Investigation of 2-Sulfanylethyl Acetate Cysteine-*S*-Conjugate as a Potential Precursor of Free Thiols in Beer

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ABSTRACT

J. Am. Soc. Brew. Chem. 75(3):228-235, 2017

The compounds 2-sulfanylethyl acetate (2SEA, roasted- or burnt-like flavor) and 3-sulfanylpropyl acetate (also with roasted- or burnt-like flavor), mainly issued from the Ehrlich pathway (in this case, the catabolism by yeast of cysteine and homocysteine, leading to the formation of 2-sulfanylethan-1-ol and 2-sulfanylpropan-1-ol, which are subsequently acetylated), are found in all fresh beers, at levels strongly depending on the yeast strain used in fermentation. Yet, even in the absence of yeast, their production is still seen to occur in the bottle during the first months of storage. As for other thiols, *S*-cysteine adducts have been suspected to be hydrolyzed during beer aging. The commercially unavailable *S*-cysteine conjugate of 2SEA (Cys-2SEA, 93% purity), never described before in the literature, was synthesized by acetylation of the *S*-cysteine conjugate of 2-sulfanylethan-1-ol (Cys-2SEol). Cys-2SEA was solubilized in model aqueous media (pH 4.2) and in beer before aging. 2SEA was further extracted and analyzed by gas chromatography coupled to a pulsed flame photometric detector (GC-PFPD), and high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI(+)-MS/MS) allowed us to quantify the undegraded Cys-2SEA (specific *m/z* ions at 120, 148, and 191). Cys-2SEA was shown to be relatively stable in both media, although some beer constituents were noted as being able to slightly facilitate the chemical degradation (molar conversion into free thiol up to 0.3% at room temperature). A modified "Strecker degradation" mechanism is proposed here, explaining the release of thiols from the Schiff bases created between the cysteine adducts and beer diketones. Up to now, tests have failed to reveal evidence for the presence of Cys-2SEol and Cys-2SEA in hops by direct HPLC-ESI(+)-MS/MS analyses, although 2SEol was released from cysteine-*S*-conjugate extracts in the presence of apotryptophanase.

Keywords: Beer aging, Citra hops, Cysteine adducts, Polyfunctional thiols, Ehrlich pathway

Hops contain a large number of odorant polyfunctional thiols (41 reported to date) (1,2). These compounds are composed of a sulfhydryl (SH) function together with an additional chemical group (alcohol, aldehyde, or ester function), often separated by three carbon atoms. Yet it is not the case for 2-sulfanylethanol (2SEol) and its ester 2-sulfanylethyl acetate (2SEA). Huge variations in the concentrations of 2SEol, 2SEA, 3-sulfanylpropan-1-ol (3SProl), and 3-sulfanylpropyl acetate (3SPrA), all with empyreumatic (roasted/grilled/gas/burnt-like) flavors, have been observed between hop varieties, with the highest values measured for the Citra dual-purpose cultivar (up to 867 µg/kg of free 2SEA) (5).

2SEol, 3SProl, and their corresponding acetates were recently investigated in 14 commercial Belgian beers (14). The Ehrlich pathway (in this case, the catabolism by yeast of cysteine and homocysteine, leading to the formation of both alcohols that are subsequently acetylated) was suspected to contribute to their content in fresh beer (14). Yet synthesis of 2SEA and 3SPrA continued during the first three months of storage even when yeast was absent from the investigated bottles. Therefore, chemical degradation of precursors coming from raw materials was suggested.

Cysteine-*S*-conjugates have been shown to be involved in the thiol potential of hops, together with glutathione adducts (2–5). Such precursors have been extensively studied in other matrices such as onion (8), bell pepper (9), and grape (12,13). The cysteine and glutathione *S*-conjugates of 3-sulfanylhexan-1-ol (Cys-3SHol and G-3SHol) have recently been identified by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI[+]-MS/MS) in the Amarillo, Mosaic, and Hallertau Blanc dual-purpose hop varieties (2,4), and the presence of other cysteine adducts has been demonstrated by analyzing hop purified hydroalcoholic extracts subjected to apotryptophanase β-lyase activity (3,5). These molecules explain why the volatile thiol profiles strongly differ from wort to beer. The major increase of free thiols takes place during fermentation (2).

The cysteine adduct of 2SEA has never been described in the literature, and the *S*-cysteine conjugate of the corresponding alcohol (Cys-2SEol), was only once reported, in *Petiveria alliacea* (6). The aim of the present work was first to design a synthetic pathway for producing the commercially unavailable Cys-2SEA from Cys-2SEol. After having characterized both cysteine adducts (reversed-phase HPLC [RP-HPLC] retention time, ESI[+] mass spectrum, and nuclear magnetic resonance [NMR]), the contents of 2SEA in three pilot beers produced with Amarillo or Citra hops (two distinct yeasts) were compared. The stability of Cys-2SEA was further determined in aqueous and beer media. Finally, the occurrence of Cys-2SEA and Cys-2SEol was investigated in the Amarillo and Citra cultivars by RP-HPLC-ESI(+)-MS/MS.

EXPERIMENTAL

Chemicals

p-Hydroxymercuribenzoic acid (*p*HMB), tris(hydroxymethyl)-aminomethane, *N*-boc-L-cysteine (98.5%), L-cysteine HCl (97%), 2-acetylthiophene (98%), 2SEA (number 38), 2-sulfanylethan-1-ol (number 36), 3-sulfanylpropyl acetate (number 1), 3-sulfanylpropan-1-ol (number 14), and 3-sulfanyl-3-methylbutan-1-ol (number 17) were purchased from Sigma-Aldrich (Bornem, Belgium). 4-Methoxy-2-methyl-2-butanethiol (internal standard, IST) was purchased from Endeavour (Daventry, U.K.). 4-Sulfanyl-4-methylpentan-2-one was purchased from Frutarom (Hartlepool, U.K.). 3-Methylbut-2-en-1-thiol (number 37), and 3-sulfanylhexan-1-ol (number 23) were obtained from Oxford Chemicals (Oxford, U.K.). A strongly basic Dowex resin 1 × 2, Cl^- form (Sigma-Aldrich) was stored in hydrogen chloride (0.1M). Anhydrous sodium sulfate was obtained from Merck (Darmstadt, Germany).

