

Occurrence of polyfunctional thiols in sorghum beer 'ikigage' made with *Vernonia amygdalina* 'umubirizi'

Francois Lyumugabe,^{a,b*} Jacques Gros,^c Philippe Thonart^a and Sonia Collin^{c*}

ABSTRACT: Several polyfunctional thiols have been previously identified in beers made from barley and hops. These compounds have not been investigated in beers brewed with 'non-Western' raw materials. Here we have performed a thiol-specific extraction with *p*-hydroxymercuribenzoic acid on a traditional *ikigage* sorghum beer from Rwandese peasants (use of *Vernonia amygdalina* just for yeast propagation), and on two pilot beers with addition (or not) of *V. amygdalina* in the boiling kettle, instead of hops. Gas chromatography–olfactometry, gas chromatography–mass spectrometry and gas chromatography with pulsed flame photometric detection analyses of the extracts enabled us to identify 14 polyfunctional thiols. The well-known hop constituent 3-methyl-2-buten-1-thiol emerged as a key flavour in the unhopped beers containing *V. amygdalina* (flavour dilution > 262 144). The addition of *V. amygdalina* during boiling also resulted in the presence of 1-butanethiol, but the production of 2-sulfanylethanol and 2-sulfanylethyl acetate was inhibited. Complementary data are required to understand how *V. amygdalina* leaves are able to impact upon the Ehrlich pathway leading to cysteine and homocysteine-derived thiols. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: polyfunctional thiols; aroma; sorghum beer; *ikigage*; *Vernonia amygdalina*

Introduction

In sub-Saharan Africa, traditional sorghum beer is a popular alcoholic beverage for African ceremonies. Sorghum beer is known as *ikigage* or *amarwa* in Rwanda,^[1] *tchoukoutou* in Benin and Togo,^[2] *pito* in Ghana and Nigerian,^[3] *bili bili* in Chad^[4] and *impeke* in Burundi.^[5] These beers made from sorghum malt or millet are unhopped.

Hop is a temperate crop that cannot be successfully grown in tropical African countries. Several studies reported the possibility of using *Vernonia amygdalina* instead of hop in African sorghum beers.^[6–10] *V. amygdalina*, known as 'bitter leaf', is a green shrub found all over Africa and belonging to the *Asteraceae* family. It mimics hop not only by its antimicrobial properties^[11,12] but also by its bitter taste.^[10,13,14]

In European beers made of malted barley and hop, many studies have shown the key role of polyfunctional thiols.^[15–17] Characterized by extremely low threshold values, these compounds are also important contributors to the characteristic aroma of various fruits,^[18] and wines.^[19] Gas chromatography–olfactometry (GC-O) and gas chromatography with pulsed flame photometric detection (GC-PFPD) analyses of thiol specific beer extracts [*p*-hydroxymercuribenzoic acid (*p*-HMB)] have led to the identification of 2-sulfanylethanol (**2** in Figure 1), 3-methyl-2-buten-1-thiol (**4**), 3-sulfanylpropanol (**5**), 2-sulfanylethyl acetate (**6**), 4-sulfanyl-4-methylpentan-2-one (**9**), 3-sulfanylpropyl acetate (**11**), 3-sulfanylhexanol (**15**), 2-sulfanyl-3-methylbutanol and 3-sulfanyl-3-methylbutanol.^[16] Among these compounds, the onion-like 2-sulfanyl-3-methylbutanol^[20] is suspected of having arisen through the reaction of hop allylic alcohol with yeast hydrogen sulfide.^[21] In these 'Western-like' beers, Takoi observed a strong effect of the hop variety, claiming that this ingredient could be the main source of certain polyfunctional thiols.^[22] Recently, Gros *et al.* identified 41 free thiols in

several hop varieties and suspected them of being released from bound precursors through wort boiling and fermentation.^[23]

Surprisingly, polyfunctional thiols have not yet been studied in beers brewed with non-Western raw materials. Here we have performed a thiol-specific extraction with *p*-HMB^[24] on a traditional *ikigage* sorghum beer from Rwandese peasants (IKT; use of *Vernonia amygdalina* for yeast propagation), and on two pilot beers made with (RV6.V) or without (RV6) addition of *V. amygdalina* (instead of hop) in the boiling kettle (use of a dominant strain selected from traditional leaven, referred to as RV6). Thiols were analysed with different gas chromatography detectors (olfactometry, pulsed-flame photometric detector, and mass spectrometry), and compared with various references available in our combinatorial libraries.^[25–28] The well-known aroma extract dilution analysis (AEDA) methodology^[29] was applied in order to assess the relative contribution of traces undetected by usual detectors.

