

The Redox Behavior of Dimethyl Sulfide and Dimethyl Sulfoxide in Malting and Brewing

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Abstract

Dimethyl sulfide (DMS) is a small thioether that can be found in miscellaneous foodstuffs. In beer it can evoke distinctive off-flavors, which are often ascribed as “cooked-vegetable”-like. Dimethyl sulfoxide (DMSO) reduction by yeast is a potential source for DMS in beer. However, the contribution of DMSO to the final levels of DMS in beer is not entirely resolved. The aim of this dissertation work was to initially re-evaluate the impact of DMSO as a precursor of DMS in beer. Therefore, an analytical method for its quantification in malt, wort and beer was developed to investigate DMSO reduction and DMS formation during fermentation as well as in bottled beer. Moreover, the method was applied for the assessment of yet undiscovered reaction mechanisms of DMS oxidation and DMSO formation during malt- and wort production. Findings on the “redox behavior” of DMS and DMSO from this dissertation work are summarized in Figure 1.

During mashing, DMSO was rapidly extracted from the malt particles whereas its entirety remained constant throughout conventional wort production. In the course of wort boiling DMS was evaporated subsequently after its formation from thermal hydrolysis of S-methylmethionine (SMM) (Figure 1, reaction 1), and was therefore not subject to oxidation. Yet, during heat holding of wort below boiling temperature, significant DMS oxidation was observed. However, as compared to the input of DMSO into the brewing process from the malt, the contribution of DMS oxidation to the levels of DMSO in pitching wort was estimated as negligible. In the course of fermentation, yeast absorbed a part of the DMSO present in pitching wort whereas this quantity was only reduced partly to DMS by methionine sulfoxide reductases (MSRA) (Figure 1, reaction 2). Still, top-fermenting yeast reduced more DMSO than bottom-fermenting yeast leading to DMS formation of up to 38 µg/L. Moreover, it was demonstrated that DMSO can be reduced to DMS in beer by the antioxidants sulfite (Figure 1, reaction 3) and thiols (Figure 1, reaction 4). These findings present another source of DMS formation in beer that has not been reported before.

On basis of the shown relevance of DMSO as a DMS precursor, reaction mechanisms responsible for DMS oxidation were investigated as a prerequisite to minimize DMSO formation in malt. Main focus was to test the applicability of selected malt-derived antioxidants as potential antagonists towards reactive oxygen species (ROS) that may potentially oxidize DMS. In model systems, molecular oxygen (O₂) was incapable of DMS oxidation under absence of transition metal ions (Mⁿ⁺). Though, the combination of Mⁿ⁺ with the antioxidants ascorbic acid (Asco) (Figure 1, reaction 5), gallic acid (GA) (Figure 1,

reaction 6), thiols (Figure 1, reaction 7) as well as sulfite (Figure 1, reactions 8 & 9) had significant prooxidative effects on DMS oxidation and increased the levels of DMSO. For the reactions 5-7, the effects were explained by donation of electrons from antioxidants to M^{n+} and ultimately, to molecular oxygen (O_2) thus leading to H_2O_2 formation, which was consequently proposed as the primary DMS oxidant in these systems (Figure 1, reaction 10). The prooxidative effect of oxygenated sulfite- M^{n+} systems on DMS oxidation was demonstrated to be initiated through the oxidation of bisulfite by M^{n+} whereupon bisulfite radicals were generated (Figure 1, reaction 8). The latter reacted with O_2 to form peroxymonosulfate radicals (Figure 1, reaction 9), which in turn revealed the highest capability of DMS oxidation (Figure 1, reaction 10). The relevance of the prooxidative effects of oxygenated antioxidant- M^{n+} systems towards DMS oxidation was verified in malting experiments where significant higher levels of DMSO were detected in malts enriched with antioxidant-copper²⁺ combinations than in malts where no additions were made. However, as probably resulting from interactions of the supplemented compounds with other malt ingredients, the effects were smaller as compared to the model systems. Based on these findings, the addition of antioxidants for minimization of DMSO in malt cannot be suggested.

Besides its autoxidative origin, ensuing experiments provided evidence for respiration-derived H_2O_2 accumulation in germinating barley seeds (Figure 1, reaction 12), indicating its potential to oxidize DMS during malt kilning. The reaction of H_2O_2 with DMS was therefore further characterized under consideration of physical-chemical parameters. Because M^{n+} are also present in malt, they are likely to react with H_2O_2 via “Fenton-like” reactions to form hydroxyl radicals ($\cdot OH$) (Figure 1, reaction 13). The reaction of $\cdot OH$ with DMS also resulted in DMSO formation (Figure 1, reaction 14), whereas the rate was significantly higher as compared to H_2O_2 .

In conclusion the findings from this work clarified pathways yielding DMSO and thus contributed to the knowledge of DMS formation in beer. Therefore, the data can help the malting and brewing industry to control the levels of DMS in end products. Besides DMS, the prooxidative behavior of antioxidant- M^{n+} systems as well as the respiration-derived H_2O_2 formation during barley germination, as discovered in this work, indicates that those reactions may be involved in further oxidative processes in malting and brewing which are vital for processability as well as beer quality.

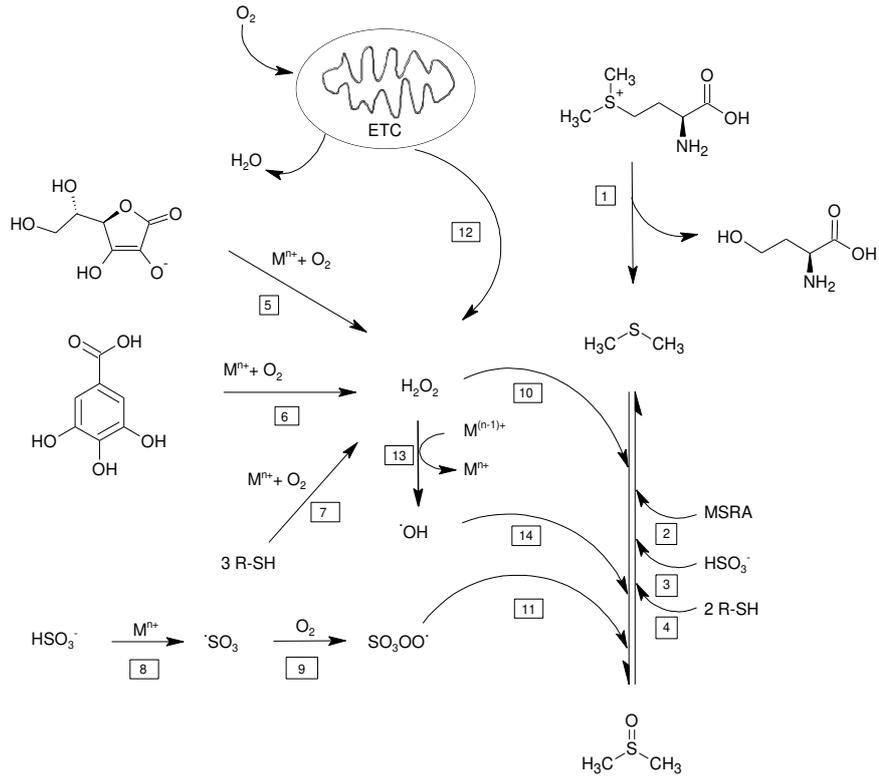


Figure 1: Summary of the findings on the redox behavior of DMS and DMSO in malting and brewing (ETC: mitochondrial electron transport chain, M^{n+} : transition metal ions).