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http://dx.doi.org/10.1094/ASBCJ-2017-3276-01

2-Bromoethanol (97%) and sodium acetate were purchased from Acros Organics (Geel, Belgium). Acetyl chloride (98%) was supplied by Alfa Aesar (Schiltigheim, France). Ethanol (99.9%), acetone, acetonitrile, sodium hydroxide, hydrochloric acid (37%), and dichloromethane were obtained from VWR (Oud-Heverlee, Belgium). Glacial acetic acid (99–100%) was supplied by Chem-Lab (Zedelgem, Belgium). D_2O (99.9%) was purchased from Euriso-Top (Saint-Aubin, France). 1-Sulfanylpentan-3-ol (number 19), 3-sulfanylpentanal (number 28), 3-sulfanylpentan-1-ol (number 21), 3-sulfanylheptan-1-ol (number 25), and 3-sulfanylhexyl acetate (number 11) were produced by combinatorial synthesis according to the methods of Vermeulen et al. (16–18). 3-Sulfanyl-4-methylpentan-1-ol (number 26) was synthesized according to the method of Takoi et al. (10).

Hop Samples

Citra and Amarillo hops (harvest 2012 for beer production and harvest 2015 for hop analyses), bred in the United States, were provided by Yakima Chief (Louvain-la-Neuve, Belgium).

Syntheses of Cysteine Adducts

Synthesis of *S*-2-(1-hydroxyethyl)-cysteine (Cys-2SEol). The procedure used was adapted from the method of Shiraiwa et al. (7). L-Cysteine HCl (2.20 g) was dissolved in ethanol (15 mL) under argon atmosphere. NaOH aqueous solution (2M) was added to the cysteine solution dropwise to raise the pH to 7. A white precipitate was then formed and removed by filtration. NaOH aqueous solution (10 mL, 2M) was added to the cysteine solution. The mixture was stirred and cooled to 0°C. 2-Bromoethanol (1.4 mL) was then added dropwise over 15 min. After 3 h at 0°C, the reaction continued at room temperature for 24 h. HCl aqueous solution (6M) was then added dropwise to lower the pH to 6–7 at the end of the reaction. Acetone addition (2 × 80 mL) to the reaction mixture allowed the formation of a white precipitate. This product was recovered by filtration. Residual solvents were removed from the solid product by evaporation under reduced pressure.

Synthesis of *S*-2-(ethylacetate)-cysteine (Cys-2SEA). Synthesized Cys-2SEol (150 mg) was added to dichloromethane (5 mL) at 0°C with glacial acetic acid (62 µL) and acetyl chloride (155 µL). After 3 h at 0°C, the reaction was continued overnight at room temperature. Dichloromethane, acetic acid, and residual acetyl chloride were removed from the solid product by evaporation under reduced pressure.

^{1}H NMR Spectra of Synthesized Compounds

NMR spectra were recorded using the solvent D_2O on a Bruker Avance spectrometer (Bruker, Billerica, MA, U.S.A.) operating at 300 MHz. After simple pulse NMR experiments, 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).

Heat Treatments of Cys-2SEA in Model Media

Model media. Cys-2SEA (2.5 mg) was solubilized in 11 mL of sodium acetate buffer, pH 4.2. The samples were then incubated at 100°C for 1 h or at 40, 60, or 80°C for 5 days.

Liquid-liquid extraction procedure. Samples (10 mL) spiked with 33.5 µg/L of 4-methoxy-2-methyl-2-butanthiol (IST; spiking of 50 µL of the 6.7 mg/L stock solution) were extracted with 2 × 5 mL of dichloromethane. The organic phase was dried on anhydrous sodium sulfate. The sample was first concentrated to 0.5 mL in a Kuderna-Danish concentrator (2-acetylthiophene added as external standard; spiking of 1 mL of the 0.2 mg/L stock solution before concentration) and to 70 µL in a Dufton apparatus.

Brewing Process

Three beers (CIT212, CIT214, and AMA212) were successively produced in our 50 L microbrewery (CoEnCo, Oostkamp, Belgium). In the brewing process, 12 kg of pilsen malt (two rows, Malterie du Château, Beloeil, Belgium) was brewed in 50 L with the mashing program as follows: 30 min at 50°C, 30 min at 63°C, and 30 min at 72°C. The wort was heated to 82°C, filtered through the lauter tun, and adjusted to 11°P. The wort thus obtained was then boiled with 33 mg/L of Tomahawk CO_2 extract for 75 min (8–11% evaporation), and 10 min before the end, the selected hop variety (Citra or Amarillo pellets) was added at 1.78 g/L. The fermentation was conducted in cylindroconical fermenters with ale-type yeast (INBR Bras 212 or Bras 214). Yeast was pitched at 7.5×10^6 cells/mL. The fermentation was carried out at 22°C for 4 days under an inner-tank pressure of 0.01 MPa. The maturation was held at 2°C for 7 days with periodical purge of excess yeast from the bottom of the fermenter. After filtration on plates (0.5 µm pore size, Buon Vino, Cambridge, ON, Canada), the beer was stored under carbon dioxide until extraction the next day.

Beer Aging

To study the chemical degradation of Cys-2SEA during beer aging, 227 mg/L of Cys-2SEA was spiked in a commercial fresh lager beer that was stored for 3 months in a dark room at 20°C (sampling after 1, 2, and 3 months). Accelerated aging of the same beer was achieved after storage for 5 days at 40 or 80°C.

Pilot beers were stored for 6 months in a dark room at 20°C (sampling after 1, 3, and 6 months).

*p*HMB Extraction of Thiols from Beers

Thiols were selectively extracted from beer according to the method of Vermeulen et al. (19), adapted from Tominaga et al. (11). The IST was 4-methoxy-2-methyl-2-butanethiol (added at the first extraction step, 0.67 µg/L in beer).

Gas Chromatography Coupled to a Pulsed Flame Photometric Detector (GC-PFPD)

Thiol extracts (2 µL) were analyzed on a ThermoFinnigan Trace GC 2000 gas chromatograph equipped with a splitless injector maintained at 250°C and connected to a ThermoFinnigan Trace PFPD detector (600 V, 250°C, 18 ms gate width, 6 ms gate delay, 3.45 Hz pulse frequency); the split vent was opened 1 min postinjection. Compounds were analyzed with a polar free fatty acid phase (FFAP) capillary column (25 m × 0.32 mm i.d., 0.3 µm film thickness). The carrier gas was helium at a flow rate of 1.3 mL/min (pressure set at 45 kPa). The oven temperature was programmed to rise from 36 to 85°C at 20°C/min, to 145°C at 1°C/min, and finally to 220°C at 3°C/min.

