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Increasing Dimethylsulfide and Polyfunctional Thiols, an Opportunity to Enhance the Fruity Flavors of NABLABs

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ABSTRACT

At present, non-alcoholic beers and low-alcoholic beers (NABLABs) suffer from premature oxidation when fresh, and from a lack of fruity fermentation aromas. In wines, dimethylsulfide (DMS) is known to enhance truffle nuances and the fruity character. This offers an opportunity to improve the flavor of NABLABs. In this work, levels of free DMS and its potential precursors (S-methylmethionine/SMM and dimethylsulfoxide/DMSO) were assessed in eleven commercial NABLABs, in parallel with their amounts of fruity esters and polyfunctional thiols. Except in two dry-hopped samples and a fruity beer, free DMS was detected at very low levels in all fresh NABLABs (four samples even displayed no detectable GC peak), likely because of dealcoholization or too-short fermentation. Through NABLABs aging, the free DMS concentration increased (+63% on average after two years), at a degree correlated with the initial SMM level (2–118 µg/L DMS eq.; R^2 = 0.79). This SMM amount revealed to be also correlated with the group I amino acid residual content (more consumed through traditional fermentations). Unlike DMSO, SMM showed significant release of free DMS after aging in spiking experiments (4% degradation after 30 days at 20 °C). As fruity fermentation esters are found in NABLABs at much lower concentrations than in conventional lagers, increasing both DMS and polyfunctional thiols by dry hopping (or DMS-enriched extracts) emerges as an opportunity to improve them.

Abbreviations: NABLABs: non-alcoholic beers and low-alcoholic beers; IST: internal standard; GC-MS: gas chromatography with electronic impact mass spectrometry; SIM: single ion monitoring; EI: electron ionization; WCOT: wall-coated open tubular; RT: retention time; GC-PFPD: gas chromatography with pulsed flame photometric detection; GC-FID: gas chromatography with flame ionization detector; HPLC: high performance liquid chromatography; HS: headspace; DMS: dimethylsulfide; EMS: ethylmethylsulfide; SMM: S-methylmethionine; DMSO: dimethylsulfoxide; PDMS: dimethylsulfide precursors; 2SEol: 2-sulfanylethan-1-ol; 3SProl: 3-sulfanylpropan-1-ol; 2SEA: 2-sulfanylethyl acetate; 3SPrA: 3-sulfanylpropyl acetate; 3SHol: 3-sulfanylhexan-1-ol; 3SHA: 3-sulfanylhexyl acetate; 3S4MPol: 3-sulfanyl-4-methylpentanol; 4S4M2Pone: 4-sulfanyl-4-methylpentan-2-one; 3SPol: 3-sulfanylpentan-1-ol; 3S4MPA: 3-sulfanyl-4-methylpentyl acetate; AA: amino acids; UPLC-UV: ultra performance liquid chromatography with ultraviolet detection; BBD: best before date; DH: dry hopped beers.

KEYWORDS

Aging; DMS; DMSO; flavor enhancer; NABLABs; SMM

Introduction

Dimethylsulfide (DMS) is a volatile sulfur compound known to play a key role in the flavor of a range of beverages (beer, wine, tea, milk, etc.)[1–4] and other foodstuffs (cheese, corn, asparagus, etc.).[5–7] It occurs naturally in beer, at concentrations ranging from 5 to 90 µg/L.[1,8] Its perception threshold is close to 30–50 µg/L.[8–10] At excessive levels, DMS can induce off-flavors described as "cooked-vegetable", "canned corn", "cooked cabbage", or onion.[1,9,11,12] At 30–100 µg/L, it is an essential contributor to the typical aroma of European lagers.[8,9,13] Its analysis is very easy in quality control, thanks to its high volatility (boiling temperature: 37 °C), either by headspace (HS) or solid phase microextraction (SPME) hyphenated to GC-FID, -MS, or -PFPD.[14–15]

Beer dimethylsulfide results from two main precursors: S-methylmethionine (SMM) and dimethylsulfoxide (DMSO), known in the literature as "potential DMS" or PDMS (derived from both malt[9,16,17] and hop[18]).

SMM is synthesized by S-adenosyl-L-methionine: L-methionine S-methyltransferase during the germination of barley grain (at about 30–60 mg/kg).[16,17,19] It is heat-labile and decomposes to DMS and L-homoserine by thermal degradation during malt kilning (Figure 1).[1,20–22] Malting conditions (steeping and germination temperature, use of

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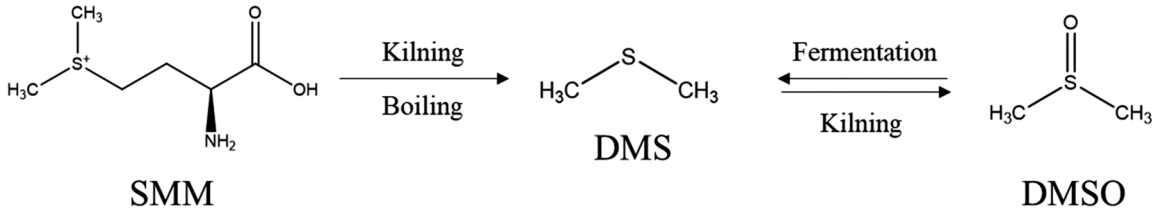

Figure 1. Formation pathways for dimethylsulfide in beer.

gibberellic acid, etc.) and particularly kilning design are crucial factors affecting the SMM level and DMS volatilization.[1,9,16,23] SMM also generates DMS during mashing (especially decoction mashing)[9] and wort boiling.[1,24] In any case, part of this DMS evaporates quickly during the boiling step.[1,9,24] A crucial step is therefore wort clarification, during which SMM continues to be degraded, without subsequent evaporation. This contributor to PDMS is usually determined in wort by heat-alkali treatment (100 °C, 1 h, 1 M NaOH), which converts SMM to DMS.[21–22] It can also be obtained by HPLC quantification methods.[25]

During kilning, dimethylsulfoxide is formed above 75 °C by DMS oxidation (Figure 1).[1,9,12,19,22,26] It is then extracted from the grist during mashing. With a boiling temperature of 189 °C, it is unfortunately not lost during boiling.[1,9,12] To an extent depending on the choice of yeast (*S. cerevisiae* more efficient than *S. pastorianus*),[27] DMSO can be reduced to DMS during fermentation by the enzyme methionine sulfoxide reductase (Figure 1).[1,9,14,28] Its conversion can be increased by several factors:[1,9,16,28] a low free amino nitrogen (FAN) content[29–30] (and hence the use of unmalted cereals), a high-gravity wort,[27] a low fermentation temperature,[27,31] a high pH, a high cylindroconical fermenter,[32] etc. DMS can be lost further during fermentation, through CO_2 stripping.[9,14] Note that DMSO can also be degraded to DMS by wort(beer)-spoilage bacteria, as in the case of lambic products.[1,9,33,34] GC-analysis of DMSO is either direct (MS detector[35]) or indirect (reduction to DMS by $Na_2S_2O_5$ at 50 °C, 90 min or by $SnCl_2$/HCl at 100 °C, 30 min).