Zusammenfassung

Dimethylsulfid (DMS) ist eine schwefelhaltige Verbindung, die im Bier zum Auftreten von Fehlparomen führen kann, welche häufig mit denen von gekochtem Gemüse verglichen werden. Dimethylsulfoxid (DMSO) kann während der Bierfermentation zu DMS reduziert werden und somit das Bieraroma signifikant beeinflussen. Jedoch ist die Rolle von DMSO und dessen Beitrag zum DMS Gehalt im Bier nicht eindeutig geklärt. Ziel der Dissertation war es zunächst die Relevanz von DMSO als Vorläufersubstanz von DMS im Bier zu überprüfen und ggf. den diesbezüglichen Wissenstand zu aktualisieren. Dazu wurde eine Analysemethoden zur quantitativen Bestimmung von DMSO in Malz, Bierwürze und Bier entwickelt, welche im weiteren Verlauf der Arbeit eingesetzt wurde, um die DMSO Reduktion während der Bierfermentation sowie der Bierlagerung zu untersuchen. Darüber hinaus wurden bislang nicht erforschte Mechanismen sowie technologische Einflussfaktoren der DMS Oxidation untersucht.

Im Bereich der Maische- und Würzproduktion wurde DMSO zunächst zeitnah extrahiert, erfuhr in konventionellen Würzereibereitungsprozessen jedoch keine ausgeprägte chemische Veränderung. Nach hydrolytischer Spaltung des primären DMS-Präkursor, S-methylmethionin (SMM) (Abbildung 1, Reaktion 1), wurde DMS im Verlauf der Würzekochung vollständig durch Verdampfung ausgetrieben. Bei der Würzeheißhaltung unterhalb dessen Siedetemperatur, wurde DMS speziell in der Aufheizphase, zu DMSO oxidiert. Das Ausmaß der dadurch bedingten Zunahme an DMSO bezogen auf die Gesamtkonzentration in der Anstellwürze wurde jedoch als vernachlässigbar eingestuft. Demnach bestimmt maßgeblich der Mälzungsprozess den Gehalt an DMSO in Bierwürze. Während der Fermentation konnte demonstriert werden, dass kommerziell verwendete Hefestämme in der Lage sind DMSO aufzunehmen, diesen Anteil jedoch nicht vollständig durch Methioninsulfoxidreduktasen (MSRA) zu DMS reduzieren (Abbildung 1, Reaktion 2). Dennoch reduzierten obergärige Bierhefen wesentlich mehr DMSO als untergärige Bierhefen, welches eine DMS-Bildung von bis zu 38 µg/L im Bier bewirkte. Weiterhin konnte belegt werden, dass DMSO während der Bierlagerung durch die in Bier vorkommenden Antioxidantien Sulfid (Abbildung 1, Reaktion 3) sowie Thiolen (R-SH) reduziert werden kann (Abbildung 1, Reaktion 4). Diese Reaktionen stellen weitere, bislang unbekannt, potentielle Quellen von DMS im Bier dar. Aufgrund der Relevanz von DMSO als DMS-Präkursor wurden im weiteren Verlauf Reaktionsmechanismen der DMSO Bildung untersucht, um somit Grundlagen für technologische Minimierungsmaßnahmen zu erarbeiten. In Modelllösungen stellte sich heraus, dass molekularer Sauerstoff (O₂) in Abwesenheit von

Katalysatoren, wie Übergangsmetallionen (M^{n+}), keine DMS Oxidation bewirkte. Es wurde weiterhin getestet, ob Antioxidantien, die potentiell in der Lage sind reaktive Sauerstoffspezies (ROS) zu inaktivieren, dadurch ebenfalls die DMS Oxidation verringern können. Die Zugabe der malzspezifischen Antioxidantien Ascorbinsäure (Abbildung 1, Reaktion 5), Gallussäure (Abbildung 1, Reaktion 6), von Thiolen (Abbildung 1, Reaktion 7) sowie Sulfit (Abbildung 1, Reaktionen 8, 9) hatte jedoch in jedem Fall eine katalytisch prooxidative Wirkung auf die DMS Oxidation sowie die DMSO Entstehung. Im Falle der Reaktionen 5-7 konnten diese Effekte auf die Einbringung von Elektronen auf M^{n+} und schließlich auf O_2 unter Bildung von Wasserstoffperoxid (H_2O_2) zurückgeführt werden. H_2O_2 wurde folglich als zentrale DMS Oxidans in diesen Systemen eingestuft. Der Effekt von Sulfit- M^{n+} -Systemen konnte auf die Bildung von Sulfitradikalen zurückgeführt werden (Abbildung 1, Reaktion 8), welche durch Reaktion mit O_2 (Abbildung 1, Reaktion 8) Peroxomonosulfatradikale erzeugen (Abbildung 1, Reaktion 9). Letztere wiesen das größte Oxidationsvermögen von DMS auf. Die Relevanz der prooxidativen Verhaltensweise der Antioxidantien bezüglich der DMSO Bildung konnte im Malz verifiziert werden, wenngleich die Effekte dort geringer ausfielen. Aufgrund dieser Erkenntnisse konnte die Anreicherung der erwähnten Antioxidantien im Malz im Hinblick auf die Verringerung der DMSO Menge nicht empfohlen werden. In weiteren Versuchen konnte eine respirationsbedingte H_2O_2 -Akkumulation (Abbildung 1, Reaktion 12) in Braugerstenkeimlingen nachgewiesen werden, welches dessen Reaktion mit DMS während des Darrprozesses nahelegt. Auf Basis dessen wurde die Reaktion von DMS und H_2O_2 unter Berücksichtigung physikalisch-chemischer Parameter näher charakterisiert. Darüber hinaus zeigte sich, dass die DMS Oxidation durch Hydroxylradikale ($\cdot OH$) (Abbildung 1, Reaktion 14), die durch die Fenton Reaktion aus H_2O_2 in Anwesenheit von M^{n+} entstehen, deutlich schneller verläuft.

Zusammenfassend wurde in dieser Dissertation das Verhalten von DMSO im Brauprozess charakterisiert, wodurch der Wissenstand bezüglich des Beitrags zum DMS Gehalt in Bier deutlich erweitert werden konnte. Weiterhin konnten bislang unbekannte Reaktionsmechanismen der DMS Oxidation im Malz aufgeklärt werden. Das in dieser Arbeit beschriebene prooxidative Verhalten von Antioxidantien sowie die zuvor nicht erforschte respirationsbedingte H_2O_2 Bildung während der Keimung von Braugetreide deutet darauf hin, dass derartige Reaktionen ebenfalls in diversen anderen oxidativen Reaktionen in Malz, Würze und Bier involviert sind, welche zu erheblicher Qualitätsverminderung führen.