The following general equation was used for quantifications (7): concentration (A, ng/L) = concentration (IST, ng/L) × (molecular weight [A]/molecular weight [IST]) × (area [A]/area [IST]) × (molar response coefficient [IST]/molar response coefficient [A]) × (recovery factor [IST]/recovery factor [A]).

For commercially available thiols, complete calibration curves relative to the IST were used. For commercially unavailable compounds (substance names carrying an IST superscript in Table I), the good equimolarity response factor of the PFPD detector enabled the setting of the IST-relative molar response coefficients at 1. The IST-relative recovery factor was set at 1 for all compounds (experimental values from 0.8 to 1.2, previously determined by standard addition) except for 2-sulfanylethan-1-ol (which showed poor recovery at the first dichloromethane extraction; approximate concentrations were given by applying a ratio of 0.1 assessed by standard addition) (2). The variation coefficients for thiols (extraction and analysis) were below 15%.

GC Coupled to an Electronic Impact Mass Spectrometer (GC-MS)

Electronic impact mass spectra were recorded at 70 eV (full scan with a mass range from 40 to 380 *m/z* and selected ion monitoring mode for each suspected thiol) on a ThermoFinnigan Trace MS simple quadrupole mass spectrometer connected to a ThermoFinnigan Trace GC 2000 gas chromatograph equipped with the aforementioned polar column (FFAP) and a splitless injector (220°C). Thiol extracts (1 µL) were injected. The split vent was opened 0.8 min postinjection. The oven temperature program was the same as described for GC-PFPD. The carrier gas was helium, and pressure was set at 45 kPa. Spectral recording was automatic throughout separation (Xcalibur software was used).

GC Coupled to an Olfactometric Detector (GC-O)

To assess the olfactory potential of each beer thiol, the FFAP column was connected to a GC-O port (250°C) of a Chrompack CP9001 GC equipped with a splitless injector maintained at 250°C; the split vent was opened 0.5 min postinjection. The carrier gas was nitrogen, and the pressure was set at 30 kPa. The oven temperature program was the same as described for GC-PFPD. The effluent was diluted with a large volume of air (20 mL/min) prehumidified with an aqueous copper (II) sulfate solution. Aroma extract dilution analysis was performed on 1 µL of *p*HMB extracts by two trained panelists. The extracts were diluted stepwise with dichloromethane. Flavor dilution (FD) is defined as the highest dilution at which the compound could still be detected (FD = 2^n, with $n + 1$ = number of dilutions applied on the extract until no odor was perceived).

Extraction of Cysteine Adducts from Hops

A thiol precursor extraction procedure adapted from Gros et al. (3) and Tominaga et al. (12) was applied to the Amarillo and Citra hop varieties. *S*-Benzyl cysteine was used as an IST at 8 mg/kg of hops. Milled pellets (100 g) were extracted with 1 L of hydroalcoholic solution ($EtOH/H_2O/HCOOH$ = 49.5:49.5:1, v/v/v) for 2 h. After centrifugation for 30 min at 1,200 × *g*, the polyphenols present in the supernatant were partially removed by addition of 30 g of polyvinylpyrrolidone and stirring for 30 min. After a second centrifugation, the extract was purified on the IR-120 (H^+) cation exchange resin (100 g) conditioned with 100 mL of 6% HCl followed by 1 L of ultrapure water. After washing with 500 mL of water, sequential fractions were recovered by eluting with aqueous ammonia solutions (100 mL each) at the following concentrations: 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, and 2.1M. The fractions 1.2 and 1.5M were pooled and concentrated to dryness under reduced pressure. The obtained extract was dissolved in 2 mL of 0.1% formic acid aqueous solution for analysis by RP-HPLC-ESI(+)-MS/MS.

RP-HPLC-ESI(+)-MS/MS or Multiple Reaction Monitoring (MRM) Analyses of Cysteine Adducts

Analyses were performed on a 150 × 2.1 mm, 2 µm C18 Prevail column (Grace, Deerfield, IL, U.S.A.). The elution solvents were water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). Gradient elution was as follows for solvent A: 95 to 80% in 4 min, 80 to 50% in 2 min, 50 to 42% in 3 min, and 42 to 0% in 3 min. Solvent B was then eluted at 100% for 4 min. The flow rate was 200 µL/min. Hop extracts or beer samples (5 µL) were injected onto the column at room temperature. A system equipped with an autosampler and a quaternary pump (1200 series, Agilent Technologies, Santa Clara, CA, U.S.A.) was used. The system was controlled with Agilent ChemStation software. Mass spectra were acquired using a Bruker Daltonics Esquire 3000 ion trap mass spectrometer equipped with an electrospray ion source (Bruker) operated in positive mode for cysteine-*S*-conjugates. The ESI inlet conditions were as follows: source voltage, 4.5 kV; capillary temperature, 300°C; and the nebulizer was nitrogen at 12 psi. Nitrogen was also used as a dry gas, at a flow rate of 8 mL/min. Collision-induced dissociation spectra were recorded at a relative collision energy of 0.2 V. Cys-2SEol and Cys-2SEA were analyzed in MRM mode by monitoring the fragmentations *m/z* = 166 → 149 and *m/z* = 208 → 148, respectively. Cys-3SHol was analyzed in MS/MS mode (*m/z* = 222). Standard solutions (25, 50, 100, 250, and 500 mg/L) of Cys-2SEA were injected for calibration. Conversion of cysteine adducts to free thiols was calculated as follows: molar conversion ratio (%) = concentration of released thiol (µmol/L) × 100/initial concentration of *S*-cysteine precursor (µmol/L).

RESULTS AND DISCUSSION

Chemical Synthesis of Cys-2SEol and Cys-2SEA

Cys-2SEA was obtained at 93% purity by acetylation of its corresponding alcohol Cys-2SEol (Fig. 1). The two compounds were fully characterized by HPLC-ESI(+)-MS/MS (Fig. 2) and NMR.

