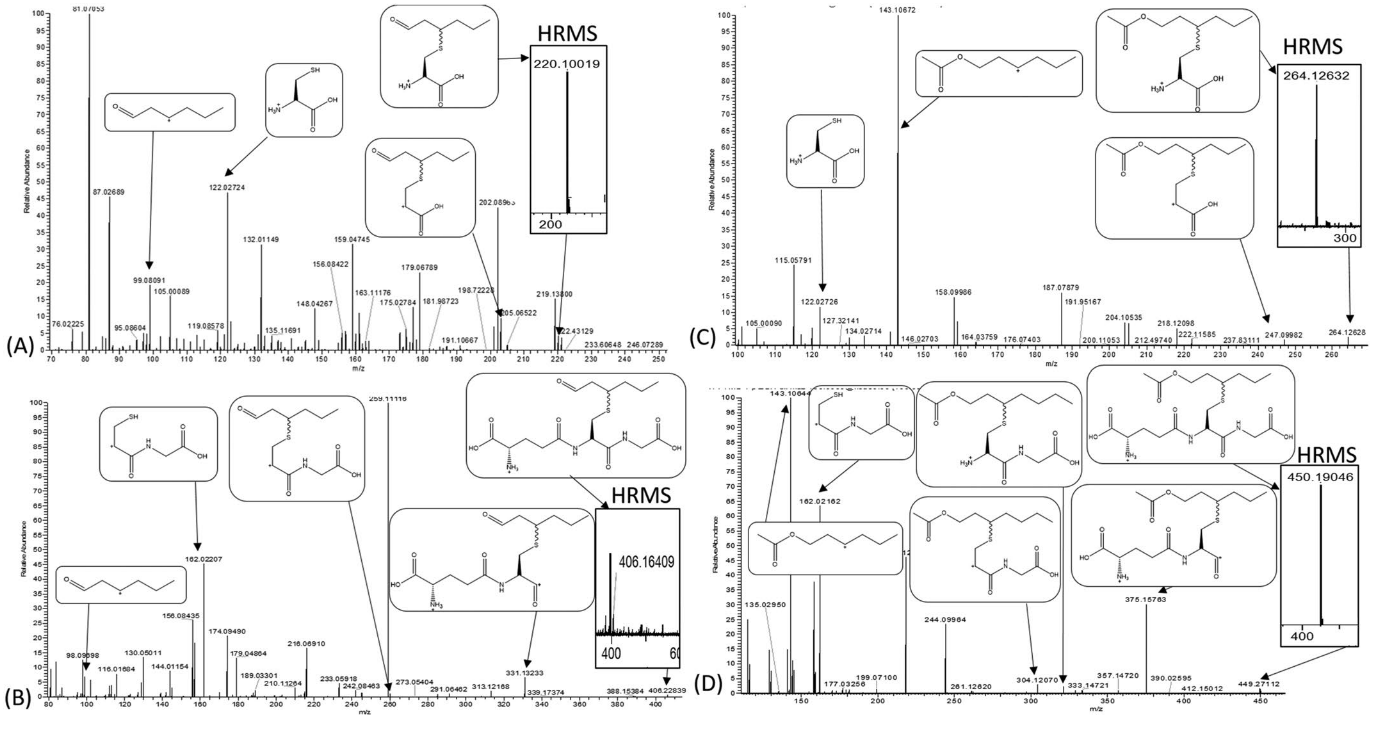


Figure 2. MS/MS-ESI(+) and HRMS mass spectra of (A) Cys-3SHal; (B) G-3SHal; (C) Cys-3SHA; (D) G-3SHA.

for RP-HPLC-ESI(+)-MRM quantitation. A peak was observed at 21-23.5 min after injection onto the Hypersil GOLD aQ column (Program B).