DMS is also present in hop,[36–37] but not at sufficient levels to contribute to the flavor of the final beer when it is not dry-hopped.[21,38,39] Yet recent studies have reported that hop DMS precursors can contribute to the final level of DMS in American commercial IPAs. In experimental aged (40 days at 40 °C) dry hopped beers (10 g/L), DMS concentrations up to 275 µg/L were measured, suspected by the authors of having derived from PDMS (not clearly identified as either SMM or DMSO).[18]

DMS is also reported to contribute indirectly to wine flavor,[40] either positively (by accentuating truffle notes) or negatively (by producing unpleasant odors of hay or green olives), depending on its concentration and on wine type (grape variety and age).[35,44–47] In freshly bottled wines the level of DMS is low, but during aging it increases significantly[35,41,42,46,47] (e.g.: from 3 µg/L in younger wines to 711 µg/L in older vintages).[44,48] When present above its threshold (e.g.: 100 µg/L), DMS may confer blackcurrant and raspberry notes to young wines, sweet-fruity or green olive notes to dearomatized wines, and truffles with undergrowth nuances to older wines.[41,43,44,49–51] Recent data show that DMS could also act as a natural enhancer in wine, modulating the perception of fruity flavors.[41,43,44,52] Through complex synergistic effects, it interacts with other volatiles and modifies their aromatic perception (e.g.: perception of "vegetable" notes with methionol and hexanol, accentuation of fruity notes with ethyl esters or C13-norisoprenoids).[40,50,53,54] It is suspected that wine DMS derives from SMM,[2,35,42,47,55] DMSO,[35,42,56] or glutathione.[41]

The aim of this study was to make better use of recent wine researches to propose new opportunities to enhance the fruity flavors in NABLABs. The lack of esters[57–58] and the presence of oxidation-related stale odorants[59] are indeed pointed out as their main defects, whatever the process. DMS (here determined by an accurate HS-GC-PFPD method), fruity esters (HS-GC-MS) and polyfunctional thiols (silver cartridge extraction-GC-PFPD) were quantitated in a panel of eleven commercial NABLABs (including lager, amber, white, acidic, and dry-hopped beers) issued from different technological processes. Dealcoholization methods based on removing alcohol from conventional beer by vacuum distillation could lead to significant or total loss of DMS, while biological processes, through adjustment of the fermentation conditions, could limit its formation from DMSO.[60–61] Changes in parameters such as temperature and the use of special yeasts/microorganisms could also alter the degree of DMSO reduction to DMS. Moreover, the relatively low density of the worts generally used to produce NABLABs (about 5°P) can limit DMSO reduction and the availability of SMM in the wort. In comparison with the level of residual group I amino acids (a good indicator of the fermentation process), both potential DMS contributors were further quantitated in the samples. The fate of them was also checked in spiked media through natural and forced aging.

Experimental

Chemicals

Acetonitrile, dichloromethane, methanol, sodium chloride, sodium hydroxide, and absolute ethanol (99%) were purchased from VWR International (Leuven, Belgium). Dimethylsulfide, ethylmethylsulfide, dimethylsulfoxide, dimethylsulfoxide-d_6 (99.9 atoms % D), DL-methionine methylsulfonium chloride, 2-pentanol, ethyl acetate, 3-methylbutanol, 3-methylbutyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, 2-acetylthiophene, 6-mL Discovery Ag-ion SPE tube, > 98% L-cysteine hydrochloride monohydrate, 4-methoxy-

2-methylbutane-2-thiol, 2-sulfanylethan-1-ol (2SEol), 3-sulfanylpropan-1-ol (3SProl), 2-sulfanylethyl acetate (2SEA), 3-sulfanylpropyl acetate (3SPrA), 3-sulfanylhexan-1-ol (3SHol), 3-sulfanylhexyl acetate (3SHA), and 4-sulfanyl-4-methylpentan-2-one (4S4M2Pone) were obtained from Sigma-Aldrich (St-Louis, U.S.A.). Anhydrous sodium sulfate was purchased from Acros Organics (Geel, Belgium). AccQ•Tag Ultra Reagent derivatization (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, AQC), AccQ•Tag Ultra Eluent I, AccQ•Tag Ultra Eluent II, AccQ•Tag Ultra borate buffer, and amino acid hydrolysate standard were purchased from Waters Corporation (Milford, U.S.A.). Milli-Q water was used (Millipore, Bedford, U.S.A.).

Beer samples

Eleven commercial NABLABs were investigated: Star Light (A), Energibajer (B), Pico Bello (C), Leopold 7 Road Trip (D), Palm N.A. (E), Maes 0.0% (F), Hoegaarden rosée 0.0% (G), Carlsberg 0.0% (H), Jupiler 0.0% (I), Leffe Blonde 0.0% (J), and Brugse Sport Zot alcoholvrij (K). The beers, either received from brewers or bought at Belgian markets (freshly released), were analyzed in duplicate. All the beers were also stored for a long period (two years at 20 °C in the dark) to mimic a wine aging.

Quantification of free dimethylsulfide by static headspace – gas chromatography – pulsed flame photometric detection (HS-GC-PFPD)

Prior to analysis, the beers were stored at 4 °C for 2 h. To 5 mL beer in a headspace vial were added at 4 °C 300 µL ethylmethylsulfide (EMS) solution (500 µg/L; final concentration in beer = 30 µg/L) as internal standard (IST) and NaCl in excess (2 g), and the vial was immediately closed before analysis. The vials were incubated at 45 °C and automatically shaken for 15 min before injection of 500 µL headspace (Gerstel automatic injector, MultiPurposeSampler MPS2, Gerstel 2.5-mL syringe at 50 °C). Dimethylsulfide was analyzed with a wall-coated open tubular (WCOT) apolar capillary column (CP-Sil 5 CB, 50 m × 0.32 mm, 1.2 µm film thickness) on an Agilent 6890 N gas chromatograph equipped with a splitless injector maintained at 250 °C. The carrier gas was helium, and the pressure was set at 90 kPa. The oven temperature was programmed to start at 40 °C for 10 min, to rise from 40 to 85 °C at 20 °C/min and then held at 250 °C for 10 min (RT = 7.2 and 11.5 min for DMS and EMS, respectively). The column was connected to an OI Analytical PFPD detector (model 5380, combustor, internal diameter: 2 mm). The following parameters were selected for the PFPD detector: 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. The ChemStation software was used to process the resulting data. A standard addition procedure for DMS was applied (preparation at 4 °C) to each type of NABLABs (lager, amber, or dry-hopped beer). The standard addition slope A was used according to the following equation: DMS concentration (in µg/L) = 1/A × IST concentration (in µg/L) × (DMS area/IST area).