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Abbreviations Used

<i>ABTS^{•+}</i>	<i>Azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation</i>
<i>•DPPH</i>	<i>2,2-diphenyl-1-picrylhydrazyl radical</i>
<i>1-HER</i>	<i>1-hydroxyethyl radical</i>
<i>Asco</i>	<i>ascorbic acid</i>
<i>C m</i>	<i>Coulomb-meter</i>
<i>CCD-RSM</i>	<i>central composite design - response surface methodology</i>
<i>cp.</i>	<i>compare</i>
<i>Cys</i>	<i>L-Cysteine</i>
<i>DMPO</i>	<i>5,5-Dimethyl-1-pyrroline N-oxide</i>
<i>DMS</i>	<i>dimethyl sulfide</i>
<i>DMS</i>	<i>dimethyl sulfide</i>
<i>DMSO</i>	<i>dimethyl sulfoxide</i>
<i>E</i>	<i>equation</i>
<i>e.g.</i>	<i>exempli gratia</i>
<i>EDTA</i>	<i>ethylenediaminetetraacetic acid</i>
<i>ESR</i>	<i>electron spin resonance</i>
<i>et al.</i>	<i>et alia</i>
<i>etc.</i>	<i>et cetera</i>
<i>FA</i>	<i>ferulic acid</i>
<i>FAN</i>	<i>free amino nitrogen concentration</i>
<i>ff.</i>	<i>folio (and following pages)</i>
<i>GA</i>	<i>gallic acid</i>
<i>GC</i>	<i>gas chromatography</i>

<i>GSH</i>	<i>L-Glutathione</i>
<i>GSSG</i>	<i>glutathione disulfide</i>
<i>ICP-OES</i>	<i>inductively coupled plasma optical emission spectrometry</i>
<i>MetSO</i>	<i>methionine sulfoxide</i>
<i>Mⁿ⁺</i>	<i>transition metal ions</i>
<i>MS</i>	<i>mass spectrometry</i>
<i>MSRA</i>	<i>methionine sulfoxide reductase</i>
<i>PBN</i>	<i>N-t-butyl-α-phenylnitrone</i>
<i>PFPD</i>	<i>pulsed flame photometric detection</i>
<i>POBN</i>	<i>α-(4-pyridyl-1-oxide)-N-tert-butyl-nitrone</i>
<i>R</i>	<i>reaction</i>
<i>ref.</i>	<i>reference</i>
<i>ROS</i>	<i>reactive oxygen species</i>
<i>SMM</i>	<i>S-methylmethionine</i>
<i>TU Berlin</i>	<i>Technische Universität Berlin</i>

1 Introduction

1.1 Dimethyl sulfide – properties and abundance in nature

Dimethyl sulfide (DMS) is the smallest existing organic volatile thioether. In its pure form it is a straw colored liquid with a disagreeable odor. It reveals a boiling point of 37°C and a melting point of -98.3 °C.¹ Its chemical structure is shown in Figure 2.

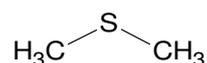


Figure 2: Chemical structure of DMS.

Both non-polar methyl groups as well as the less-hydrophilic sulfur atom are responsible for a poor water solubility (<300mM), whereas it is soluble in ethanol or diethyl ether.² DMS is considered as highly volatile in aqueous solutions and its odor threshold in water was reported to 1 µg/L,³ which already elucidates its significance in olfactory perception, in general. DMS plays a number of important roles in nature. It is formed by algae from dimethyl sulfonium propionate whereupon it is released into the biosphere in marine areas.⁴ Andreae⁵ reported that DMS and its oxidation products present ~50 percent of the total sulfur emitted to the atmosphere. In 1987, the “CLAW-hypothesis” by Charlson et al.⁶ aroused huge attention as DMS emission by phytoplankton was postulated to directly correlate with the global climate. They reported that aerosols of DMS and its oxidation products are the major source of cloud condensation nuclei that influence cloud formation as well as climate regulation. Recently, DMS was reported to function as chemical attractant for reef fish larvae that use DMS to locate phytoplankton-rich areas as settlement habitats.⁷ As aquatic cranial animals use dimethyl sulfonium propionate-rich algae as nutrient source, DMS is also a major aroma compound of diverse seafoods.^{8,9} In a plethora of other foodstuffs the hydrolysis of S-methylmethionine (SMM, cp. 1.2.1.) is the main source of DMS where it contributes positively to the aroma of many thermally treated vegetables, notably tomatoes,^{10,11} asparagus¹²⁻¹⁴ and cabbages.¹⁵⁻¹⁷ DMS was reported as one of the most important aroma compounds in black truffle.^{18,19} In this context Talou et al.²⁰ showed that it is actually the olfactory perception of DMS that leads dogs and pigs to the location of underground black truffles. Aroma-relevant levels of DMS are also present in wine.²¹⁻²³

1.2 DMS in beer

While Ahrenst-Larsen and Hansen²⁴ were the first to discover DMS in beer in 1963 Sinclair et al.²⁵ reported that DMS was a characteristic flavor component of lager beers. The flavor threshold of 25-30 µg/L was reported later by Anderson et al.,²⁶ whereas off-flavors ascribed as “vegetable-like” were only recognized in lager beers possessing DMS levels above 80 µg/L. They also noted that lower DMS concentrations may contribute positively to beer flavor. Nowadays it is well established that DMS also plays an important role in the flavor of other beer types,^{27,28} whereas its flavor contribution is highly affected by masking effects of other beer aroma compounds.^{29,30} The following section will outline the current knowledge of the origin of DMS in beer.

1.2.1 Formation of DMS in malting and brewing

White and Parsons³¹ showed that the origin of DMS in beer can be primarily retraced to a heat-labile precursor that is formed during germination of brewing cereals. It was postulated that S-methylmethionine (SMM),³² a compound reported before to release DMS upon heating in sweet corn,³³ is a constituent of the DMS precursor in malt. Lastly, Dickenson³⁴ identified SMM in green malt as only substance being responsible for DMS formation upon heating. The chemical structure of SMM is illustrated in Figure 3.

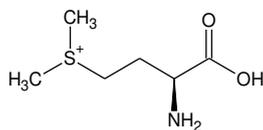


Figure 3: Chemical structure of SMM.

SMM, like dimethyl sulfonium propionate in algae, is a thermally unstable, water-soluble sulfonium compound (S^+R_3) which is synthesized in a plethora of flowering plants, including brewing cereals. However, its role in plant physiological processes is still poorly understood. It is known that SMM synthesis takes place via methyl transfer from S-adenosyl methionine (Adomet) to methionine (Met) catalyzed by methionine S-methyl transferase (MMT). Demethylation of SMM only takes place via the transfer of the methyl group to L-homocysteine (Hcy) as catalyzed by homocysteine methyl transferase (HMT).^{35,36} An overview of the SMM synthesis and its cycle in plants is presented in Figure 4.