Cys-3SPA

The mass spectrum is depicted in supplemental material 2 online. The fragmentation pathway was similar to that of Cys-3SHA, with an m/z difference of 14 (one CH_2 less) for the fragments containing the thiol moiety (fragments corresponding to the amino acid moiety fragmentation were identical). The m/z 233 fragment was used for RP-HPLC-ESI(+)-MRM quantitation. A peak was observed at 14.5-16.5 min after injection onto the Hypersil GOLD aQ column (Program B).

G-3SHA

As shown in Figure 2D, the MS/MS-ESI(+) spectrum contained the pseudo-molecular ion (m/z 450), the ion corresponding to glycine loss (m/z 375), and the ion corresponding to glutamyl loss (m/z 321), which can further lose an NH_3 (resulting m/z 304), the dipeptide cysteinylglycine ion (m/z 162) and the carbocation of the volatile thiol (m/z 143). The m/z 321 fragment was used for RP-HPLC-ESI(+)-MRM quantitation. A double peak was observed at 22-25 min after injection onto the Hypersil GOLD aQ column (Program C).

RP-HPLC-ESI(+)-MRM investigations of cysteinylated and glutathionylated sulfanylalkyl aldehydes in grapes and hops

As depicted in Table 2 and Figure 3, neither Cys-3SPal nor Cys-3SHal was detected in any of the hop extracts. On the other hand, G-3SPal was detected (signal/noise > 3) and quantified (signal/noise > 9) in the Citra, Mandarina Bavaria, Mosaic, Nelson Sauvin, and Saaz hop varieties (2.95 to 15.37 mg/kg). These concentrations are within the range of G-3SPol, while G-3SHol is always much more concentrated (close to 100 mg/kg in Amarillo, Citra, Hallertau Blanc, Polaris). Since in plant, glutathionylated sulfanylalkyl aldehydes (resulting from glutathione addition on a toxic alpha, bêta-unsaturated compound) are at the top of every possible biotransformation pathway, we suspect that a huge amount of G-3SHol is obtained through extensive reduction of the aldehyde precursor at the expense of its content. This probably explains why G-3SHal was detected only in trace amounts (below the quantitation limit) in a few hop varieties (Mosaic, Nelson Sauvin, Polaris, and Saaz). Our quantifications are consistent with the fact that free 3SPal has been found in several hops, while 3SHal was usually absent (or hardly detected).[20]

In grapes, even though noticeable amounts of free 3SPal had been found in several Belgian wines, no trace of Cys- or G-3SPal was evidenced.[19] No trace of G-3SHal (nor Cys-3SHal) could neither be observed here (unlike in *Vitis vinifera* L. cv. Sauvignon Blanc).[17]

RP-HPLC-ESI(+)-MRM investigation of cysteinylated and glutathionylated sulfanylalkyl acetates in grapes and hops

Cys-3SPA was quantified (at 4.32 mg/kg) in Mosaic hop and detected as traces in Citra and Nelson Sauvin (Table 2 and Figure 4). This means that in the former, Cys-3SPA reaches the level of the ubiquitous Cys-3SHol. On the other hand, G-3SHA and Cys-3SHA were not detected in any of the hop samples, suggesting that beer 3SHA essentially originates from 3SHol esterification through fermentation.[9,18]

In grapes, neither Cys-3SPA nor Cys-3SHA was detected, while G-3SHA was hardly detected in Chardonnay and Johanniter extracts.

Levels of pre-synthesized cysteinylated and glutathionylated sulfanylalkyl alcohols in uninvestigated interspecific grape varieties

Cys-3SHol and G-3SHol were sought here, for the first time, in Solaris and Johanniter (two interspecific varieties), by

Table 2. Concentrations (mg/kg) of Cys- and G- sulfanylalkyl aldehydes and acetates in (A) grapes and (B) hops, determined by RP-HPLC-ESI(+)-MRM.