Quantification of SMM by heat-alkali treatment and measurement of released DMS

Prior to analysis, free DMS was removed from the sample by nitrogen gas stripping. The sample was then subjected to thermal treatment under alkaline conditions:[17,35,62] 200 mg sodium hydroxide was added to the 5-mL sample to obtain a 1 M solution. The headspace vial was heated at 100 °C for 1 h and then allowed to cool. Next, 300 µL EMS solution (500 µg/L; final beer concentration: 30 µg/L) as IST, and NaCl in excess were added to the sample and the DMS released by the reaction (SMM given in DMS eq.) was quantitatively determined as described above for free DMS.

Quantification of DMSO by gas chromatography – electron impact mass spectrometry (GC-MS)

To a 10-mL beer sample was added 75 µL [$^2H_6$]-DMSO (40 mg/L; final concentration in beer: 300 µg/L) as IST. The sample was adsorbed at room temperature onto a Chem Elut S cartridge (Agilent, Santa Clara, CA, U.S.A.). DMSO was then recovered with 2 × 25 mL bidistilled dichloromethane. The eluate was concentrated to 500 µL in a Danish-Kuderna distillation apparatus for GC-MS analysis. One microliter of each DMSO extract was analyzed with an Agilent Technologies 7890 NB Gas Chromatograph System equipped with a splitless injector maintained at 250 °C. DMSO was separated from the mixture with a WCOT apolar capillary column (CP-Sil 5 CB, 50 m × 0.32 mm, 1.2 µm film thickness). The carrier gas was helium, and the pressure was set at 100 kPa. The oven temperature was programmed to rise from 36 °C to 85 °C at 20 °C/min, then to 145 °C at 1 °C/min, and at last to 250 °C at 30 °C/min, and was then held at this temperature for 30 min. The column was connected to a single quadrupole mass spectrometer (Agilent 5977B MSD) operating in single ion monitoring (SIM) mode with electron ionization (EI) at 70 eV. The following *m/z* values were monitored: 66 and 84 for dimethylsulfoxide-d_6 (IST), 63 and 78 for dimethylsulfoxide. Chromatograms were recorded throughout elution. Agilent OpenLab software was used to record the resulting data. A standard addition slope was constructed and the following equation was used for quantitation (IST relative recovery factor set at 1): DMSO concentration (in µg/L) = IST concentration (in µg/L) × (DMSO area/IST area) × (IST response coefficient/DMSO response coefficient). A factor of 0.8 was used to translate DMSO in DMS eq.

Quantification of group I amino acids by ultra performance liquid chromatography - UV detection (UPLC-UV)

10 µL of a degassed beer sample, filtered through a Chromafil polyester filter (0.22 µm, Macherey-Nagel, Düren, Germany),

was mixed with 70 µL of borate buffer and 20 µL of AQC derivatization reagent. The mixture was then heated at 55 °C for 10 min. An ACQUITY UPLC liquid chromatography system (Waters Corporation), equipped with a degasser, an autosampler, an oven, a quaternary pump and a UV detector set at 210 nm was used. Separation was carried out on ACQUITY UPLC BEH C18 (100 × 2.1 mm, 1.7 µm column - Waters Corporation) at a flow rate of 0.65 mL/min, with a mixture of A (Eluent I), B (10% Eluent II in water), C (water) or D (Eluent II). Gradient elution was as follows: 0.0–0.29 min, 10–9.9% A and 90–90.1% C; 0.29–5.49 min, 9.9–9% A, 0–80% B and 90.1–11% C; 5.49–7.10 min, 9–8% A, 80–15.6% B, 11–57.9% C and 0–18.5% D; 7.10–7.30 min, 8% A, 15.6% B, 57.9% C and 18.5% D; 7.30–7.69 min, 8–7.8% A, 15.6–0% B, 57.9–70.9% C and 18.5–21.3% D; 7.69–7.99 min, 7.8–4% A, 70.9–36.3% C and 21.3–59.7% D; 7.99–8.59 min, 4% A, 36.3% C and 59.7% D; 8.59–8.68 min, 4–10% A, 36.3–90% C and 59.7–0% D; 8.68–10.20 min, 10% A and 90% C. One microliter of mixture was injected into the column kept at 42 °C. Chromatograms were recorded throughout elution using Empower 2 software. Group I amino acid identification was done by injection of a commercial mixture of standards. Quantification was achieved using the calibration curves.

Chemical conversion of SMM to DMS in beer

To investigate in beer the potential degradation of SMM to DMS, several trials were conducted. A beer sample (Jupiler 0.0, 330 mL) was spiked with 100 µg/L SMM (diluted in water) and directly recapped (after manual purging by foaming). The DMS level was quantified by HS-GC-PFPD (as described above) directly after SMM addition, after forced aging (5, 8, and 30 days at 45 °C), and after natural aging (5, 8, and 30 days at 20 °C). A control sample (without SMM addition) was also analyzed after 5, 8, and 30 days at 45 °C. Experiments were done in duplicate.

Chemical conversion of DMSO to DMS in beer

To investigate whether DMSO can be chemically reduced to DMS in beer, several trials were conducted. A beer sample (Jupiler 0.0, 330 mL) was spiked with 250 µg/L DMSO (diluted in water) and directly recapped (after manual purging by foaming). The DMS level was quantified by HS-GC-PFPD (as described above) directly after DMSO addition, after forced aging (5, 8 and 30 days at 45 °C), and after natural aging (5, 8 and 30 days at 20 °C). A control sample (no DMSO added) was also analyzed after 5, 8 and 30 days at 45 °C. Experiments were done in duplicate.