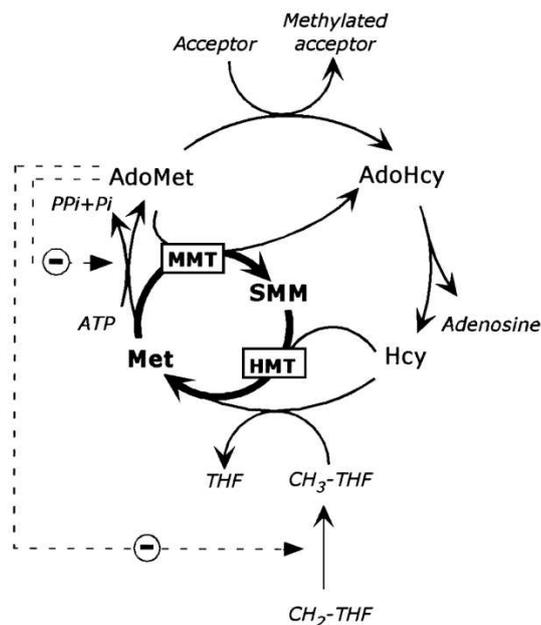


Figure 4: SMM cycle in flowering plants according to Ranocha et al.³⁶

It was assumed that SMM serves as storage compound for Met, which is transported via the phloem from the leaves to the seedling, where it can be reconverted to methionine and implemented into protein biosynthesis.³⁷ However, this assumption cannot explain SMM accumulation in germinating barley seeds, for example. A more reasonable explanation for SMM synthesis in brewing cereals would be a protective role against methionine depletion by the formation of AdoMet as proposed by Mudd and Datko.³⁵ In barley, SMM synthesis mainly takes place in the embryo, especially in rootlets, acrospire and scutellum.³⁷⁻³⁹ Factors enhancing SMM formation during germination are generally those that trigger modification, notably high temperatures,^{40,41} and high steeping degrees.^{38,39,41} Also barley variety has a decisive impact on the levels of SMM being formed during germination,^{39,41} whereas this is strongly related to the respective nitrogen content of the grain.⁴² During malt kilning SMM decomposes hydrolytically thereby forming DMS and L-homoserine.^{31,34,40-45} The formed DMS either remains in the grain, evaporates into the ventilation air and/or is oxidized to form dimethyl sulfoxide (DMSO) and minor amounts of dimethyl sulfone (DMSO₂).^{10,12,13} The aforementioned reactions are summarized in Figure 5.

The higher the kilning temperature is set the more SMM will be degraded and consequently, also more DMSO is formed.^{41,46}

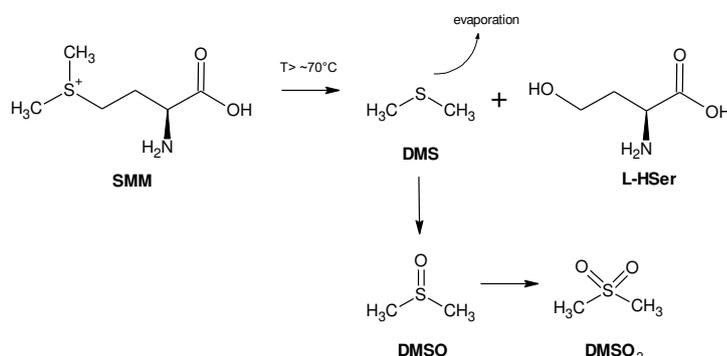


Figure 5: Formation of DMS and DMSO in malting and brewing.

DMSO reveals a unique role in brewing as it can be reduced back to DMS by yeast during fermentation, thereby acting as a secondary precursor of DMS (cp. page 6 ff.). Compared to DMS, pure DMSO is odorless and reveals a much higher boiling point (189°C). As a result of its high molecular dipole moment (13.03×10^{-30} Cm) DMSO is readily miscible in water and other organic solvents. Also, based on its amphiphilicity, DMSO is capable of solvating miscellaneous organic and inorganic compounds, which makes it a highly used solvent in chemical applications.¹

Up to $14.5 \mu\text{g/g}$ ^{10,11,15,16} of SMM remain in the malt and enter the brewhouse as a so-called thermal DMS precursor, which is generally degraded and removed via wort boiling. But also DMS and significant amounts of DMSO are introduced into the brewing process. The role of free DMS from malt was declared as irrelevant as the majority would be evaporated during brewhouse operations.⁴⁷

Owing to its high water-solubility SMM is rapidly extracted from the malt particles into the aqueous mash phase. At common mash pH (5.5—5.7), SMM is essentially stable below 60°C , whereas upon heating to mashing-off temperature ($\sim 78^\circ\text{C}$), SMM hydrolysis and accompanied DMS and HSer formation becomes observable. Though, the majority of SMM degradation takes place above 80°C .^{44,48} A main reason for the expensive wort boiling process is to hydrolyze SMM and to evaporate the generated DMS and other undesirable volatile aroma compounds. There is a general agreement about the reaction mechanism of SMM decomposition and its contribution to DMS formation in the literature.^{6,7,12,13} Thermal SMM degradation follows a 1st order mechanism according to Equation 1.

$$SMM = SMM_{t=0} \times e^{-kt}$$

E1

The hydrolysis increases with rising temperature and rising pH. A compilation of rate constants of the SMM degradation in wort is presented in Table 1.

Table 1: Compilation of 1st order rate constants (k) of SMM hydrolysis in wort (*calculated from half-life).

Temperature [°C]	pH [-]	k [min ⁻¹]	source
65	-	0.000385	Dickenson ⁴⁹
70	-	0.000958	Hysert et al. ⁵⁰
70	-	0.000301	Zürcher et al. ^{51*}
80	-	0.00093	Zürcher et al. ^{51*}
80	5.2	0.0009	Scheuren et al. ⁴⁸
85	-	0.00648	Hysert et al. ⁵⁰
85	5.2	0.0015	Scheuren et al. ⁴⁸
86	5.2	0.0023	Dickenson ^{44*}
90	5.2	0.0049	Dickenson ^{44*}
90	-	0.0029	Zürcher et al. ^{51*}
90	-	0.010	Mitani et al.
90	5.2	0.0039	Scheuren et al. ⁴⁸
95	5.2	0.0099	Dickenson ^{44*}
95	5.2	0.01	Scheuren et al. ⁴⁸
98.5	5.2	0.0227	Scheuren et al. ⁴⁸
98.5	-	0.018	Felgenträger ⁵²
100	-	0.03	Hysert et al. ⁵⁰
100	-	0.0087	Zürcher et al. ^{51*}
100	-	0.0345	Mitani et al. ⁵³
101	5.5	0.0213	Dickenson ^{44*}

Once knowing the 1st order rate constant (T, pH = const.) and the concentration of SMM at t=0 minutes, SMM degradation can be predicted. The temperature dependence of the SMM hydrolysis is illustrated in Figure 6 by using rate constants of Scheuren et al.⁴⁸ (80°C), Mitani et al.⁵³ (90 °C) and Hysert et al.⁵⁰ (100°C) under consideration of $SMM_{t=0}=350 \mu\text{g/L}$.