		Concentrations (mg/kg)						
		Adducts of sulfanylalkyl aldehydes				Adducts of sulfanylalkyl acetates		
Matrix		Cys-3SPal	Cys-3SHal	G-3SPal	G-3SHal	Cys-3SPA	Cys-3SHA	G-3SHA
A. Grapes	Chardonnay	nd	nd	nd	nd	nd	nd	d
	Johanniter	nd	nd	nd	nd	nd	nd	d
	Solaris	nd	nd	nd	nd	nd	nd	nd
B. Hops	Amarillo	nd	nd	nd	nd	nd	nd	nd
	Citra	nd	nd	11.51	nd	d	nd	nd
	Columbus	nd	nd	nd	nd	nd	nd	nd
	Hallertau Blanc	nd	nd	nd	nd	nd	nd	nd
	Mandarina Bavaria	nd	nd	10.04	nd	nd	nd	nd
	Mosaic	nd	nd	15.37	d	4.32	nd	nd
	Nelson Sauvin	nd	nd	13.88	d	d	nd	nd
	Polaris	nd	nd	d	d	nd	nd	nd
	Saaz	nd	nd	2.95	d	nd	nd	nd
	Sorachi Ace	nd	nd	nd	nd	nd	nd	nd

nd: not detected (<0.01 mg/kg in grape; <0.02 mg/kg in hop); d: detected at trace levels (up to 3 times the detection limit).

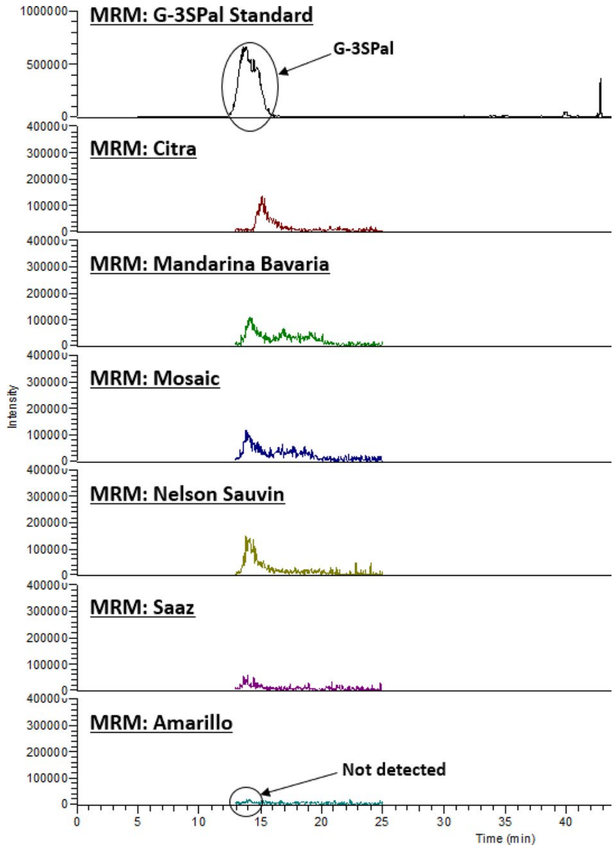


Figure 3. RP-HPLC-ESI(+)-MRM (m/z 392 → 245) of G-3SPal in Citra, Mandarina Bavaria, Mosaic, Nelson Sauvin, Saaz, and Amarillo hops with the Hypersil Gold aQ column.