Quantification of esters and higher alcohols by static headspace – gas chromatography – electron impact mass spectrometry (HS-GC-MS)

Prior to analysis, the beers were stored at 4 °C for 2 h to avoid excessive foaming. Then, 40 µL 2-pentanol solution (2500 mg/L; final beer concentration: 20 mg/L) as IST and NaCl in excess (2 g) were added to 5 mL beer in a headspace vial, which was immediately closed before analysis. The vials were incubated at 60 °C and automatically shaken for 30 min before injection of 500 µL of headspace (automatic injector CTC Analytics Combipal, Hamilton 2.5-mL syringe at 70 °C). Esters and higher alcohols were analyzed with the column described above for DMS, in this case on an Agilent Technologies 7890 NB GC hyphenated to a single quadrupole mass spectrometer (Agilent 5977B MSD) operating in SIM mode with EI at 70 eV. The carrier gas was helium, and the pressure was set at 65 kPa. The oven temperature was programmed to start at 32 °C for 5 min and then to rise from 32 to 140 °C at 8 °C/min, from 140 to 180 °C at 15 °C/min, and was finally held at 180 °C for 30 min. The following *m/z* ions were analyzed: 45 and 55 for 2-pentanol (IST), 61 and 70 for ethyl acetate, 55 and 70 for 3-methylbutanol, 70 and 87 for 3-methylbutyl acetate, 88 and 99 for ethyl hexanoate, 88 and 127 for ethyl octanoate, and 88 and 101 for ethyl decanoate. Chromatograms were recorded throughout elution. Agilent OpenLab software was used to record the resulting data. A standard addition procedure was applied for each compound.

Quantification of polyfunctional thiols by GC-PFPD after selective Ag cartridge extraction

As previously optimized by Chenot et al.,[63] 2 µg/L 4-methoxy-2-methylbutane-2-thiol was added as IST to 150 mL beer, which was then saturated with NaCl and stirred with 50 mL dichloromethane for 15 min. The mixture was centrifuged at 4500 rpm for 15 min. The recovered organic phase was loaded onto a Discovery Ag-ion SPE cartridge conditioned beforehand with 10 mL dichloromethane. The cartridge was rinsed with 10 mL dichloromethane, then with 20 mL acetonitrile, and finally with 10 mL ultrapure water (reversed cartridge in this last case). Free thiols were released from the Ag cartridge by percolating 20 mL washed cysteine solution (4 × 20 mL dichloromethane for washing 215 mg cysteine in 20 mL water). The eluent was extracted twice with bidistilled dichloromethane (5 mL for 5 min and 10 mL for 10 min). The resulting organic phase was dried on anhydrous sodium sulfate and concentrated to 250 µL in a Danish-Kuderna distillation apparatus and to 70 µL on a Dufton column at 45 °C. 2-Acetylthiophene was added as external standard (EST, 0.5 mL at 200 µg/L added before concentration).

One microliter of free thiol extract was analyzed with a ThermoFinnigan Trace GC 2000 gas chromatograph equipped with a splitless injector maintained at 250 °C. Compounds were analyzed with the column described above for DMS and esters. The helium pressure was set at 90 kPa. The oven temperature was programmed to increase from 36 to 85 °C at 20 °C/min, then to 145 °C at 1 °C/min, and finally to 220 °C at 3 °C/min, and was held at this temperature for 30 min. The column was connected to the OI Analytical PFPD detector described for DMS analysis, with the same operational parameters and the same Chemstation software.

The following equation was used for quantitation of the commercially available standards 2SEol, 3SProl, 2SEA, 3SPrA, 3SHA, 3SHol, and 4S4M2Pone: thiol concentration (in µg/L) = IST concentration (in µg/L) × (thiol area/IST area) × (IST molar response coefficient/thiol molar response coefficient) × (thiol molar weight/IST molar weight) × (IST recovery factor/thiol recovery factor). For the commercially unavailable standards, 3-sulfanylpentan-1-ol (3SPol), 3-sulfanyl-4-methylpentanol (3S4MPol), and 3-sulfanyl-4-methylpentyl acetate (3S4MPA), the good equimolarity of the PFPD detector enabled us to set the IST-relative molar response coefficients at 1 and to apply only the corrective molar weight ratio. For all thiols, the IST-relative recovery factor was set at 1 (experimental values from 0.8 to 1.2, determined beforehand by standard addition).

Beer sample microbiological controls

Microbiological analyses of the beer samples were performed according to Section 4.0 of the European Brewery Convention-Analytica Microbiologica for detecting contaminants in beer.[64] For aerobic bacteria, WLD (Wallerstein Laboratory Differential Agar) was used with cycloheximide to inhibit yeast growth. To isolate Gram-positive lactic acid bacteria, selective mMRS medium (Manosa Rogosa, Sharpe Agar and Raka Ray) was selected. To prevent yeast growth and inhibit growth of Gram-negative bacteria, 10 mL/L of a sterile cycloheximide solution (40 mg/100 mL) was added to these media. All determinations were performed under sterile conditions in a vertical laminar flow hood. All the samples seeded in the culture media were incubated at 27 °C for two weeks. At the end of the incubation, the results of the plates were recorded by direct visual examination.

Statistical analyses

All analytical measurements were carried out in duplicate. Multiple comparisons of means were performed with Student-Newman-Keuls tests (JMP Program). Values sharing no common letter in the same row of a table are significantly different ($p < 0.05$).

Results and discussion

HS-GC-PFPD determination of DMS levels in fresh commercial NABLABs

In most of the fresh commercial NABLABs investigated here (A, D, F, G, K), whatever the process, dimethylsulfide was evidenced only in trace amounts (< 22 µg/L, Figure 2 and Table 1), far below its perception threshold (30–50 µg/L).[60–61] No free DMS at all was found in beers H, I, and J, likely because it was lost during the dealcoholization process (DMS boiling temperature: 37 °C). DMS was also undetectable in beer E, for which a cold contact process was used. In this case, it can be assumed that its low original gravity brought little SMM, and that its short cold fermentation step didn't allow DMSO reduction. The dry hopping process emerges here as a potential way to significantly increase the DMS level of NABLABs, as can be seen for beers B and C, the only samples in which 50 and 78 µg/L were observed.