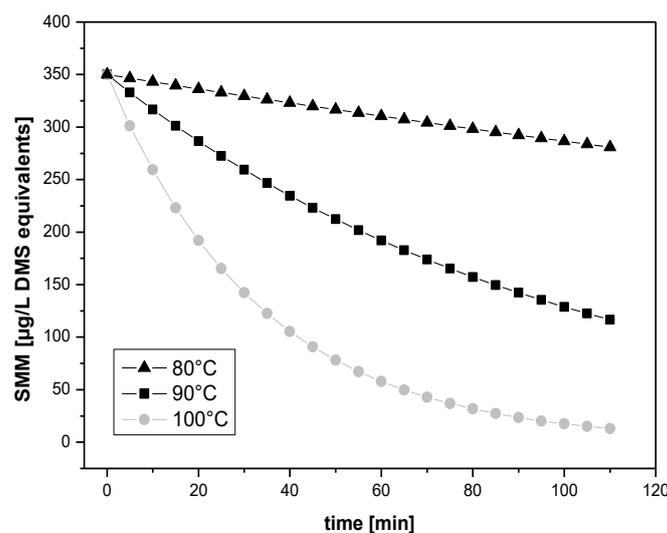


Figure 6: Kinetic modeling of SMM hydrolysis.

By the use of such degradation models the total boiling time necessary to degrade SMM below a commonly accepted value of 100 µg/L (SMM+DMS)^{54,55} can be calculated. Potential oxidation of DMS to DMSO is not considered by these models. Also, it is important to mention that sufficient isomerization and protein coagulation has to be ensured as base premises for wort and beer quality. However, insufficient degradation of SMM during wort boiling can be accompanied by a re-formation of DMS during the hot break separation (e.g. in a whirlpool) as a result of missing evaporation. This may lead to significant higher levels of DMS in the final beer. During fermentation yeast degrades SMM, whereas it is not metabolized to DMS.^{49,56}

Formation of DMS during fermentation

The formation of DMS during fermentation was first reported by Anderson et al.²⁶ They found that DMS formation was increased by using malts that have been kilned at higher temperatures and that *Saccharomyces cerevisiae* produces more DMS during fermentation than *Saccharomyces uvarum* (nowadays *Saccharomyces pastorianus*). They have also shown that the DMS content was higher in beers produced in a closed fermentation system compared to an open fermentation system, which was explained by a more pronounced removal of the volatile DMS via the CO₂ desorption. This observation was verified several times.^{42,43,46} White and Wainwright³² proposed that there are two precursors of DMS present in malt, one that forms DMS upon heating (as later identified as SMM³⁴) and one that can be metabolized to DMS by yeast. Wilson and Boer⁵⁷ found that DMS formation during fermentation was

independent of SMM as they also found DMS formation in its absence. In 1979, Annes et al.⁴⁶ identified DMSO as being the precursor of DMS during fermentation. They present an evident connection between the DMSO content of the malt and DMS formation during fermentation. They also found minor amounts of DMSO₂ in malt, whereas it was shown to be not convertible to DMS by yeast. Further research by Annes⁵⁶ elucidated that yeast reduces 13-21% of the DMSO content during fermentation, which was accompanied by significant DMS formation. DMSO reduction was enhanced at low fermentation temperatures, which likewise reduce DMS desorption by CO₂. Also, high gravity and low pitching wort pH was leading to a higher rate of DMSO reduction.⁴²

It was shown by Bamforth⁵⁸ that the enzyme responsible for DMSO reduction revealed a higher affinity for methionine sulfoxide (MetSO) and thus, was similar to the enzyme methionine sulfoxide reductase (MSRA). Around 20 years later Hansen⁵⁹ isolated the encoding gene of MSRA in *Saccharomyces cerevisiae* and thereby confirmed that this enzyme is responsible for DMS formation during fermentation. It is therefore assumed that DMSO reduction by yeast is a side-effect of MSRA activity (Figure 7).

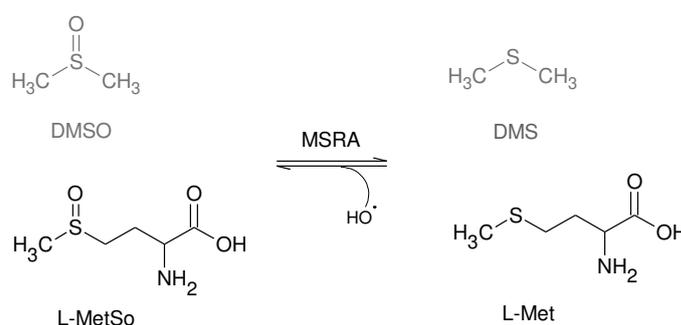


Figure 7: Pathway of the MRSA mediated reduction of DMSO by yeast.

Other research sectors reported that MSRA are essential enzymes that play an important role in regulation of protein biosynthesis and in the defense against oxidative stress.^{60,61} Methionine is oxidized in the cell to methionine sulfoxide (MetSO) by reactive oxygen species (ROS). The antioxidative functionality of MSRA is mainly based on the regeneration of free- and protein-bound methionine thereby maintaining protein functionality. Regarding the contribution of DMSO reduction during fermentation on final DMS levels in beer Dickenson⁴⁹ concluded from his investigations that yeast is indeed capable of DMSO reduction, whereas the reduction has no big influence on final DMS levels in beer.⁴⁹

On the contrary, Leemans et al.⁶² reported that DMSO was the most potential source for DMS in beer as they have shown that 80% of the total DMS present in beer derived from spiked DMSO-d₆. Even though this assumption implies sufficient SMM degradation and DMS evaporation during wort production these findings seem reasonable considering DMSO levels in pitching wort of about 400-800 µg/L.⁴⁶ In context to the low flavor threshold of DMS (cp. 1.2), even minor DMSO reduction during fermentation may be crucial for beer flavor.

1.3 Oxidation of DMS – Origin of DMSO in malt, wort and beer

Undoubtedly, it is the oxidation of DMS during malt kilning that yields DMSO.^{41,42,46,63–65} Lloyd⁶³ was the first to report that DMS is subject to oxidation while migrating through the kiln bed and that consequently, low kiln bed heights lead to lower levels of DMSO in malt.⁶⁴ Though, the levels of DMSO in malt are mainly influenced by the amount of SMM formed during germination in the green malt as it determines the levels of DMS that can potentially be oxidized on the kiln.^{42,46,63,65} High temperature-kilned malts, like ale- or dark malts contain significant higher levels of DMSO than Lager- or Pilsner type malts, for example. Annes et al.⁴⁶ tested the oxidation of DMS in ethanol as well as in an ethanol-barley grist mixture to simulate kilning conditions. After 4 h of incubation at 105°C less than 1% of the DMS was oxidized to DMSO in pure ethanol, whereas in combination with barley grist approximately 34% of the spiked DMS was recovered as DMSO. Also Yang and Schwarz⁶⁵ could not find significant DMSO formation during heating of aqueous DMS solutions.