* Correspondence to: Francois Lyumugabe, National University of Rwanda, Faculty of Science, B.P. 117 Butare, Rwanda. E-mail: flyumugabe@nur.ac.rw

Sonia Collin, Université catholique de Louvain, Earth and Life Institute ELIM, Unité de Brasserie et des Industries Alimentaires – Croix du Sud 2 Box L7.05.07, B-1348 Louvain-la-Neuve, Belgium. E-mail: sonia.collin@uclouvain.be

^a Université de Liège, Gembloux Agro – Bio Tech, Centre Wallon de Biologies Industrielles, Passage des Déportés 2, B-5030 Gembloux, Belgium

^b National University of Rwanda, Faculty of Science, B.P. 117 Butare, Rwanda

^c Université catholique de Louvain, Earth and Life Institute ELIM, Unité de Brasserie et des Industries Alimentaires, Croix du Sud 2 Box L7.05.07, B-1348 Louvain-la-Neuve, Belgium

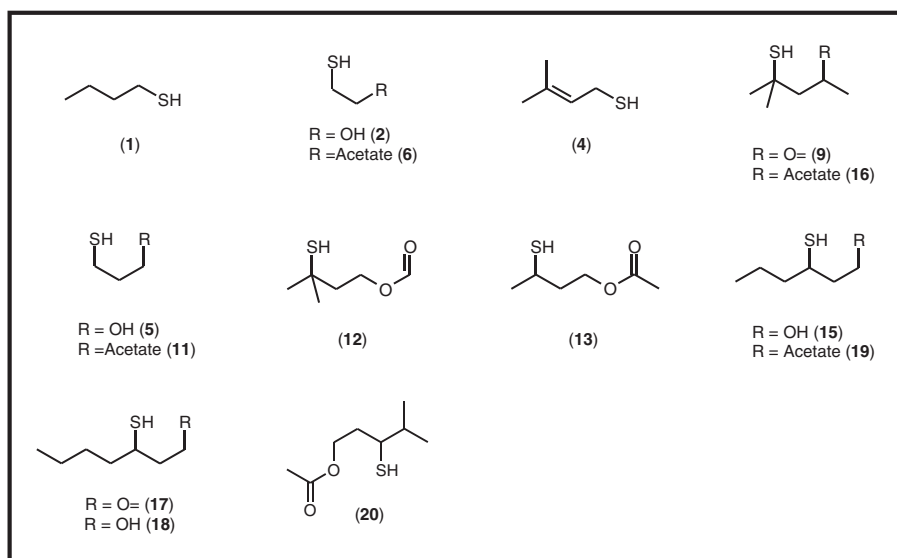


Figure 1. Chemical structures and numbering of thiols

Materials and Methods

Reagents

p-Hydroxymercuribenzoic acid (*p*-HMB), L-cysteine hydrochloride monohydrate and hydrochloric acid (37%) were purchased from Sigma-Aldrich (Bornem, Belgium). Dichloromethane (99.9%), obtained from Romil (Cambridge, UK), was distilled before use. 4-Methoxy-2-methylbutan-2-thiol was obtained from Oxford Chemicals (Oxford, UK). Milli-Q water was used (Millipore, Bedford, MA, USA). NaOH and Na₂SO₄ (99%) were supplied by Janssen (Geel, Belgium). A strongly basic Dowex resin 1 × 2, Cl⁻ form (Sigma-Aldrich, Bornem, Belgium) was stored in hydrogen chloride (0.1 M). Anhydrous sodium sulfate was obtained from Merck (Darmstadt, Germany) and tris(hydroxymethyl)aminomethane (TRIS) from USB (Cleveland, OH, USA). 1-Butanethiol (**1**), 2-sulfanylethan-1-ol (**2**), 3-sulfanylpropan-1-ol (**5**), 2-sulfanylethyl acetate (**6**) and 3-sulfanylpropyl acetate (**11**) were purchased from Sigma-Aldrich (full chemical structures are given in Figure 1). 4-Methoxy-2-methylbutan-2-thiol, 3-sulfanylhexas-1-ol (**15**) and 3-methyl-2-buten-1-thiol (**4**) were obtained from Oxford Chemicals. 4-Sulfanyl-4-methylpentan-2-one (**9**) was from Frutarom (Hartlepool, UK). 3-Sulfanyl-3-methylbutyl formate (**12**) was obtained from Endeavour (Northamptonshire, UK).