Fruity fermentation ester (and alcohol) quantitation in NABLABs

As depicted in Figure 3, fermentation ester concentrations were much lower than in conventional lagers. All dealcoholization technologies led to significant losses of these compounds, while

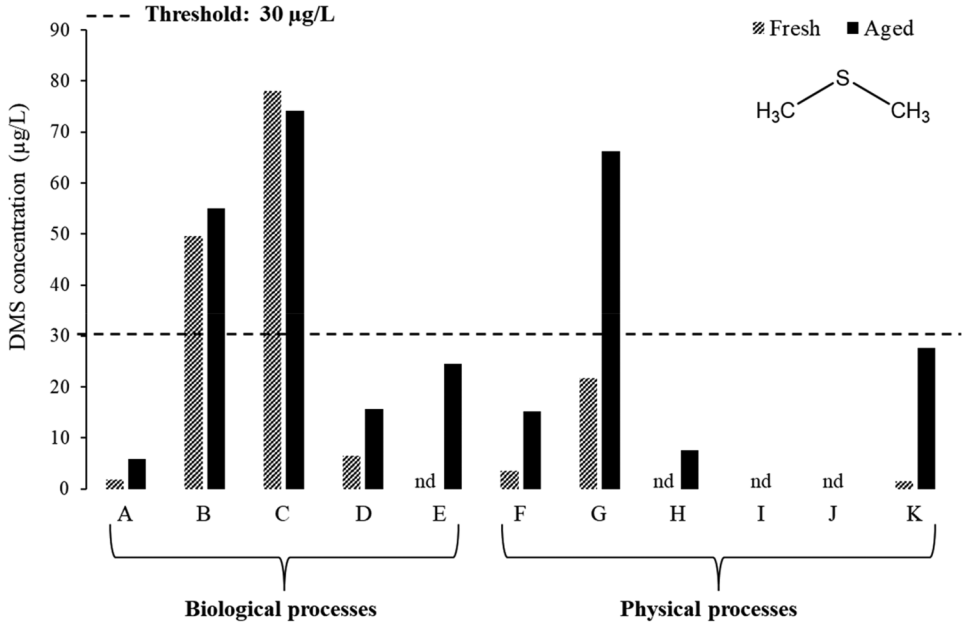

Figure 2. Concentration (µg/L) of free dimethylsulfide in NABLABs, fresh and after two years of storage at 20 °C in the dark (nd: not detected; inferior to LOD of 1 µg/L). Threshold corresponds to that of a conventional beer.[8] Means of duplicates (coefficient of variation <10%).

Table 1. Free DMS, SMM and DMSO levels (µg/L) in eleven fresh NABLABs. Means of duplicates (coefficient of variation <10% both for the DMS/SMM HS-GC-PFPD and DMSO GC-MS analyses).

	Biological processes					Physical processes					
	Special yeast			*Mixed fermentation*	*Cold Contact*	*Distillation*					*Membrane filtration*
		Dry-hopped									
Beer	A	B	C	D	E	F	G	H	I	J	K
Alcohol content (% ABV)	0.5	0.3	0.2	0.8	0.1	< 0.1	< 0.1	0.1	< 0.1	< 0.1	0.5
Free DMS (µg/L) (in bold when exceeding the flavor threshold of 30–50 µg/L)[8–10]	1.8^{e}	**49.6**^{b}	**78.0**^{a}	6.5^{d}	nd^{e}	3.5^{d,e}	21.7^{c}	nd^{e}	nd^{e}	nd^{e}	1.5^{e}
SMM (µg/L DMS eq.)	nd^{d}	64.3^{b}	60.8^{b}	1.7^{d}	29.0^{c}	6.2^{d}	117.5^{a}	5.2^{d}	nd^{d}	nd^{d}	6.4^{d}
DMSO (µg/L DMS eq.)	83^{e}	191^{c,d}	159^{d}	74^{e}	260^{a,b}	273^{a,b}	87^{e}	304^{a}	185^{c,d}	234^{b,c}	300^{a}

Within a line, values with different letters are significantly different ($p < 0.05$) according to the Student-Newman-Keuls test; nd: not detected (inferior to LOD of 1 µg/L both for GC-PFPD and GC-MS).