Those observations indicate that barley or green malt contains substances that are acting as catalysts for DMS oxidation. It was also proposed that DMSO may be subject to oxidation in mash at elevated temperatures.⁶⁶ However, a detailed literature research did not allow for further deductions on DMSO formation in malt, mash or wort. It is therefore proposed that the mechanisms of DMS oxidation and DMSO formation have not been investigated in malt or any other related foodstuffs that contain DMS.

1.4 Oxygen and reactive oxygen species - relevance in malting and brewing

Oxygen plays an important role in malting as it is required for respiration and accompanied germination in order to achieve a desirable rate of proteolysis, cytolysis as well as enzyme formation. In the brewing process oxygen is necessary for the yeast's synthesis of ergosterol, which is indispensable for cell growth. However, in other process steps of malting and brewing the ingress of oxygen should be minimized as it triggers a plethora of reactions that lead to quality deterioration of beer.

During germination, kilning and mashing fatty acids, like linoleic acid and linolenic acid are oxidized to the corresponding hydroperoxy fatty acids (HPODs) in the presence of molecular oxygen and enzymes called lipoxygenases (LOX).⁶⁷ HPODs are ultimately transformed into aldehydes which have been established to evoke strong off-flavors, when present in beer.^{68,69}

Other aldehydes, like 2- and 3-methylbutanal, benzaldehyde as well as phenylacetaldehyde derive from the reaction of dicarbonyls with amino acids, the so-called Strecker reaction.⁷⁰ Methner et al.⁷¹ observed that the formation of Strecker aldehydes was promoted at elevated levels of oxygen. Hoffmann and Schieberle⁷² demonstrated that these aldehydes can also be formed via oxidative degradation of Amadori compounds. Wietstock et al.⁷³ reported another mechanism by which Strecker aldehydes are formed via direct oxidation of amino acids. In general, the majority of those aldehydes are highly volatile and are mostly lost during wort boiling. However, as demonstrated by Baert et al.,^{74,75} the same aldehydes, irrespective of their origin, can be bound to the thiol L-cysteine via the formation of 2-substituted 1,3-thiazolidine-4-carboxylic acids. The latter are essentially stable and non-volatile. Consequently, they survive the brewing process and enter the final beer, where the aldehydes are released as a result of pH drop. The consequence is off-flavor formation. Besides their release, aldehydes can also be formed *de-novo* via amino acid oxidation in beer.⁷³ Further consequences of oxidation are decelerated mash filtration rates,⁷⁶ haze formation,^{77,78} or loss of hop bitter substances in beer.⁷⁹ In contrast to DMS oxidation those reactions are mostly established.

As precondition for the understanding of oxidative reactions in general, one has to consider that molecular oxygen in its ground state, also referred as triplet oxygen (henceforth assigned as O₂), possesses 2 unpaired electrons with the same spin quantum, located in a different antibonding 2p orbital, thereby fulfilling the lowest energy configuration according to Hund's rules. As a result of this configuration O₂ is rather unreactive (redox potential E₀=-0.33).^{80,81} In order to overcome the spin restriction and increase the reactivity, O₂ needs to be activated

via reduction (Figure 8). The first product of the univalent reduction of O_2 is the superoxide radical (O_2^-/HO_2). Further acceptance of electrons yields peroxide anion (O_2^{2-}), hydrogen peroxide (H_2O_2) and the hydroxyl radical $\cdot OH$. The reduction of O_2 to the hydroxyl radical increases the redox potential E_0 from -0.33 V to $+2.32$ V,⁸² indicating that reactions of reduced O_2 species become endergonic and thus proceeding at much higher rates.

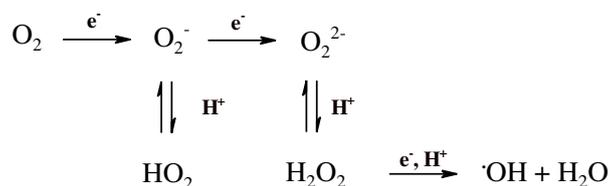
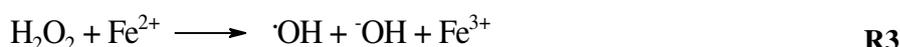
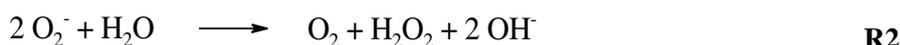
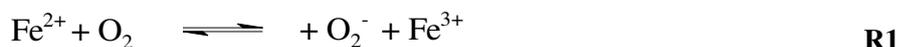


Figure 8: Oxygen activation through electron transfer.^{80,81}

In nature as well as in food various substances are present that can act as electron donors for O_2 reduction, especially enzymes (cytochrome-c-oxidase or peroxidases) together with reducing substances, like NADH. In thermally processed foodstuffs, such as malt and beer transition metal ions (M^{n+}), such as copper (Cu^+/Cu^{2+}), iron (Fe^{2+}/Fe^{3+}) and manganese (Mn^{2+}/Mn^{3+}) were shown to be relevant electron donors for the activation of oxygen yielding the aforementioned ROS. The reduction of O_2 by Fe^{2+} is considered as key-step for the initiation of ROS formation in malting and brewing (Reaction 1).^{73,83-90}



O_2^- may undergo dismutation to form H_2O_2 (Reaction 2), which can form $\cdot OH$ in the presence of Fe^{2+} or related transition metal ions via the Fenton reaction (Reaction 3). This reaction is considered as most significant contributor to beer quality deterioration as it forms $\cdot OH$ at diffusion control rates. $\cdot OH$ are considered as most reactive radicals found in nature. In beer they predominantly react with ethanol to 1-hydroxyethyl radicals (1-HER). The latter are responsible for quality issues as mentioned before.

1.5 Antioxidants

The earth's atmosphere consists to ~21% of O₂. In aerobic organisms the successive reduction of O₂ to water via the respiratory chain is the foundation of their existence. However, incomplete reduced oxygen species, like O₂⁻, H₂O₂ or [•]OH can accumulate in cells as a result of stress conditions,⁹¹⁻⁹⁶ but they may also act as signaling- or growth regulators.⁹⁷⁻¹⁰⁰ An imbalance or uncontrolled release of ROS can lead to enormous cellular damage.^{81,81,101} As the formation of ROS is nearly inevitable, nature provides complex defense systems acting antagonistic towards an overshoot formation of ROS. Holistically, such systems can be assigned as antioxidants. The following sections will describe the classification of antioxidants and the current level of knowledge regarding a selected group of important food antioxidants.