Reference compounds synthesized in our laboratory

3-Sulfanylbutyl acetate (**13**), 3-sulfanyl-2-methylpentyl acetate (**20**), 4-sulfanyl-4-methyl-2-pentyl acetate (**16**) and 3-sulfanylhexas-1-ol acetate (**19**) were synthesized in our laboratory prior to the study (reagents and complete procedure are given by Vermeulen and Collin^[27]). 3-Sulfanyloctanal (**17**) and 3-sulfanyloctanol (**18**) had been previously produced according to Vermeulen *et al.*^[26,28]

Ikigage beer samples

Production of pilot beers with or without Vernonia amygdalina leaves (RV6 and RV6.V)

The production process for pilot beers is described in Figure 2. Two hundred grams of malt (60 g *Eleusine coracana* and 140 g

red sorghum) from peasants were mashed in 1000 ml distilled water at 45°C for 30 min. Thereafter, 150 ml of the clear 'enzymatic supernatant' was removed, while the remaining mash was heated at 90°C, held at the same temperature for 30 min, and cooled below 50°C at which time the clear 'enzymatic supernatant' was re-added.^[30] This mixture was then brewed according to the following mashing programme: 1 h at 63°C, 10 min at 75°C. The wort was centrifuged and then heated to boiling for 50 min. For production of the RV6.V pilot beer, leaves of *V. amygdalina* (2 g/l) were added after 10 min boiling. Fermentation was conducted with dominant yeast strains (*Saccharomyces cerevisiae*, referred to as RV6) from Rwandese traditional 'umusemburo' leaven in a light-protected Erlenmeyer flask, equipped with a gas trap. Yeast was pitched at 10⁷ cfu/ml and fermentation was carried out at 30°C for 3 days. After centrifugation (13 000 × *g*, 25 min), the beer was kept at -20°C until thiol-specific extraction.

Beers from Rwandese peasants (IKT)

Traditional *ikigage* beer was collected from the Tumba market site, in the southern province of Rwanda. Samples were taken from opaque tanks, in bottles (500 ml), preserved from light with aluminium sheets, stored in the freezer (-20°C) and then extracted immediately after opening. The traditional process of manufacture of *ikigage* has been described by Lyumugabe *et al.*^[11] *V. amygdalina* is used only during the production process of traditional 'umusemburo' leaven.

Extraction of polyfunctional thiols

This extraction method is based on the specific affinity of thiols for *p*-HMB.^[24] Beer (500 ml) was stirred with distilled CH₂Cl₂ (200 ml) for 30 min. After decantation (± 15 min), the lower phase and the interfacial emulsion were centrifuged for 20 min at 1200 *g*. The organic phase was then extracted by 2 × 20 ml of a *p*-HMB solution (360 mg of *p*-HMB, 24.6 g of Tris in 1 litre of Millipore water) for 5 and 10 min, respectively. The combined aqueous phases were loaded into a strongly basic anion exchanger column (Dowex 1WX2-100 resin from Aldrich Chemicals), washed beforehand by NaOH 2 M, HCl 2 M, and, in between, was rinsed by ultrapure water.

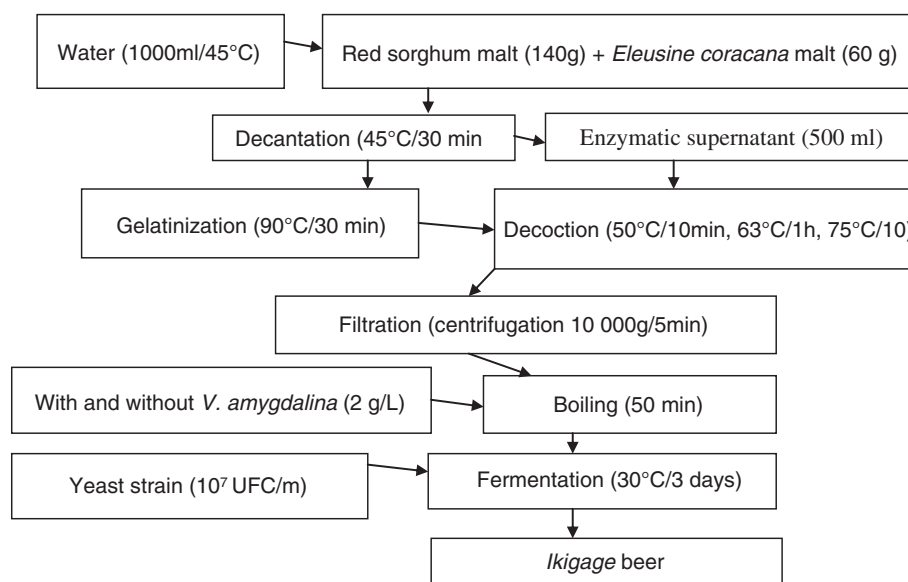


Figure 2. Production process of pilot *ikigage* beers (RV6.V and RV6)

Then 50 ml of sodium acetate buffer (0.1 M, pH 6) was poured on the resin to remove impurities. Volatile thiols were released by percolating a purified cysteine solution (640 mg of L-cysteine hydrochloride monohydrate in 60 ml of Millipore water; this solution was washed with 2×5 ml of distilled CH_2Cl_2 before use). The eluate containing the volatile thiols was collected and extracted by 4 ml and 3 ml of distilled CH_2Cl_2 using magnetic stirring (5 min each time). The organic phases were pooled, dried on anhydrous Na_2SO_4 , and finally concentrated in a Kuderna to 250 μl , to be stored at -80°C . 4-Methoxy-2-methyl-butan-2-thiol (Oxford Chemicals), added at the first extraction step, and thiazole 99% (Aldrich Chemicals), added before the final concentration step, were used, respectively, as internal and external standards (IST and EST).