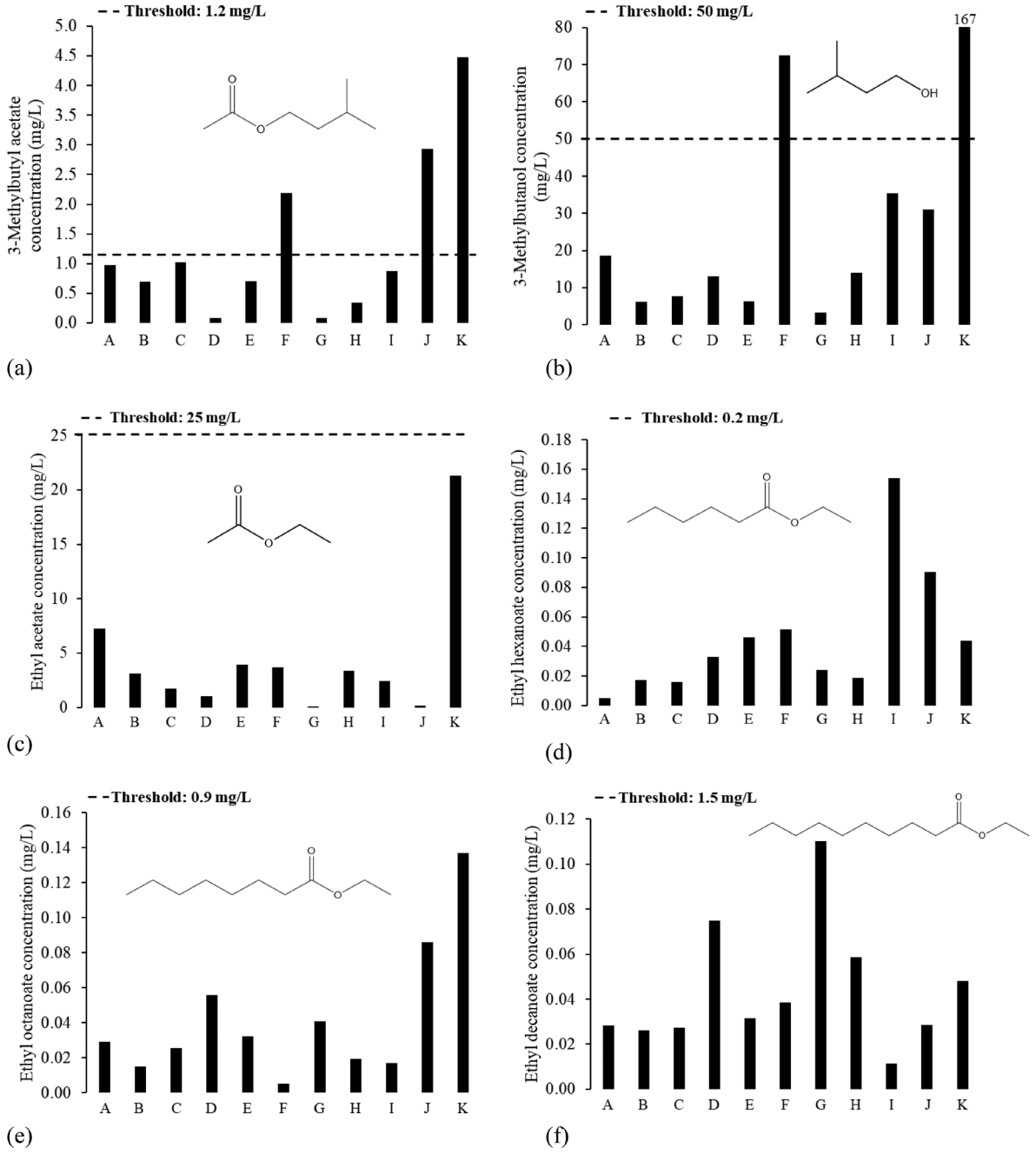

Figure 3. Concentration (mg/L) of (a) 3-methylbutyl acetate, (b) 3-methylbutanol, (c) ethyl acetate, (d) ethyl hexanoate, (e) ethyl octanoate, and (f) ethyl decanoate in fresh NABLABs. (LOD was to 0.01 mg/L). Thresholds correspond to those of a conventional beer.[66] Means of duplicates (coefficient of variation <10%).

biological processes were limiting for their biosynthesis.[65] 3-Methylbutyl acetate (banana flavor, Figure 3a) was found above its sensory threshold (1.2 mg/L[66]) only in beers F, J, and K (up to 2.2–4.5 mg/L), two which also emerged as richest in 3-methylbutanol (up to 73–167 mg/L, Figure 3b). As stated by,[67] increasing level of this higher alcohol enables a NAB to better mimic the conventional beer. All ethyl esters (Figure 3c–f) were also detected far below their perception thresholds.

Fruity polyfunctional thiol quantitation in NABLABs

Unlike fusel oils, many pleasant hop-derived polyfunctional thiols were found above their thresholds in fresh NABLABs, especially 3-sulfanylhexan-1-ol (3SHol, rhubarb, 0.07–1.3 µg/L in beers A, B, C, D, F, G, H, and K for a threshold of 0.055 µg/L) (Figure 4a). Also found was its ester 3-sulfanylhexyl acetate (3SHA, passion fruit, 0.1–0.5 µg/L in beers A, C, D, E, H, I, J, and K for a threshold of 0.005 µg/L) (Figure 4b). Despite their high volatility (therefore potentially lost during dealcoholization) and likely less biosynthesis from cysteinyl precursors (especially in short/cold fermentation), thiols concentration was interestingly slightly higher than in previously studied lagers, in contrast to DMS, and comparable to dry-hopped beers.[68–69] The selection of hops or flavor extracts has likely been carefully optimized to reach such amounts. 3-Sulfanyl-4-methylpentanol (3S4MPol, grapefruit), known to be more hop-variety-dependent, was evidenced in five fresh NABLABs (B, C, D, H, and K), three of which were dry-hopped (0.1–0.6 µg/L in B, C and D for a threshold of 0.07 µg/L) (Figure 4c). Two other polyfunctional thiols were also found above their threshold in a few samples, depending on the hop variety used: 4-sulfanyl-4-methylpentan-2-one (4S4M2Pone, blackcurrant, catty, 0.1–0.6 µg/L in beers B, D, I, and J for a threshold of 0.0015 µg/L) (Figure 4d), and 3-sulfanyl-4-methylpentyl acetate (3S4MPA, grapefruit, melon, 0.1–0.3 µg/L in beers E, F, I, and K for a threshold of 0.16 µg/L) (Figure 4e). In some beers, 3-sulfanylpentan-1-ol (3SPol) could also impact the fruity/citrus flavor by synergy (0.03–0.3 µg/L in beers C, D, J, and K for a threshold of 0.62 µg/L) (Figure 4f). A recent study[61] showed that even low concentrations of hop-derived aromas added in late kettle hopping and/or dry hopping could mask worty flavors and compensate for low levels of fermentation esters.

As for traditional lager beers, Ehrlich-derived thiols were detected well below their sensory thresholds in all samples (Figure 4g–j). Yet relatively high concentrations of 2-sulfanylethan-1-ol (2SEol, grilled, 6.5 and 7.7 µg/L), 3-sulfanylpropan-1-ol (3SProl, popcorn, 3.4 and 6.5 µg/L), 2-sulfanylethyl acetate (2SEA, toasted, 16.5 and 41.9 µg/L), and 3-sulfanylpropyl acetate (3SPrA, grilled, 4.4 and 13.8 µg/L) could be emphasized in beers I (lager beer) and J (abbey blond beer).

DMS fate through storage and relationships with SMM, DMSO and group I amino acids measured in fresh beer

As DMS was recently shown to increase through wine aging, it was interesting to investigate if similar evolution occurred through NABLABs aging. DMS was quantified in the 11 commercial beers after two years of storage in the dark (longer than most BBD but shorter than the time a great wine can be stored) (Figure 2). In parallel, DMSO (Table 1; measured by GC-MS), SMM (Table 1 and Figure 5a; quantitated by DMS released after heat-alkali treatment) and residual amino acids from group I (as defined by Jones:[70] Arg, Asn, Asp, Glu, Gln, Lys, Ser and Thr – Figure 5b; UPLC-UV method) were determined.

DMS levels were found to be increased in almost all the NABLABs after this long storage (+63% on average), confirming that the potential precursors are present in the beers. In beer G, DMS even exceeded its threshold after aging, reaching up to 66.2 µg/L. Microbiological analyses (WLD under aerobic conditions and MRS under both aerobic and anaerobic conditions) of the aged NABLABs confirmed that the DMS increase was not related to beer spoilage. DMS remained undetectable only in beers I and J. The 24.5 µg/L quantitated in beer E indicates that some precursors totally untransformed through the cold contact process can be degraded over a long time in the bottle.

The DMSO content ranged from 74 (beer D) to 304 (beer H) µg/L DMS eq. in fresh NABLABs, with no obvious relationship according to the process used. SMM (in DMS equivalents) was also detected in almost all the NABLABs (ranging from 2–29 µg/L), the exceptions being beers A, I, and J, where the free DMS concentration was very low to undetectable. Interestingly, beer G (a fruity wheat beer, 118 µg/L DMS eq.) reached the highest SMM level. As observed for free DMS, fresh dry hopped beers B and C also showed higher SMM levels, up to 61–64 µg/L.