Classification of Antioxidants

“Antioxidant” is a far-ranging term, including enzymatic-, molecular-, dietary- and chelative antioxidants. According to Halliwell and Gutteridge¹⁰² an antioxidant is defined as *“any substance, that when present at low concentrations compared to an oxidisable substrate, significantly delays or prevents oxidation of that substrate.”* It is therefore important to determine the target molecule(s), which is in the present dissertation work. Commonly, antioxidants are classified according to their mode of action into primary- and secondary antioxidants.¹⁰³

Primary antioxidants

Primary- or chain-breaking antioxidants are mostly radical scavengers as they donate electron(s) (e⁻/H⁺) to a designated harmful radical (X[•]) (Reaction 4). Thereby the harmful radical gets inactivated (XH) whereas an antioxidant radicals (A[•]) is generated. In the best case the latter is less reactive than the designated radical to be inactivated. Also, efficient primary antioxidants should generate A[•] that quickly react with themselves, mostly via disproportionation (Reaction 5).



Through this reaction the radical chain mechanism is terminated. Common examples of food-derived primary antioxidants are for example ascorbic acid, (poly-)phenols, tocopherol, carotenoids, thiols or sulfite.^{104,105} In malt, wort and beer, antioxidative behavior was demonstrated for sulfite,¹⁰⁶⁻¹⁰⁸ (poly-)phenols,¹⁰⁹ thiols,^{86,110} ascorbic acid^{111,112} and other

Maillard reaction-derived reductones.^{83,113} As the impact of primary antioxidants on DMS oxidation is a major subject of this dissertation work, the following section will address the current knowledge regarding the relevance of each group of potential antioxidants in malt, wort and beer together with findings from related research sectors.

Reductones and other Maillard reaction products

A representative example of a reductone is ascorbic acid (Asco). High levels of Asco occur naturally in fruits and vegetables, especially in strawberries, cherries, citrus fruits, asparagus, brussels sprout, cress, cabbage and pumpkins.^{114–116} The reductone- or enediol structure is located from C₁-C₃ and possesses strong reducing properties. The successive oxidation of Asco, here illustrated as ascorbate anion (AscH⁻) (Figure 9 a) leads to the formation of the ascorbyl radical ([•]AscH⁻) (Figure 9 b) and dehydro ascorbic acid (DHA) (Figure 9 c).^{117–119}

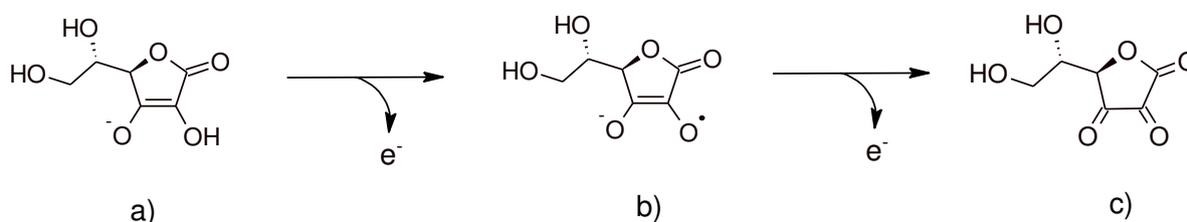


Figure 9: Oxidation of ascorbic acid.^{117–119}

The functionality of Asco as primary antioxidant is presented using the example of [•]OH scavenging. Asco donates an electron (e⁻/H⁺) to [•]OH which results in the formation of water and [•]AscH⁻^{118,119} (Reaction 6).



2 molecules of [•]AscH⁻ rapidly dismutate thereby forming AscH⁻ and DHA (Reaction 7),^{120,121} which can be regarded as the radical termination reaction. Besides its reactivity with [•]OH, Asco also scavenges singlet oxygen,¹²² O₂⁻¹²³ and H₂O₂.¹²⁴ Based on these versatile functionalities Asco is one of the most applied food additives, in general.¹²⁵ Other reductones found in food are mostly formed by thermal processing via the so-called Maillard reaction. This initially involves reducing carbohydrates, amino acids, peptides or proteins leading to glycosylamines and consecutive formation of miscellaneous final products, mostly high- and low molecular melanoidins, furans, furanones, pyrroles, pyrazines, pyrrolinones, ketones and aldehydes.^{72,126–132} Antioxidative properties were mainly reported for amino reductone

intermediates^{133,134} (Figure 10 a), non-amino reductones^{135,136} (Figure 10 b), 1,2-dicarbonyl compounds¹³⁷ (Figure 10 c) and condensed melanoidines.^{113,132}

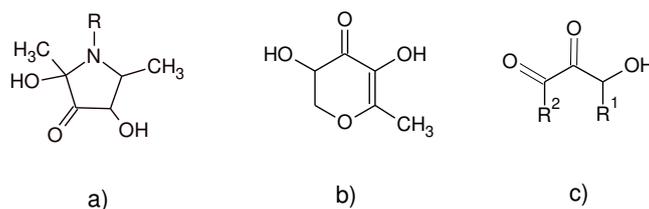
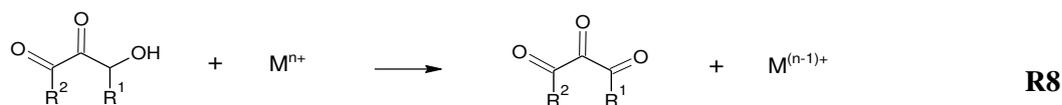


Figure 10: Chemical structure of Maillard reaction-derived antioxidants (abbreviations described in the text).

However, the reducing property of some reductones and Maillard reaction intermediates was also declared as being the source of prooxidative characteristics. These phenomena are mainly explained by their capability to donate electrons to transition metal ions (M^{n+}) making them available for oxygen activation.^{117,138,139} The mechanism is presented in Reaction 8.¹¹⁷



The reduced transition metal ion (e.g. Fe^{2+}) is then again available for $\cdot OH$ formation via the Fenton reaction, for example (cp. 1.4, Reaction 3). Because of the ambiguous pro- and antioxidative properties the role of reductones and other Maillard reaction products in oxidative reactions in malting and brewing is controversially discussed. While Chapon and Chapon¹⁴⁰ demonstrated antioxidative properties of Asco in relation to peroxide scavenging they also mentioned potential prooxidative effects of Asco. Irwin and Barker⁸⁹ demonstrated that Asco catalyzed the oxidation of 1-butanol in the presence of O_2 and Fe^{3+} or Cu^{2+} . Kunz et al.⁸⁷ reported acceleration of 1-HER formation in beer by Asco, whereas they also elucidated that such prooxidative effects of Asco are dose-dependent and may be compensated by increasing the Asco concentration. At higher Asco concentration radical formation in beer was decelerated indicating that a turnover from pro- to antioxidative properties (cp. Reactions 6 & 7) did occur. Regarding further intermediates Andersen et al.⁸⁸ and later also Kunz et al.⁸⁷ reported that the addition of Maillard reaction-derived reductones to beer increased the formation of organic radicals and hence, decreased the sulfite content. The concentration of organic radicals was greatly influenced by the kilning temperature and thus the application of melanoidin- or reductone-rich malt types for brewing was leading to beers with a reduced

oxidative stability.¹¹² All of those prooxidative effects were assigned to the interaction of reductones with M^{n+} (cp. Reaction 8).