TABLE I
Polyfunctional Thiols in Fresh Pilot Beers: PFPD Quantifications and Flavor Dilution (FD) Factors[a]

RI						PFPD concentrations in µg/L and FD AEDA					
						CIT212		CIT214		AMA212	
FFAP	CP-Sil 5	Substance	Acronym	Number	Odor (GC-O)	µg/L	FD	µg/L	FD	µg/L	FD
1,112	810	3-Methyl-2-butene-1-thiol	MBT	37	Coffee, skunky	–	≥2,048	–	≥2,048	–	≥2,048
1,381	915	4-Sulfanyl-4-methylpentan-2-one	4S4M2Pone	29	Catty, blackcurrant	–	32	–	64	–	32
1,454	880	2-Sulfanylethyl acetate	2SEA	38	Roasted, burnt	1.10	64	22.75	1,024	1.30	64
1,458	907	3-Sulfanylpentanal	3SPal	28	Flower, hoppy	–	1,024	–	1,024	–	1,024
1,538	722	2-Sulfanylethan-1-ol	2SEol	36	Grilled, gas	(2.54)	–	(10.44)	–	(1.07)	–
1,565	989	3-Sulfanylpropyl acetate	3SPrA	1	Roasted, burnt	–	–	0.74	4	–	–
1,620	849	3-Sulfanylpropan-1-ol	3SProl	14	Potatoes	–	–	+	–	–	–
1,656	944	3-Sulfanyl-3-methylbutan-1-ol	3S3MBol	17	Broth, soup	–	16	0.06	32	0.07	32
1,698	977	1-Sulfanylpentan-3-ol^{IST}	1S3Pol	19	Mushroom, nettle	–	–	0.05	–	–	–
1,727	1,215	3-Sulfanylhexyl acetate	3SHA	11	Candy, pumpkin	0.07	8	–	4	–	4
1,760	1,009	3-Sulfanylpentan-1-ol	3SPol	21	Catty, citrus	–	8	–	4	–	32
1,818	1,208	3-Sulfanyl-4-methylpentan-1-ol^{IST}	3S4MPol	26	Rhubarb, grapefruit	–	128	–	256	0.62	1,024
1,858	1,094	3-Sulfanylhexan-1-ol	3SHol	23	Rhubarb, grapefruit	–	256	–	512	–	64
1,962	1,199	3-Sulfanylheptan-1-ol	3SHptol	25	Lemon, hoppy	–	8	–	8	–	128

[a] Assays in duplicate. The concentrations of number 36 (2SEol) have been roughly assessed by applying an internal standard-relative recovery factor of 0.1 (concentrations given in parentheses). Concentrations of compounds carrying an IST superscript are measured in IST equivalents. PFPD = pulsed flame photometric detector; RI = retention index; FFAP = free fatty acid phase; GC-O = GC coupled to an olfactometric detector; AEDA = aroma extract dilution analysis; – = not detected by PFPD or GC-O; + = detected by PFPD under quantification limits; and IST = internal standard.

As depicted in Figure 2, Cys-2SEA logically eluted a bit after its analog Cys-2SEol on the reversed-phase column (retention times = 2.1 and 3.3 min for Cys-2SEol and Cys-2SEA, respectively). ESI(+)-MS/MS analyses allowed for easy differentiation of the two compounds by four major *m/z* ions: 149 for Cys-2SEol (M + 1 = 166) and 120, 148, and 191 for Cys-2SEA (M + 1 = 208) (Fig. 2).

The NMR data of the two compounds were as follows: ^{1}H NMR (300 MHz, D_2O) δ 3.93 (dd, 1 H, J = 7.3, 4.4 Hz, –S–CH_2–C**H**NH_2–COOH), 3.76 (t, 2 H, J = 5.9 Hz, HOC**H**$_2$–CH_2–S–), 3.15 (dd, 1 H, J = 14.8, 4.3 Hz, –S–C**H**$_2$–$CHNH_2$–COOH), 3.05 (dd, 1 H, J = 14.8, 7.3 Hz, –S–C**H**$_2$–$CHNH_2$–COOH), 2.77 (t, 2 H, J = 6.0 Hz, $HOCH_2$–C**H**$_2$–S–) for Cys-2SEol; ^{1}H NMR (500 MHz, D_2O) δ 4.29 (t, 2 H, J = 6.2 Hz, CH_3COOC**H**$_2$–CH_2–S–), 4.15 (dd, 1 H, J = 7.0 , 3.8 Hz, –S–CH_2–C**H**NH_2–COOH), 3.24 (dd, 1 H, J = 14.9, 4.2 Hz, –S–C**H**$_2$–$CHNH_2$–COOH), 3.12 (dd, 1 H, J = 14.9, 7.4 Hz, –S–C**H**$_2$–$CHNH_2$–COOH), 2.89 (t, 2 H, J = 6.1 Hz, CH_3COOCH_2–C**H**$_2$–S–), 2.11 (s, 3 H, C**H**$_3COOCH_2$–CH_2–S–) for Cys-2SEA.

Free 2SEA in Fresh and Aged Pilot Beers

Two pilot beers (different yeast strains) produced with the 2SEA-rich Citra hop variety were investigated, in comparison with Amarillo hopping. Polyfunctional thiols were selectively extracted by *p*HMB from the three fresh pilot beers and analyzed by GC-PFPD, GC-MS, and GC-O.

As shown in Figure 3A and B, three and six thiols were detected by GC-PFPD in CIT212 and CIT214, respectively (Citra-hopped beers fermented either with Bras 212 or Bras 214). Additionally, seven other thiols were perceived at the sniffing port (Table I). All these thiols were previously identified in beers (1,2,10,19).

As previously mentioned for commercial beers (14), the empyreumatic 2SEA (number 38) was the highest PFPD peak in all beer extracts. Yet it was revealed that yeast exerted a major impact on 2SEA concentration in fresh beer (1.1 and 22.8 µg/L in CIT-212 and CIT214, respectively; FD = 64 and 1,024). In the latter, the 2SEA level was quite close to its sensorial threshold value assessed at 40 µg/L (17). 3SPrA (number 1) was undetectable in CIT212 even at the sniffing port but was found at 0.74 µg/L (FD = 4) in CIT214. Similarly, the corresponding alcohol of 2SEA, 2-sulfanylethan-1-ol (number 36) was four times more concentrated in CIT214 (Table I).

On the other hand, all the other thiols were perceived at the sniffing port with similar FD values in both beers. Among them were found the pleasant grapefruit-like 3-sulfanyl-4-methylpentan-1-ol (3S4MPol, number 26, FD = 128–256), 3-sulfanylhexan-1-ol (3SHol, number 23, FD = 256–512), 3-sulfanylheptan-1-ol (3SHptol, number 25, FD = 8), and the blackcurrant-like 4-sulfanyl-4-methylpentan-2-one (4S4M2Pone, number 29, FD = 32–64), together with two more aggressive odorants: 3-sulfanylpentanal (3SPal, number 28, hoppy, FD = 1,024) and 3-methyl-2-butene-1-thiol (MBT, number 37, skunky, FD ≥ 2,048).