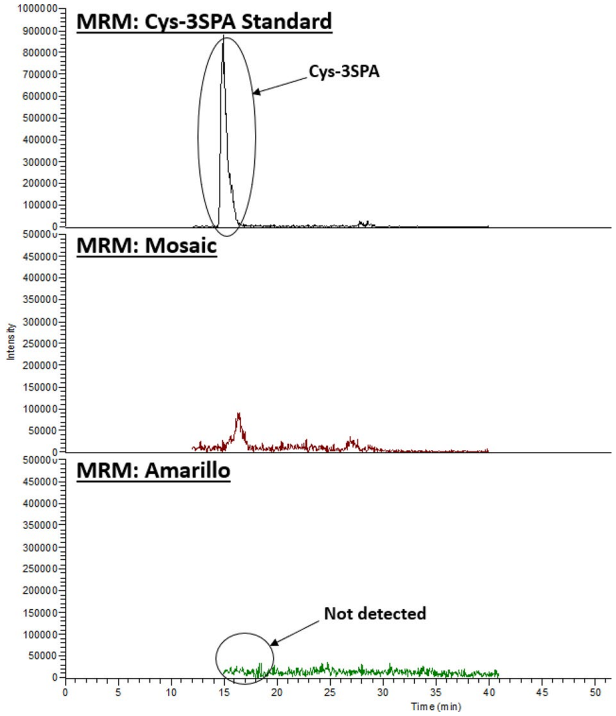


Figure 4. RP-HPLC-ESI(+)-MRM (m/z 250 → 233) of Cys-3SPA in Amarillo and Mosaic hops with the Hypersil Gold aQ column.

comparison with a pure *Vitis vinifera* Chardonnay from Belgium (Table 3). In the latter, Cys-3SHol and G-3SHol were found within higher ranges (factor 10) than those reported in literature (0.42 mg/kg Cys-3SHol versus 0.01-0.04 mg/kg in French Chardonnay and 4.42 mg/kg G-3SHol versus 0.11-0.52 mg/kg). Since 3SHol and 3S4MPol adducts co-elute from the Hypersil Gold aQ column, the apotryptophanase method confirmed that the extracts were free of Cys-3S4MPol and that our quantification by RP-HPLC-ESI(+)-MRM was accurate. Moreover, HPLC with the Cyclobond column confirmed the absence of G-3S4MPol in grapes.[8,24]

As shown in Figure 5, our Belgian Chardonnay sample remained the richest in G- and Cys-3SHol. On the other hand, first evidence of G-3SPol in grapes was obtained here with Johanniter and Solaris.

Just like in hops, it appears that S-conjugates profiles of grapes are highly variety-dependent. As precursors are, in both plants, secondary metabolites resulting from biotic or abiotic stresses, geographic localization could also impact their content.

Conclusion

Synthesis of G-3SHal and G-3SPal has enabled us to evidence these compounds for the first time in a natural matrix. In contrast to what is observed for their sulfanylalkyl alcohol counterparts, G-3SPal was found in more hop samples (Citra, Mandarina Bavaria, Mosaic, Nelson Sauvin, Polaris, and Saaz, up to 15 mg/kg) than G-3SHal (only in the four latters, between 0.02 and 0.05 mg/kg). On the other hand, no cysteinylated precursors of the sulfanylalkyl aldehydes 3SHal and 3SPal were found, whatever the hop cultivar. As for the sulfanylalkyl acetates, traces of Cys-3SPA were evidenced in Citra, Mosaic, and Nelson Sauvin (up to 4 mg/kg). Therefore, in beer, thanks to its low sensory threshold, released 3SPA could enhance the impact of this compound also produced by yeast in beer through sulfanylalkyl alcohol esterification.[25]

This first investigation of polyfunctional thiol precursors in interspecific grape varieties (7 mg/kg G-3SPol, 3 mg/kg G-3SHol and traces of G-3SHA in Johanniter) suggests that optimization of the harvest period (the later the harvest, the more cysteinyl S-conjugates while the opposite is true for hops)[16,26] could be an efficient way to improve the quality of emerging Belgian white wines. Complementary investigations are needed to quantify Cys- and G-3SPal, -3SHal, -3SPA, and -3SHA in other grape and hop varieties and to identify the yeast strains most able to release free flavors from them (known to be adduct-dependent).[25]

Disclosure statement

No potential conflict of interest was reported by the authors.

Abbreviations

G-: Glutathionylated; Cys-: Cysteinylated; 3SHal: 3-sulfanylhexanal; 3SPal: 3-sulfanylpentanal; 3SHol: 3-sulfanylhexanol; 3SPol: 3-sulfanylpentanol; 3SHA: 3-sulfanylhexyl acetate; 3SPA: 3-sulfanylpentyl acetate

Table 3. Concentrations (mg/kg) of Cys-3SHol, G-3SPol and G-3SHol in grapes, determined by RP-HPLC-ESI(+)-MRM (and confirmed by apotryptophanase-GC-PFPD for Cys-3SHol).