Gas chromatography–olfactometry

One microlitre of the *p*-HMB extract was analysed with a Chrompack CP9001 gas chromatograph (Antwerpen, Belgium), equipped with a splitless injector maintained at 250°C ; the split vent was opened 0.5 min post-injection. Compounds were analysed with a wall-coated open tubular (WCOT) apolar CP-Sil5-CB (50 m \times 0.32 mm i.d., 1.2 μm film thickness) or polar FFAP CB (25 m \times 0.32 mm i.d.) capillary column. The carrier gas was nitrogen and the pressure was set at 50 kPa (CP-Sil5-CB). The oven temperature was programmed to rise from 36°C to 85°C at $20^\circ\text{C}/\text{min}$, then to 145°C at $1^\circ\text{C}/\text{min}$, and finally to 250°C at $3^\circ\text{C}/\text{min}$ and held for 30 min. In order to assess the olfactory potential of the extract, the column was connected to a GC-O port (Chrompack) maintained at 250°C . The effluent was diluted with a large volume of air (20 ml/min) pre-humidified with an aqueous copper(II) sulfate solution. The extract was analysed immediately after extraction by three trained panellists. Complete AEDA was performed on the *p*-HMB extracts by one operator, with the CP-Sil5-CB column. The extracts were diluted stepwise with dichloromethane (1 + 1 by volume). The dilution factor (FD) is defined as the highest dilution at which the compound could still be detected ($\text{FD} = 2^n$ with $n + 1 =$ number of dilutions applied on the extract until no detection by GC-O). The precision of this AEDA analysis is $n \pm 1$ (factor 2 between FD values).

Gas chromatography–electronic impact mass spectrometry

Mass spectra ($m/z = 40$ to 380) were recorded at 70 eV on a ThermoFinnigan Trace MS mass spectrometer connected to a ThermoFinnigan Trace GC 2000 gas chromatograph (Manchester, U.K.) equipped with a splitless injector and a 50 m \times 0.32 mm i.d., wall-coated open tubular (WCOT) apolar CP-Sil 5 CB MS capillary column (film thickness: 1.2 μm). The oven temperature program was the same as that described for GC-O. Spectral recording was automatic throughout elution; Xcalibur software was used (Manchester, U.K.).

Gas chromatography with pulsed flame photometric detection

Two microlitres of the *p*-HMB extract were analysed on a ThermoFinnigan Trace GC 2000 gas chromatograph equipped with a splitless injector maintained at 250°C and connected to a O.I. Analytical Pulsed Flame Photometric Detector, model 5380 (College Station, Texas, U.S.A.). The injections were carried out in the splitless mode at 250°C , the split being turned on after 0.5 min. The carrier gas was helium at a pressure of 90 kPa. At the detector, the following parameters were selected: 250°C as the temperature, 600 V as the voltage, 18 ms as the gate width, 6 ms as the gate delay, 580 mV as the trigger level and 3.70 Hz as the pulse frequency. The oven temperature program was the same as that described for GC-O.

Identification of the compounds

Mass spectrometry identifications were done by comparing the mass spectra obtained from each sample with those obtained with pure or synthesized compounds injected under the same conditions and/or present in the NIST library. The retention indices (retention times normalized with respect to adjacently eluting *n*-alkanes – decimal numeral system) were determined by injection onto two capillary columns (CP-Sil5-CB and FFAP-CB) connected to the FID or the olfactometric detector. In the case of PFPD detections (interesting for traces giving no GS-MS peak), injection of

Table 1. Polyfunctional thiols in *ikigage* beers with (RV6.V and IKT) or without *Vernonia amygdalina* (RV6)