The thiol composition of the third pilot beer (hopped with the Amarillo cultivar; Fig. 3C) produced with the 2SEA low producer yeast strain Bras 212 confirmed that compounds number 38, 36, 1, and 14 are mainly issued in the fresh beer from the Ehrlich pathway, whereas most other thiols are hop-dependent. Indeed, as expected, similar levels of 2SEA, 2SEol, and 3SPrA were found in CIT212 and AMA212 (much less than in CIT214). On the other hand, 3-sulfanylpentan-1-ol (3SPol, number 21, citrus, FD = 32), 3S4MPol (number 26, 0.6 µg/L, FD = 1,024), and 3SHptol (number 25, FD = 128) gave rise to higher FD when the Amarillo

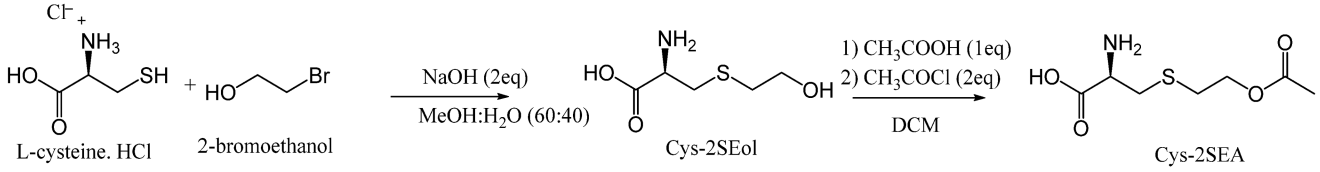

Fig. 1. Chemical synthesis pathways of *S*-cysteine conjugate of 2-sulfanylethan-1-ol (Cys-2SEol) and *S*-2-(ethylacetate)-cysteine (Cys-2SEA).

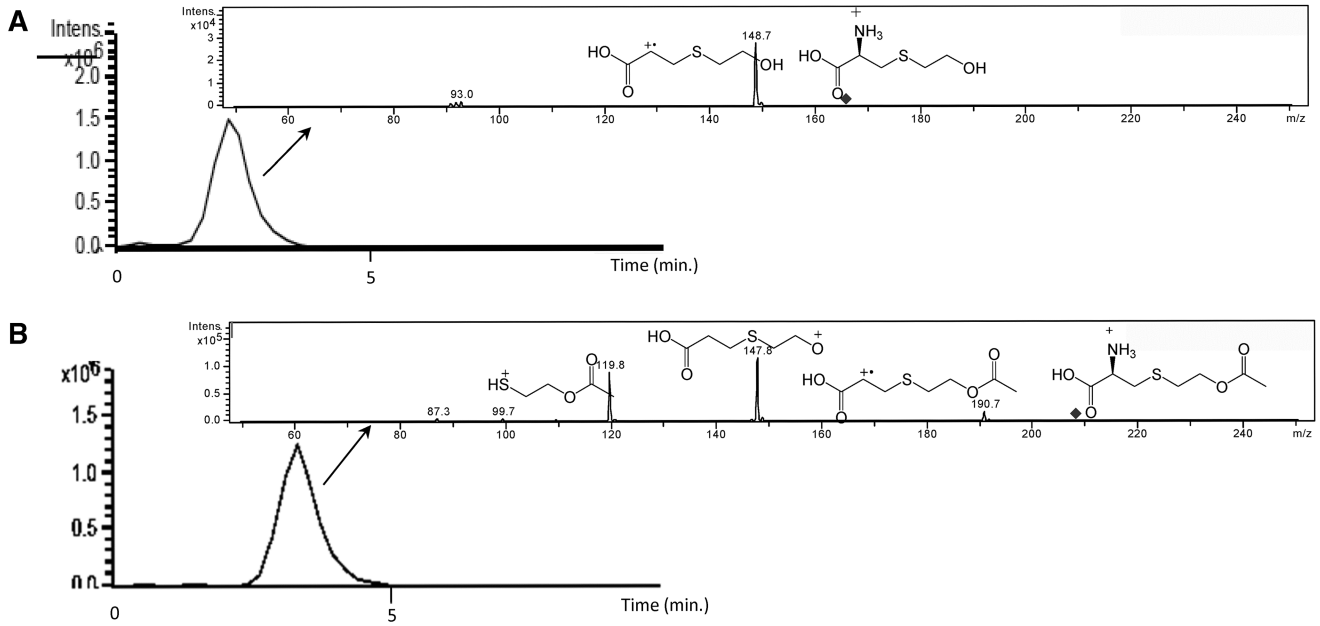

Fig. 2. HPLC chromatograms and ESI(+)-MS/MS mass spectra of 2-sulfanylethan-1-ol (**A**) and *S*-2-(ethylacetate)-cysteine (**B**).

hop was used, whereas 3SHol (number 23) was a less potent odorant in AMA212 than in both Citra beers.

The two Citra pilot beers were stored in a dark room at 20°C, and sampled after 1, 3, and 6 months. Polyfunctional thiols were selectively extracted by *p*HMB and analyzed by GC-PFPD (Fig. 4).

In CIT214, concentrations of 2SEA (Fig. 4A), which was very high in fresh beer (22.8 µg/L), quickly dropped to reach 7.25 µg/L after 6 months of storage. Oxidation most probably explains these results. In contrast, 2SEA increased quickly in CIT212 to reach 11.8 µg/L after only 1 month (Fig. 4A). In this case, degradation of the initial 1.1 µg/L of 2SEA was not significant enough to hide a new synthesis occurring in the bottle. Yet after 3 months, oxidation outweighed its release. For both beers, 3SPrA showed the same pattern as 2SEA (Fig. 4B).

Chemical Degradation of Cys-2SEA in Acetate Buffer Model Medium

The synthesized Cys-2SEA compound was solubilized in sodium acetate buffer (pH 4.2). This model medium was then heated at 100°C for 1 h or at 40, 60, or 80°C for 5 days. Prior to liquid-liquid extraction and PFPD quantification of free thiols, 1 mL of the heated medium was sampled for HPLC-ESI(+)-MS/MS quantification of cysteine adducts. All heat treatments were conducted in duplicate.

As depicted by the HPLC quantifications reported in Figure 5A, Cys-2SEA was shown to be slightly degraded after 1 h at 100°C (to mimic wort boiling; 21% degraded) or 5 days at 40, 60, and 80°C (temperatures applied for accelerated aging; ≤15% degraded). Moreover, GC-PFPD data given in Figure 5B confirmed that the conversion ratios of Cys-2SEA into free 2SEA remained low in all experiments (0.01–0.3%, Table II). Yet amounts close to the 2SEA sensorial threshold (40 µg/L [14]) were measured after 5 days at 40°C. The strongest treatment here investigated (5 days at 80°C) released up to 389 µg/L of free 2SEA (10 times higher than its sensorial threshold).