	Concentrations (mg/kg) Adducts of sulfanylalkyl alcohols		
Grapes	Cys-3SHol	G-3SPol	G-3SHol
Chardonnay	0.42	nd	4.42
Johanniter	0.26	7.33	3.06
Solaris	0.05	0.01	1.18

nd: not detected (<0.01 mg/kg)

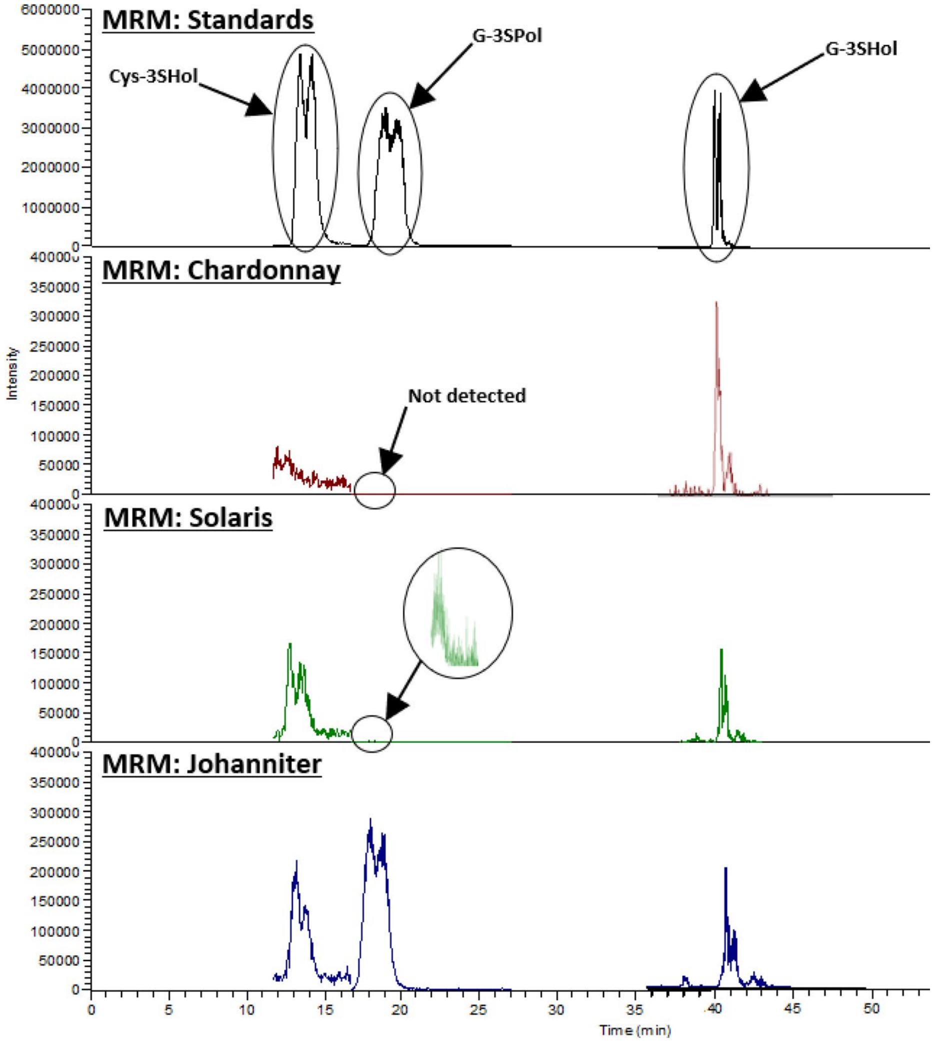


Figure 5. RP-HPLC-ESI(+)-MRM (m/z 222 → 205) for Cys-3SHol; (m/z 394 → 265) for G-3SPol and (m/z 408 → 262) for G-3SHol in Chardonnay, Solaris, and Johanniter grapes with the Hypersil Gold aQ column.

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