No.	Retention indices	Substance	Odour (GC-O)	PPFD concentration (µg/l) in beer (FD GC-O AEDA given in parentheses)		
				RV6.V	RV6	IKT
1	686	1-Butanethiol	Rot/egg	0.7* (16)	0	0
2	755	2-Sulfanylethanol	Roasted/sewage	0.7* (2)	3.1* (4)	4.0* (4)
3	788	Unknown	Cabbages/rubber	0.2 (32)	– (8)	0
4	815	3-Methyl-2-buten-1-thiol	Skunky/garlic/old tea/plastic	– (> 262 144)	– (16 384)	– (> 262 144)
5	840	3-Sulfanylpropanol	Roasted/potato	Trace (65 536 [†])	– (4096 [†])	Trace (65 536 [†])
6	872	2-Sulfanylethyl acetate	Roasted/plastic	0	10.6* (8)	11.8* (8)
7	900	Unknown	Fatty/sauce	0	0	1.3 (8)
8	905	Unknown	Flowered	0	0	0.7 (32)
9	921	4-Sulfanyl-4-methylpentan-2-one	Cat/blackcurrant	– (256)	0	– (4096)
10	939	Unknown	Flowered/rubber/caramel	– (2)	0.2 (32)	– (8)
11	984	3-Sulfanylpropyl acetate	Roasted	Trace (1)	0.3* (2)	1.5* (4)
12	991	3-Sulfanyl-3-methylbutyl formate	Blackcurrant	0.8* (64)	1* (64)	0.6* (64)
13	1031	3-Sulfanylbutyl acetate	Nettle/sesame	– (16)	– (4)	– (4)
14	1044	Unknown	Mushroom/soup	– (4)	– (4)	3.5 (32)
15	1106	3-Sulfanylohexanol	Rhubarb	– (2)	0	0.4 (8)
16	1111	4-Sulfanyl-4-methyl-2-pentyl acetate	Sesame/toast	– (64)	– (64)	– (16)
17	1113	3-Sulfanyloheptanal	Candy/citrus/almond	Trace (64)	– (2)	– (32)
18	1189	3-Sulfanyloheptanol	Cola/strawberry	– (16)	– (2)	– (64)
19	1219	3-Sulfanylohexyl acetate	Greenery/candy	– (64)	0	– (32)
20	1227	3-Sulfanyl-2-methylpentyl acetate	Blackcurrant	– (8)	– (16)	– (16)

RV6.V: Pilot beer made with *V. amygdalina*; RV6: Pilot beer made without *V. amygdalina*; IKT: Beer from Rwandese peasants; Trace, when the compound is detected under the quantification limit (0.15 µg/l); – : when not detected (<0.05 µg/l) by PFPD.

* Identifications confirmed by mass spectrometry (full scan monitoring).

† Co-elution on CP-Sil5-CB with traces of co-extracted methional.

thioesters allowed translation into the alkane-related decimal numeral system. GC-O and GC-PFPD identifications were checked by co-injection of the extracts with commercial standards and standards issued from combinatorial syntheses achieved according to Vermeulen *et al.*^[25–28]

Quantification of the compounds

Quantifications were achieved by GC-PFPD with the CP-Sil5-CB column. For commercially available thiols, complete calibration curves relative to the IST were used. For commercially unavailable thiols, quantifications are given in internal standard (IST) equivalent (4-methoxy-2-methyl-2-butanethiol added in the sample at 0.67 ng/l).

Analyses of humulones and lupulones by reverse phase high-performance liquid chromatography

Humulones and lupulones were analysed in *V. amygdalina* leaves according to the method recommended by the European Brewing Convention (EBC 7.4.1).^[31] Humulones and lupulones were extracted by a diethylether/methanol/hydrochloric acid (84:15.75:0.25, v/v) mixture. The HPLC (Waters 600 pump) was equipped with a Grace Smart RP 18 (250 × 4 mm, 5 μm) column and a Perkin-Elmer LC75 detector (314 nm) (Zaventem, Belgium).

Results and Discussion

Despite the absence of hop, many polyfunctional thiols were detected in the traditional and pilot *ikigage* beers (Table 1). As depicted in Figure 3, GC-PFPD analyses of the *p*-HMB extracts enabled us to identify six thiols in RV6.V beers [1-butanethiol (1), 2-sulfanylethanol (2), 3-sulfanylpropanol (5), 3-sulfanylpropyl acetate (11), 3-sulfanyl-3-methylbutyl formate (12) and 3-sulfanylheptanal (17)]; four in RV6 beers [(2), 2-sulfanylethyl acetate (6), (11) and (12)]; and six thiols in IKT beers [(2), (5), (6), (11), (12) and 3-sulfanylhexanol (15)]. Other polyfunctional thiols [3-methyl-2-buten-1-thiol (4), 4-sulfanyl-4-methylpentan-2-one (9), 3-sulfanylbutyl acetate (13), 4-sulfanyl-4-methyl-2-pentyl acetate (16), 3-sulfanylhexyl acetate (19) and 3-sulfanyl-2-methylpentyl acetate (20)] were detected by GC-O only. Nice GC-MS peaks made it possible to confirm the identification of compounds (1), (2), (6), (11) and (12).