Cys-2SEA Spiking Experiments in Beer

To assess if hydrolysis of cysteine adducts could explain the increase of 2SEA observed during the first month of CIT212 beer aging, spiking experiments of Cys-2SEA (227 mg/L) were also conducted in beer. Beer (1 mL) was sampled for HPLC-ESI(+)-MS/MS quantification of Cys-2SEA (Fig. 6A) before *p*HMB ex-

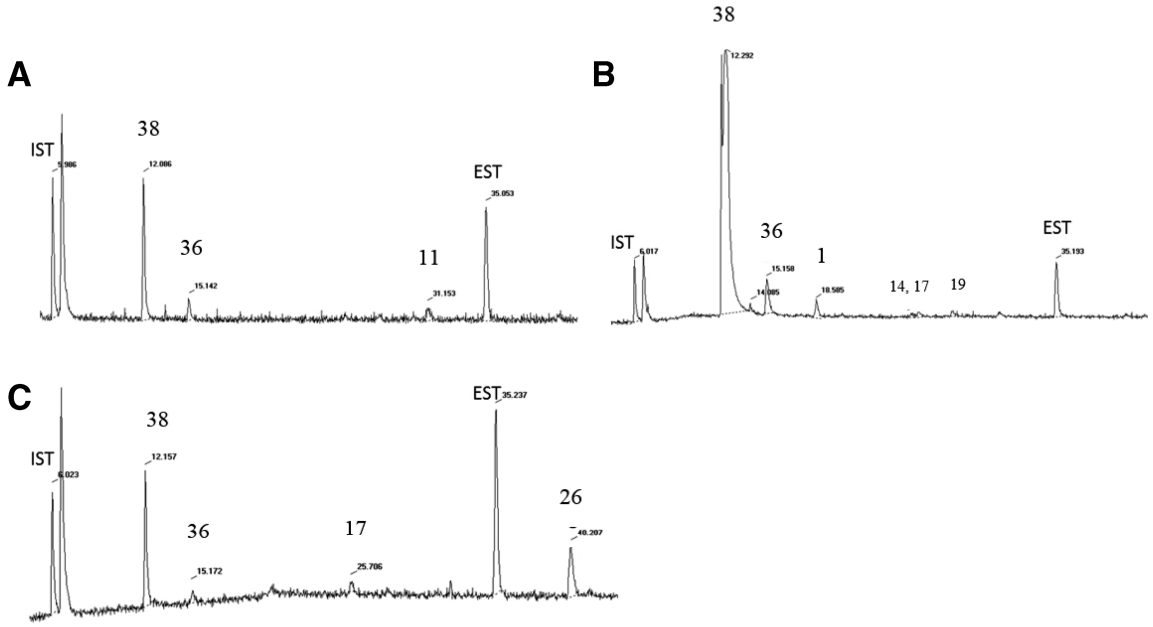

Fig. 3. GC coupled with a pulsed flame photometric detector chromatograms of *p*-hydroxymercuribenzoic acid extracts issued from fresh beers: **A,** CIT212, **B,** CIT214; and **C,** AMA212. IST = internal standard, and EST = external standard. Numbers identifying substances are listed in Table I.

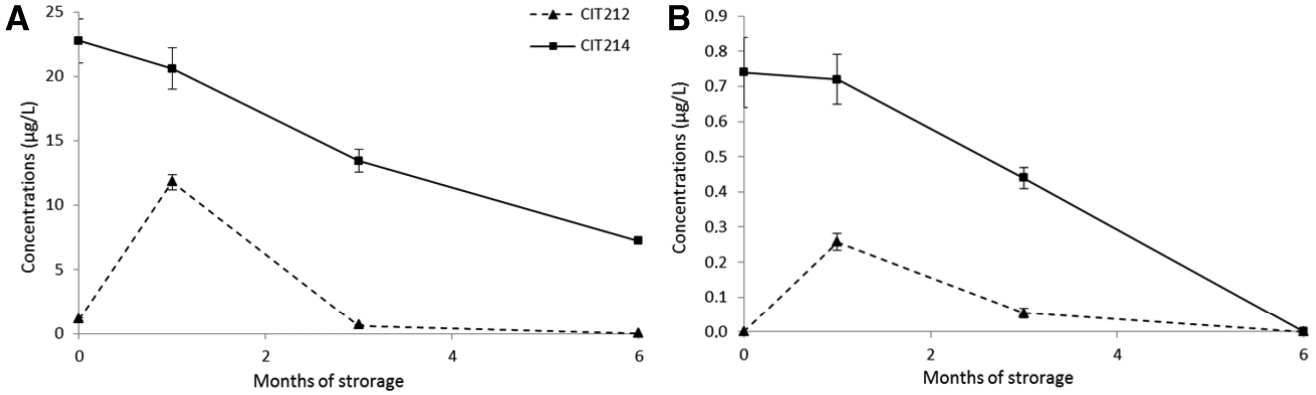

Fig. 4. Concentrations of 2-sulfanylethyl acetate (**A**) and 3-sulfanylpropyl acetate (**B**) in beers CIT212 and CIT214 through aging (quantifications using GC coupled with a pulsed flame photometric detector).

traction and PFPD quantification of free thiols. All assays were conducted in duplicate. Although only 14% were degraded in the acetate buffer after 5 days at 80°C, up to 43% had disappeared in beer after the same period (Fig. 6A). Similar spiking experiments with Cys-3SHol recently showed that other beer constituents, especially diketones, could facilitate the chemical degradation of *S*-cysteine adducts into free thiols (15). As depicted in Figure 7, the Strecker degradation intermediate issued from the addition of Cys-2SEA on a dicarbonyl could easily release free 2SEA in beer. The beers were also analyzed after 0, 1, 2, and 3 months at 20°C and after 5 days at 40°C. Although Cys-2SEA appeared relatively stable after both natural and accelerated aging (Fig. 6A), a release of 2SEA was already quantifiable after 1 month at 20°C (Fig. 6B, molar conversion of 0.3%). 2SEA was then gradually degraded, following a pattern similar to that observed in the pilot beer CIT-212 (Fig. 4A). An accelerated aging of 5 days at 40°C was revealed to well mimic the natural aging of 2 months.