As depicted in Figure 5c, a correlation was obtained between the concentration of group I amino acids and the level of remaining SMM (R^2 = 0.89 without beer G), both being indicators of the fermentation rate (group I almost completely consumed by yeast in conventional lagers - < 30 mg/L).

From all these data, another interesting correlation (R^2 = 0.79) emerged between the amount of DMS produced after two years of aging and the available SMM (Figure 5d). No similar relationship was found with DMSO.

Potential transformation of SMM and DMSO to DMS through beer aging

The ability of SMM to be a DMS precursor during NABLABs aging was assessed by subjecting a beer spiked with 100 µg/L SMM to natural (20 °C) and forced (45 °C) aging. As depicted in Figure 6, DMS production was significantly higher after SMM spiking, whatever the conditions, while its concentration remained unchanged in the control sample over the first 5 days at 45 °C (slight increases after 8 and 30 days at 45 °C, due to intrinsic SMM). As expected, more DMS was found in the spiked media after the forced aging (5% SMM degradation rate after 5 days vs. 1% at 20 °C; 18% vs. 4% after one month). Degradation of 750 µg/L SMM should be required to bring DMS above its threshold (30 µg/L) after one month at room temperature.

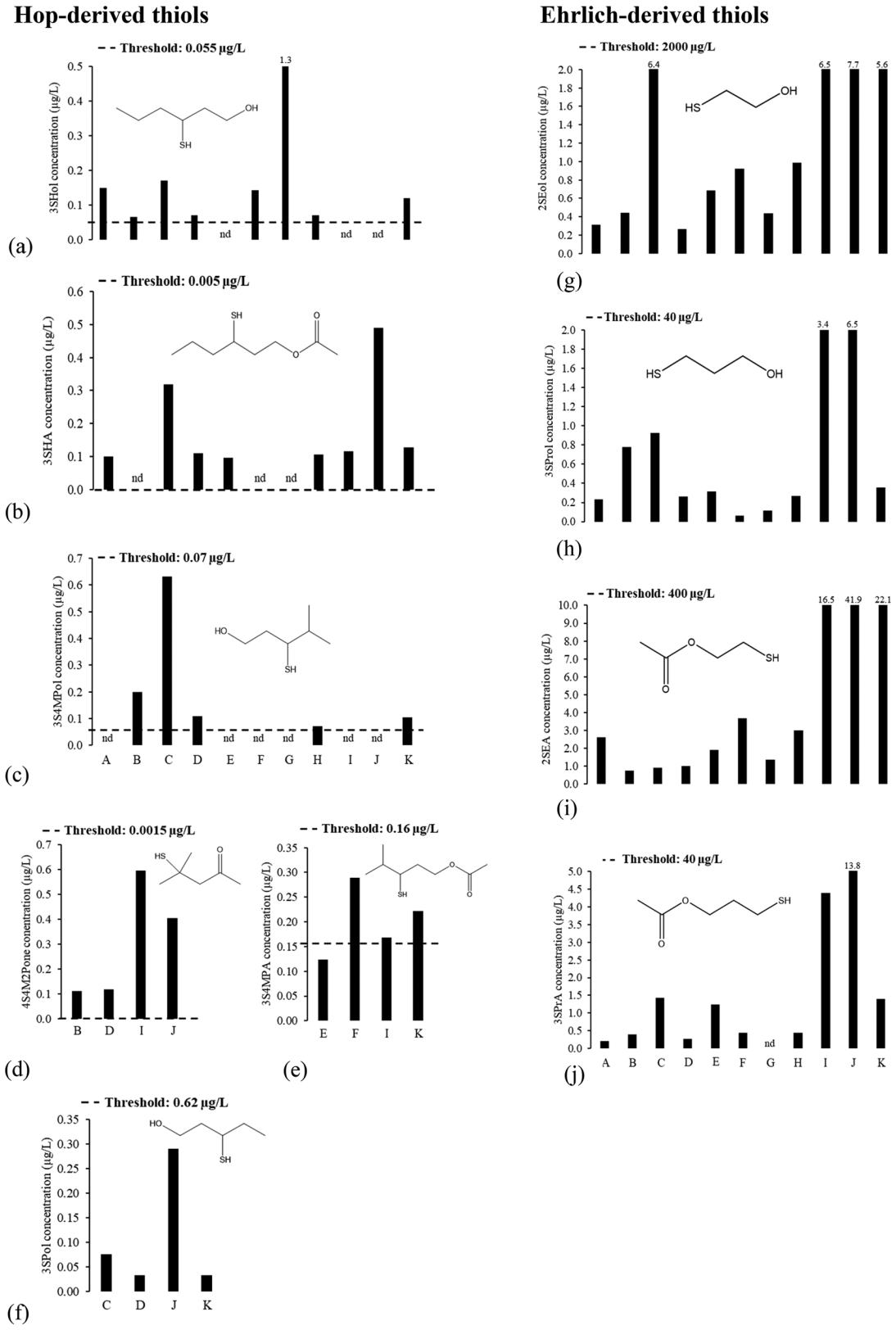

Figure 4. Concentration (µg/L) of (a) 3SHol, (b) 3SHA, (c) 3S4MPol (IST eq.), (d) 4S4M2Pone, (e) 3S4MPA (IST eq.), (f) 3SPol (IST eq.), (g) 2SEol, (h) 3SProl, (i) 2SEA, and (j) 3SPrA in fresh NABLABs (nd: not detected; inferior to LOD of 0.01 µg/L). Thresholds correspond to those of a conventional beer.[69] Means of duplicates (coefficient of variation <10%).

Next, the ability of DMSO to be a DMS precursor through NABLABs aging was also checked by subjecting the same beer, here spiked with 250 µg/L DMSO, to natural (20 °C) and forced (45 °C) aging. Over a period of 5 to 8 days (at 20 or 45 °C), no DMS was released from DMSO. Only 3 µg/L DMS (less than 2% DMSO degradation) was detected after one month at 45 °C. These results confirm that at room temperature, yeast is required to transform beer DMSO to DMS.