GC-O analyses (AEDA methodology) showed 3-methyl-2-buten-1-thiol (4, MBT, skunky, garlic, old tea) as the most odorant thiol in the extracts from *ikigage* beers produced with *V. amygdalina* (RV6.V and IKT). Compared to the reference RV6, 3-methyl-2-buten-1-thiol (4) proved much more persistent (FD > 262 144) when *V. amygdalina* was used, either during boiling (RV6.V) or for yeast propagation (IKT). In hopped Western beers, the skunky off-flavour 3-methyl-2-buten-1-thiol is known to arise through the photochemical degradation of isohumulones.^[32] In light-protected beer, 3-methyl-2-buten-1-thiol is found at lower concentration,^[33]

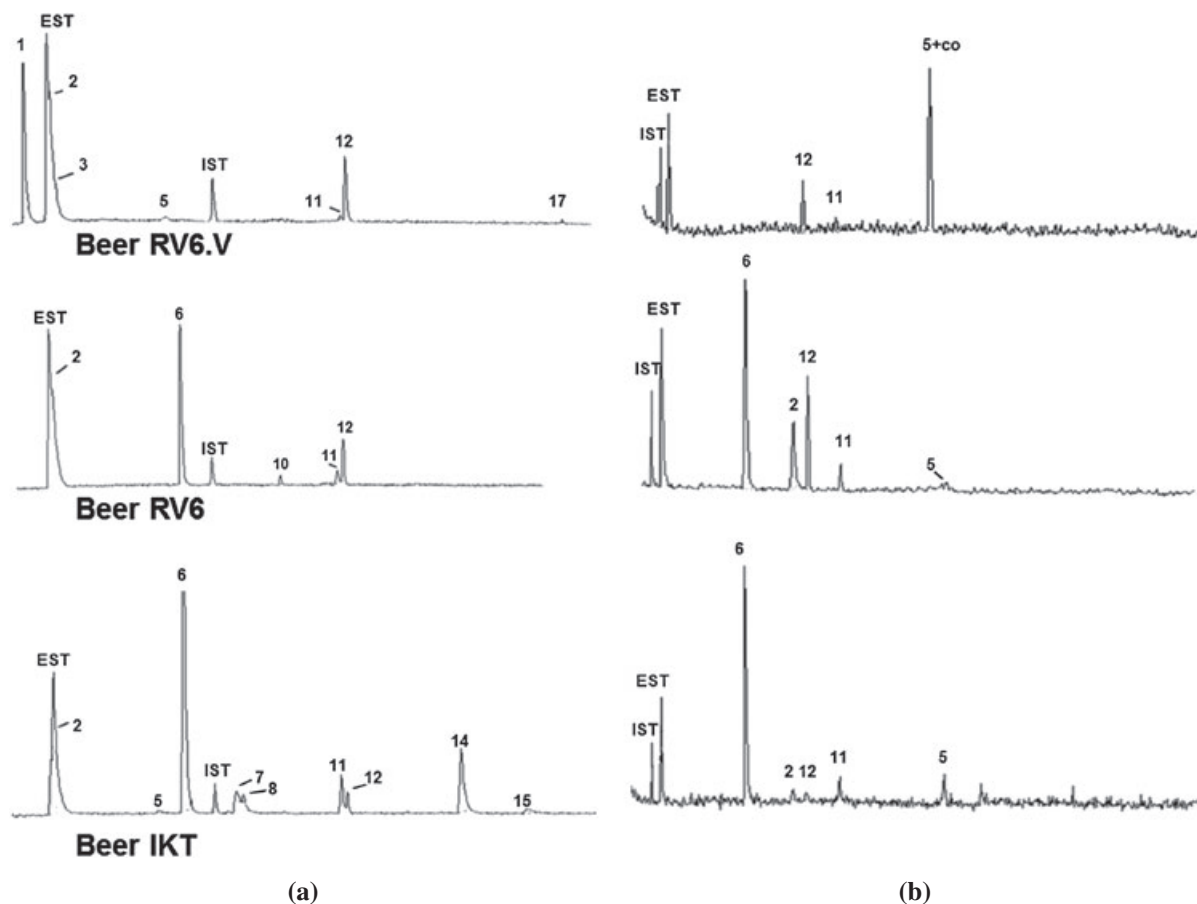


Figure 3. GC-PFPD chromatogram, (a) CP-Sil5-CB; (b) FFAP, of *p*-HMB extracts obtained from *ikigage* beer. Co = co-eluent of 3-sulfanylpropanol on the FFAP column (RI on CP-Sil5-CB > 1200; m/z = 55, 83, 141, 41, 82)

giving rise to pleasant hoppy flavours.^[34] In this case, it arises through nucleophilic substitution by hydrogen sulfide on 3-methyl-2-buten-1-ol.^[16] The amount of allylic alcohol is correlated with hop bitterness.^[35,36] Reports on previous studies carried out in Nigeria mentioned the presence of hop bitter compounds in *V. amygdalina*.^[13] In our HPLC analyses, we failed to detect any humulones or lupulones in Rwandese *V. amygdalina*. The source of this isoprenyl moiety thus remains to be identified (saponins or other plant metabolites).