Investigation of Cys-2SEol and Cys-2SEA in Hops

As depicted by the HPLC-ESI(+)-MRM chromatograms shown in Figure 8A and B, Cys-2SEol and Cys-2SEA were not found in the Citra and Amarillo cysteine adduct hop extracts. Yet the same

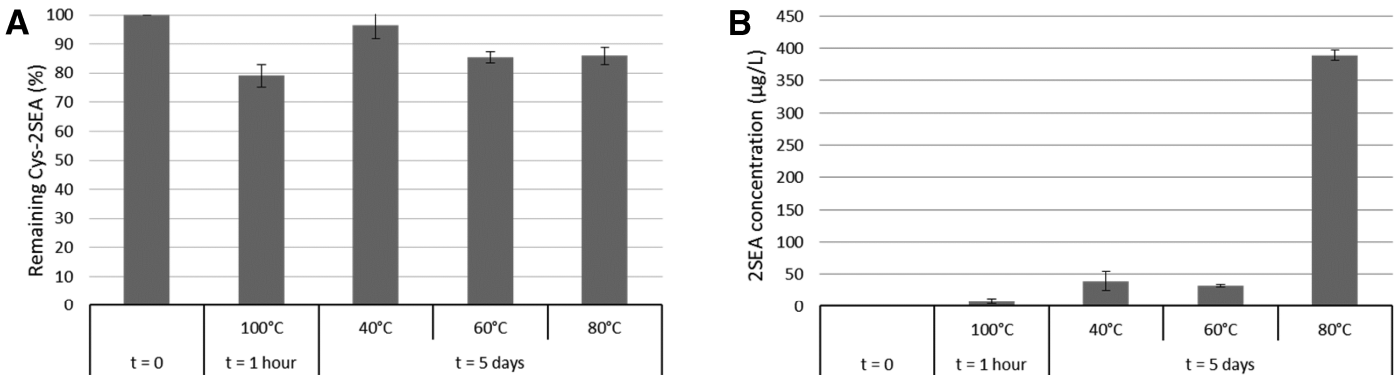

Fig. 5. A, Remaining concentrations of *S*-2-(ethylacetate)-cysteine (Cys-2SEA) (%), and **B,** concentrations of released 2-sulfanylethyl acetate (2SEA) (µg/L) in acetate buffer model medium spiked before heat treatments with 227 mg/L of Cys-2SEA.

TABLE II
Loss of *S*-2-(Ethylacetate)-cysteine (Cys-2SEA) and Released 2-Sulfanylethyl Acetate (2SEA) Through Various Heat Treatments in Acetate Buffer (pH 4.2) or Fresh Beer[a]

Heat treatments		Loss of Cys-2SEA, from 227 mg/L = 1,097 µmol/L			Released 2SEA		Molar conversion ratio (%)
Time	Temperature	mg/L	µmol/L	Degradation ratio (%)	µg/L	µmol/L	
Acetate buffer							
t = 0		0	0	0	0	0	0
t = 1 h	100°C	47.4	229	21	7	0.06	0.01
t = 5 days	40°C	nq	nq	nq	40	0.33	0.03
	60°C	33.1	160	15	32	0.27	0.02
	80°C	32.1	155	14	389	3.24	0.29
Fresh beer							
t = 0		0	0	0	0	0	0
t = 1 month	20°C	nq	nq	nq	399	3.29	0.30
t = 2 months	20°C	nq	nq	nq	338	2.79	0.25
t = 3 months	20°C	nq	nq	nq	155	1.28	0.12
t = 5 days	40°C	nq	nq	nq	330	2.72	0.25
	80°C	98	472	43	nd	nd	nd

[a] Assays in duplicate; nq = unquantifiable; and nd = not measured.

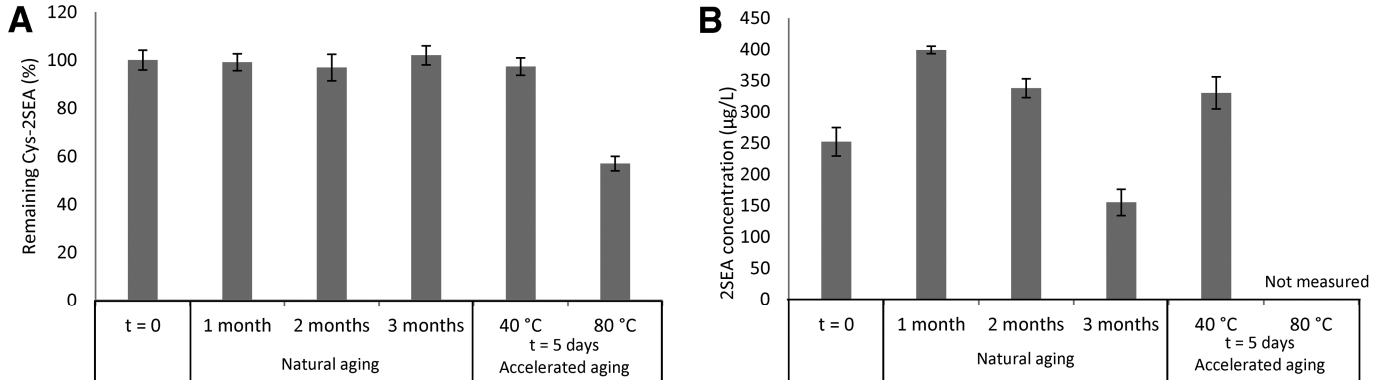

Fig. 6. A, Remaining concentrations of *S*-2-(ethylacetate)-cysteine (Cys-2SEA) (%); and **B,** concentrations of released 2-sulfanylethyl acetate (2SEA) (µg/L) in a commercial lager beer spiked with 227 mg/L of Cys-2SEA before natural or accelerated aging.