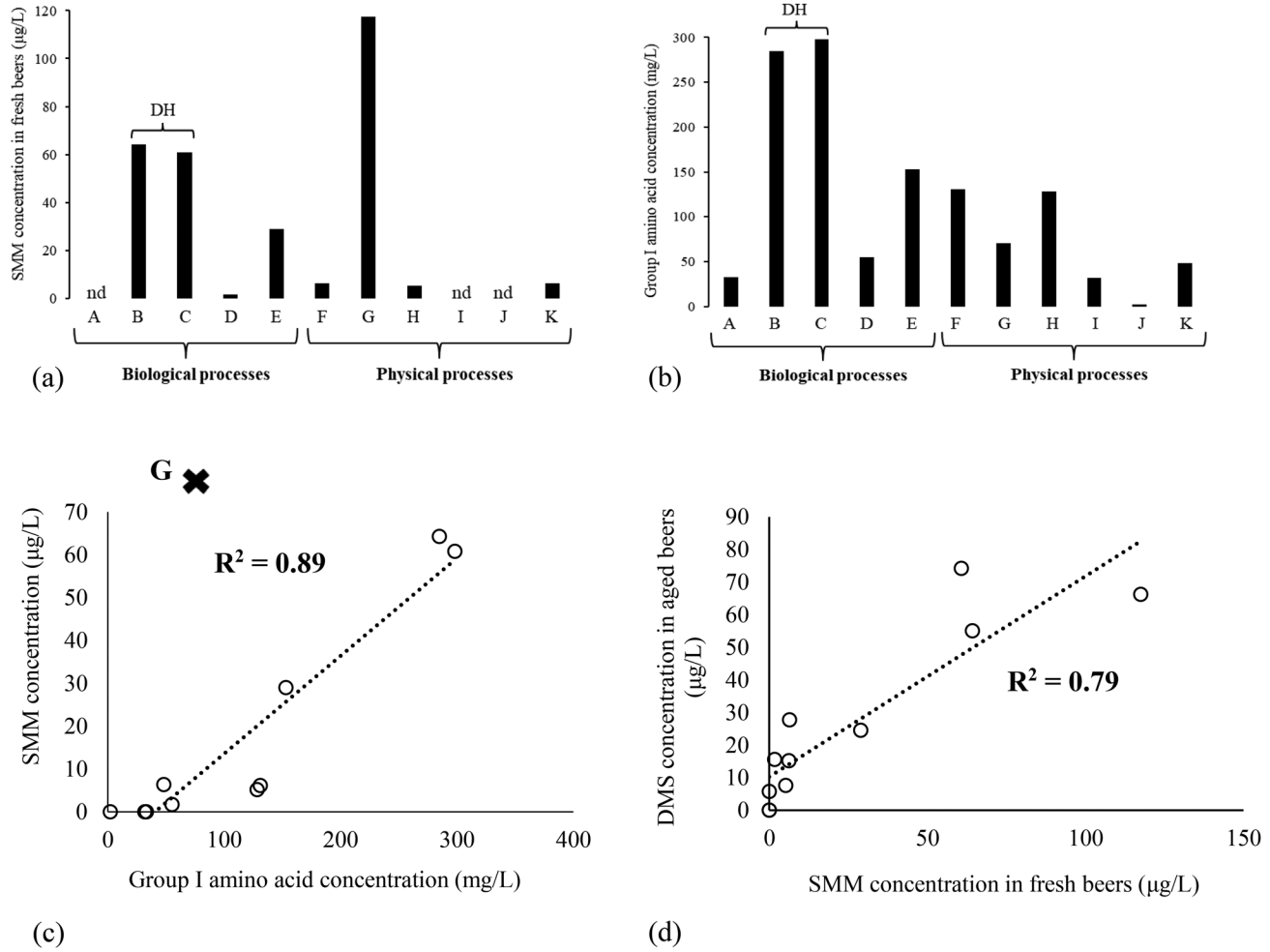

Figure 5. Concentrations of (a) SMM (µg/L) and (b) group I amino acids (mg/L) in fresh NABLABs (nd: not detected; inferior to LOD of respectively 1 µg/L and 0.1 µM, means of duplicates, coefficients of variation <10%, DH: dry-hopped beers). Correlations between (c) the concentrations of group I amino acids (mg/L) and SMM (µg/L) (excluding beer G), or (d) SMM (µg/L) in fresh beers and DMS (µg/L) in aged beers.

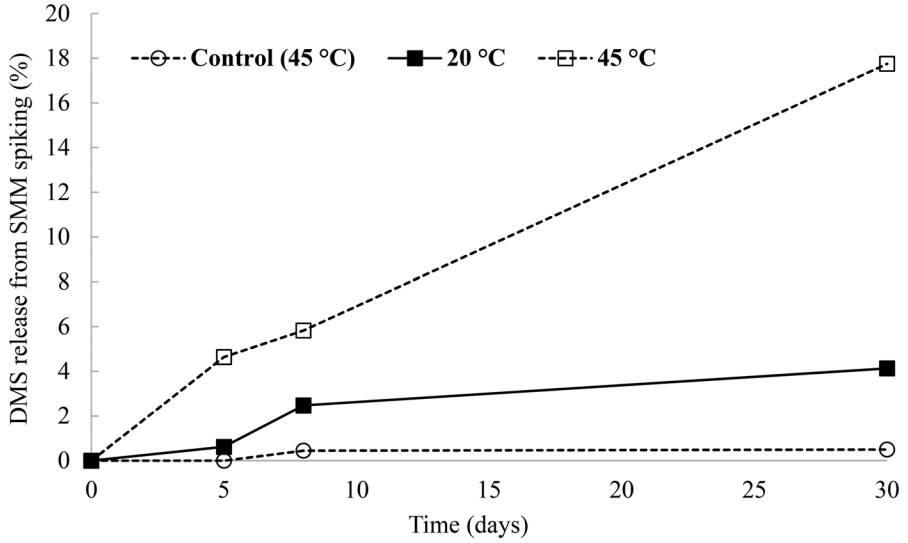

Figure 6. DMS release (%) from a 100 µg/L SMM spiking, after ▪ natural (at 20 °C) or □ forced (at 45 °C) aging. Means of duplicates (coefficient of variation <10%).

Conclusion

As expected, fruity fermentation esters are lacking in most NABLABs, while some pleasant polyfunctional thiols issued from hop are found above their sensory thresholds, especially in dry-hopped beers. Dimethylsulfide was also found below its perception threshold in most fresh NABLABs (little SMM in low-°P worts, likely lost during dealcoholization, and not formed through DMSO reduction in cold-contact fermentation). SMM (1.7–117.5 µg/L DMS eq. in NABLABs) was evidenced as the main precursor of DMS created through aging (R^2 = 0.79 between them). Despite its occurrence at higher level, DMSO showed no significant release of DMS under aging conditions. Dry hopping and/or spiking after dealcoholization of DMS-rich aroma extracts (e.g.: corn extracts,[6,9] heat-treated SMM-rich hops…) appear as interesting options for improving NABLABs flavor quality. Yet, sensorial analyses similar to those published for wines are still needed to optimize this synergy in NABLABs.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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