Together with low amounts of 2-sulfanylethanol (**2**) and 3-sulfanylpropyl acetate (**11**), the most surprising result was the total absence of 2-sulfanylethyl acetate (**6**) in RV6.V (never observed before in usual hopped beers, where (**6**) is often the major PFPD peak^[16,23]). This compound was found at the expected concentration in the IKT and RV6 beers. Addition of *V. amygdalina* in the boiling kettle proved able to inhibit the production of 2-sulfanylethanol and 2-sulfanylethyl acetate. Complementary data are needed to understand how *V. amygdalina* leaves added through boiling can have such a strong impact on cysteine transformation via the Ehrlich pathway.^[16]

1-Butanethiol (**1**) emerged as another thiol distinguishing the pilot *ikigage* beer containing *V. amygdalina* (RV6.V). It is known to contribute to some meat, sea food and cheese flavours.^[37] Compound (**1**) was previously identified in American and Mexican beers,^[38] but turned out to be absent in all following studies focusing on beer thiols.^[16,22,23] This compound could arise through nucleophilic substitution by hydrogen sulfide on butan-1-ol.

4-Sulfanyl-4-methylpentan-2-one (**9**, catty/blackcurrant) was found in both unhopped beers containing *V. amygdalina* (FD=4096 in IKT and 256 in RV6.V). This compound has been identified as a key contributor to the blackcurrant/Muscat-like aroma in Tomahawk and Cascade hopped beers.^[17,23] As in the case of *p*-HMB extracts of Japanese beer^[22] and fresh hopped lager beers,^[16] only GC-O enabled us to detect it in *p*-HMB extracts of *ikigage* beers. On the other hand, Kishimoto,^[17] Takoi^[22] and Gros^[23] were able to quantify it by PFPD or GC-MS in hop samples. In wine, (**9**) has been shown to be issued from the biolysis of a cysteine conjugate S-4-(methylpentan-2-one)-l-cysteine and from Michael addition of hydrogen sulfide on mesityl oxide.^[39,40]

3-Sulfanyl-3-methylbutyl formate (**12**, blackcurrant) was found in all three beers (Figure 3b). This compound is usually not detected in fresh industrial lager beers^[16] but it can appear through ageing.^[41] It is also found in roasted seeds such as coffee^[42,43] and white sesame,^[44] where it results from the reaction between formic acid and 3-sulfanyl-3-methylbutanol in the presence of oxygen and heat.^[42,44] The absence of hop polyphenols in the *ikigage* beers may explain the occurrence of this oxidation-derived molecule.

In conclusion, *V. amygdalina* leaves are emerging as an interesting hop substitute for tropical African countries,^[6–14] but the thiol profile of the resulting beers will be significantly different. Even when only used for leaven preparation, *V. amygdalina* brings some plant-derived polyfunctional thiols to beer. *V. amygdalina* addition during boiling also appears to strongly inhibit the production of 2-sulfanylethyl acetate. Complementary data are needed to understand which thiol precursors pre-exist in the plant, and by which mechanisms *V. amygdalina* might impact the yeast Ehrlich pathway.

Acknowledgements

The authors thank the Student Financing Agency of Rwanda (SFAR) and Walloon Center of Industrial Biology (CWBI) for financial support.