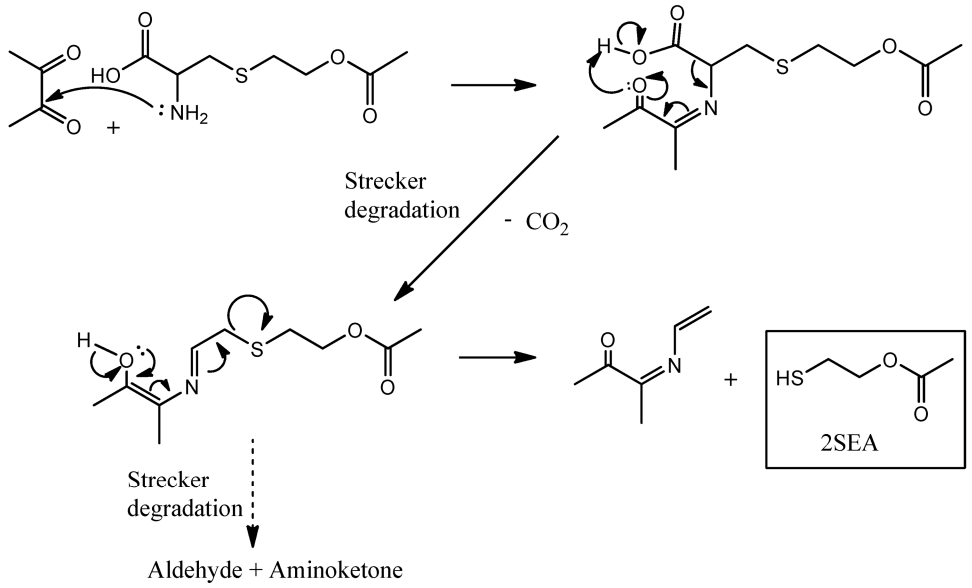

Fig. 7. Hypothetical degradation pathway of *S*-2-(ethylacetate)-cysteine in the presence of dicarbonyls (modified Strecker degradation). 2SEA = 2-sulfanyl-ethyl acetate.

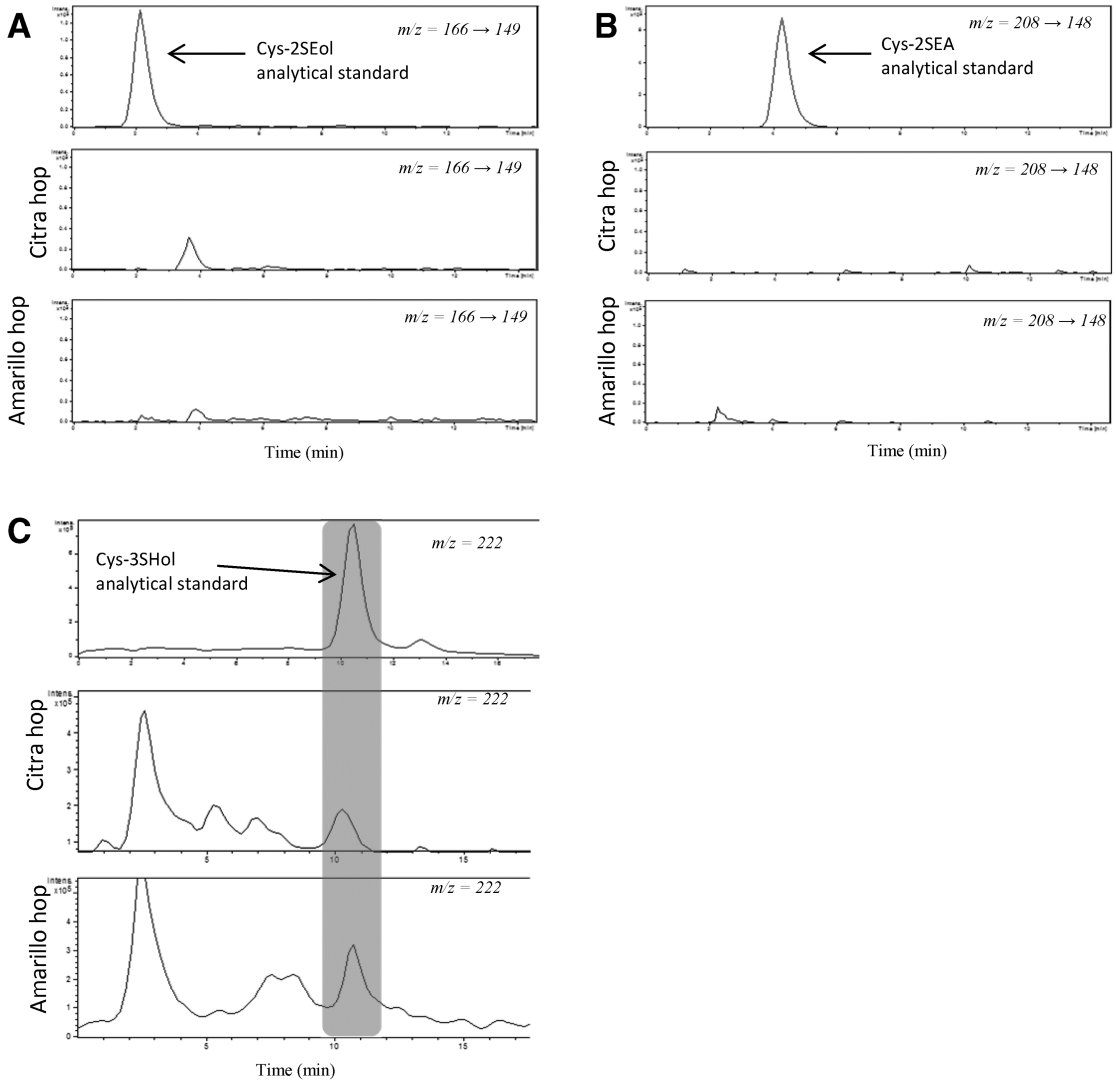

Fig. 8. HPLC-ESI(+)-MS/MS analyses, in Citra and Amarillo hop extracts: **A,** 2-sulfanylethan-1-ol (Cys-2SEol) (*m/z* = 166 →149), **B,** *S*-2-(ethylacetate)-cysteine (Cys-2SEA) (%) (*m/z* = 208 →148), and **C,** cysteine *S*-conjugate of 3-sulfanylhexan-1-ol (Cys-3SHol) (*m/z* = 222).

extraction procedure allowed for the detection, as expected, of Cys-3SHol in both cultivars (MS/MS [*m/z* = 222] chromatograms given in Figure 8C). As apotryptophanase did release 2SEol from these hop varieties (5), other adducts with a free cysteine amine (required for enzyme) should exist.

CONCLUSIONS

In conclusion, 2SEA found in fresh beer is mainly issued from cysteine, by the Ehrlich pathway (strongly yeast-dependent). On the other hand, our spiking experiments showed that amino acid adducts are the most probable precursors accounting for the release of 2SEA through beer aging even if the conversion ratio is very small. The traces of polyfunctional thiols released from these adducts do not significantly contribute to the flavor of aged beers. As for the cysteine adduct of 3SHol, whose glutathionylated analog has been recently evidenced in hops, further investigations should now be conducted on the glutathione and cysteinylglycine adducts of 2SEol and 2SEA.

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