References

1. F. Lyumugabe, G. Kamaliza, E. Bjanya, Ph. Thonart, *Afr. J. Biotechnol.* **2010**, *9*, 4241.
2. A. P. P. Kayode, A. Aégbidi, A. R. Linnemenn, M. J. R. Nout, J. D. Hounhouigana, *Ecol. Food Nutr.* **2005**, *44*, 271.
3. J. A. Ekundayo, *J. Food Technol.* **1969**, *4*, 217.
4. N. Maoura, M. Mbaiguinam, H. V. Nguyen, C. Gaillardin, J. Pourquie, *Afr. J. Biotechnol.* **2005**, *4*, 646.
5. A. Nzigamasabo, A. Nimpagaritse, *Food Res. Int.* **2009**, *42*, 588.
6. N. Okafor, G. N. Anichie, *Brew. Dist. Int.* **1983**, *13*, 20.
7. J. O. Ogundiwin, M. O. Ilori, *Lebensm. Wiss. Technol.* **1991**, *24*, 182.
8. I. A. Okoh, G. O. Babalola, M. O. Ilori, *Technol. Quart. Master Brew. Assoc. Am.* **1995**, *32*, 11.
9. O. O. Lasekan, W. O. Lasekan, J. O. Babalola, *Food Chem.* **1999**, *64*, 507.
10. P. E. Ajebesome, J. O. Aina, *Afr. J. Food Technol.* **2004**, *9*, 13.
11. C. I. Mboto, M. E. Eja, A. A. Adegoke, G. D. Iwatt, B. E. Asikong, I. Takon, S. M. Udo, M. Akeh, *Afr. J. Microbiol. Res.* **2009**, *3*, 557.
12. F. O. J. Oboh, H. I. Masodje, *Int. J. Biomed. Health Sci.* **2009**, *5*, 5.
13. C. C. Okoro, J. O. Aina, *Afr. J. Biotechnol.* **2007**, *6*, 1479.
14. W. Adenuga, O. N. Olaleye, P. A. Adepoju, *Afr. J. Biotechnol.* **2010**, *9*, 8819.
15. C. Vermeulen, S. Lecocq, S. Collin, In *Proceedings of the 29th European Brewery Convention Congress. Dublin, Ireland.* Fachverlag Hans Carl: Nürnberg, Germany, ISBN: 798-90-70143-24-4, **2003**, *91*, 930–940.
16. C. Vermeulen, I. Lejeune, T. T. H. Tran, S. Collin, *J. Agric. Food Chem.* **2006**, *54*, 5061.
17. T. Kishimoto, A. Wanikawa, K. Kono, K. Shibata, *J. Agric. Food Chem.* **2006**, *53*, 8855.
18. H. K. Engel, R. Tressl, *J. Agric. Food Chem.* **1991**, *39*, 2249.
19. T. Tominaga, A. Furrer, R. Henry, D. Dubourdieu, *Flavour Fragr. J.* **1998**, *13*, 159.
20. A. Olsen, *Carlsberg Res. Commun.* **1988**, *53*, 1.
21. J. Gros, S. Nizet, S. Collin, *Acta Hort.* **2009**, *848*, 273.
22. K. Takoi, M. Dequeil, S. Shinkaruk, C. Thibon, K. Maeda, K. Ito, B. Benetau, D. Dubourdieu, T. Tominaga, *J. Agric. Food Chem.* **2009**, *57*, 2493.
23. J. Gros, S. Nizet, S. Collin, *J. Agric. Food Chem.* **2011**, *59*, 8853.
24. T. Tominaga, M. L. Murat, D. Dubourdieu, *J. Agric. Food Chem.* **1998**, *46*, 1044.
25. C. Vermeulen, J. Pellaud, L. Gijs, S. Collin, *J. Agric. Food Chem.* **2001**, *49*, 5445.
26. C. Vermeulen, S. Collin, *J. Agric. Food Chem.* **2002**, *50*, 5654.
27. C. Vermeulen, S. Collin, *J. Agric. Food Chem.* **2003**, *51*, 3618.
28. C. Vermeulen, C. Guyot-Declerck, S. Collin, *J. Agric. Food Chem.* **2003**, *51*, 3623.
29. W. Grosch, *Flavour Fragr. J.* **1994**, *9*, 147.
30. M. A. Igyor, A. C. Ogbonnab, G. H. Palmer, *Process Biochem.* **2001**, *36*, 1039.
31. Analytica EBC. *Hops* (7.4.1), Fachverlag Hans Carl: Nürnberg, **1998**.
32. C. S. Burns, A. Heyerick, D. de Keukeleire, M. D. E. Forbes, *Chem. Eur. J.* **2001**, *7*, 4554.
33. D. Callemien, S. Dasnoy, S. Collin, *J. Agric. Food Chem.* **2006**, *54*, 1409.
34. G. Lermusieau, S. Collin, *J. Am. Soc. Brew. Chem.* **2003**, *61*, 109.
35. H. Goldstein, P. Ting, A. Navarro, D. Ryder. In *Proceedings of the 27th European Brewery Convention; Cannes, France.* IRL Press Ltd.: Oxford, England, **1999**; 53–62.
36. J. Gros, S. Collin, *Cerevisia* **2012**, *37*, 10.
37. J. A. Maga, *Crit. Rev. Food Sci. Nutr.* **1976**, *7*, 147.
38. M. C. Meilgaard, *MBAA Technol. Q.* **1975**, *12*, 151.
39. T. Tominaga, C. Peyrot des Gachons, D. Dubourdieu, *J. Agric. Food Chem.* **1998**, *46*, 5215.
40. R. Schneider, F. Charrier, A. Razungles, R. Baumes, *Anal. Chim. Acta* **2006**, *563*, 58.
41. P. Schieberle, Z. *Lebensm.-Unters. Forsch. A* **1991**, *193*, 558.
42. I. Blank, A. Sen, W. Grosch, Z. *Lebensm. Unters. Forsch.* **1992**, *195*, 239.
43. W. Holscher, O. G. Vitzthum, H. Steinhart, *J. Agric. Food Chem.* **1992**, *40*, 655.
44. H. Tamura, A. Fujita, M. Steinhaus, E. Takahisa, H. Watanabe, P. Schieberle, *J. Agric. Food Chem.* **2011**, *59*, 10211.