(Poly-)phenols

Polyphenols are secondary plant metabolites that occur widely in fruits,^{141,142} vegetables^{143,144} and cereals.^{145–147} Phenolic compounds are aromatic hydrocarbons containing phenyl- as well as hydroxyl groups. They exist in innumerable compositions and linkages ranging from monophenolic compounds (e.g. hydroxybenzoic- and hydrocinnamic acids), diphenolic-tetrahydropyrans (e.g. flavans, flavonols, flavanols, flavones, flavanoids or anthocyanidins) up to condensed higher molecular polyphenols. Some major classes of phenolic compounds are illustrated in Figure 11.

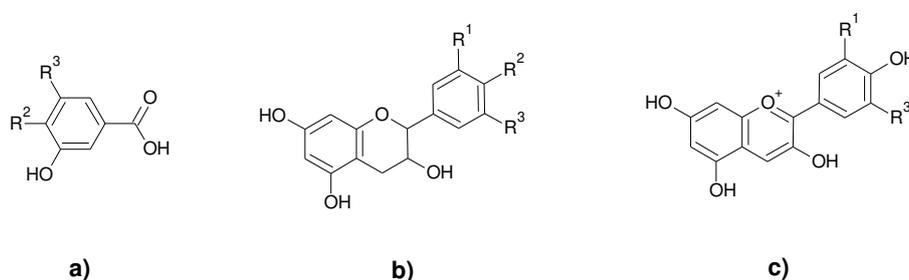
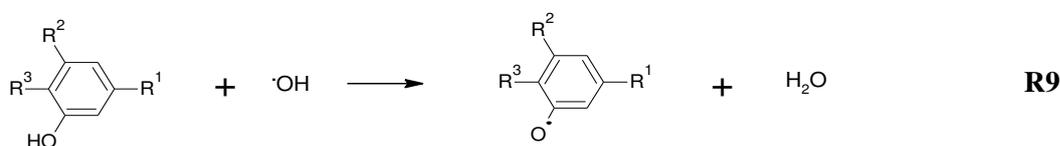
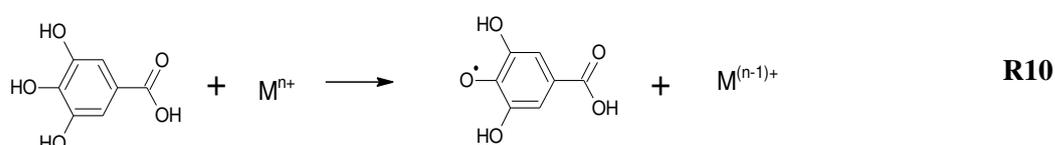


Figure 11: Chemical structures of selected phenolic compounds (a: hydroxybenzoic acid; b: flavanol; c: anthocyanidin).¹⁴⁵

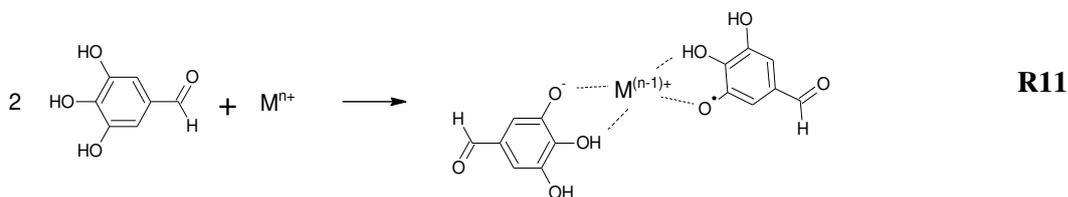
Regarding their antioxidative properties (poly-)phenols were reported to scavenge $\cdot\text{OH}$ (Reaction 9) at diffusion-controlled rates.^{148,149} Also they may suppress peroxidation in non-polar media.¹⁴⁸ The generated phenoxy radicals are considered to be unreactive as a result of resonance stabilization.¹⁵⁰



On the contrary, another study demonstrated that phenoxy radicals may also reduce O_2 and form $\cdot\text{OOH}$.¹⁵¹ Similar to Asco, (poly-)phenols may also reduce M^{n+} as shown for gallic acid in reaction 10.¹⁵²



However, there are uncertainties regarding the availability of $M^{(n-1)+}$ for O_2 reduction as (poly-)phenols, especially those bearing catechol- or galloyl groups, are also capable of $M^{(n-1)+}$ chelation (Reaction 11).¹⁵³

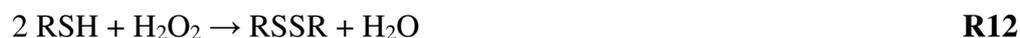


The content of lower molecular phenols was reported to increase during barley germination which in turn also increased antioxidant activity of malt as assessed by ABTS^{•+}- and [•]DPPH radical scavenging.^{154,155} In beer, phenolic compounds derive from malt and hops and their oxidation products significantly contribute to the color of wort and beer. Also, they have a positive effect on protein precipitation and cold break formation.¹⁵⁶ Polyphenols, especially those derived from hops, may also increase beer flavor stability.¹⁵⁷ However, in the final beer polyphenol-protein interactions are the major source of undesirable haze formation.^{77,78}

Regarding antioxidative effects of polyphenols in beer there are substantial disagreements in the literature. McMurrough et al.¹⁵⁸ reported that polyphenols contribute to ~60% of the overall reducing capacity of beer. It is important to mention that the measurement of the “reducing capacity” is based on the extent of Fe^{3+} reduction and that there is no evidence present for a connection between reducing capacity, flavor,- or physical chemical stability of beer. Liégeois et al.¹⁰⁹ claimed that malt- and hop-derived polyphenols are the most efficient antioxidants in beer as measured by the inhibition of linoleic acid oxidation by 2,2'-azobis(2-amidinopropane) dihydrochloride. On the contrary, Irwin and Barker⁸⁹ reported that 1,2,3-trihydroxypolyphenols catalyzed the oxidation of 1-butanol, when combined with copper, iron and O_2 . Concerning the scavenging of organic radicals in beer Andersen et al.¹⁰⁸ reported that phenolic compounds were without any effect. Also Wietstock et al.¹⁵⁹ reported that hop-derived polyphenols did not affect [•]OH and 1-HER formation as shown by POBN spin trapping and ESR spectroscopy, even though they found effects regarding the scavenging of the [•]DPPH radical.

Thiols

Thiols are organosulfur compounds that are characterized by at least one sulfhydryl- or thiol group (R-SH). Thiols occur in significant amounts in a plethora of food systems, mostly originating from the essential amino acid L-cysteine (Cys) in its molecular form as well as incorporated in low- and high molecular peptides and proteins. Besides Cys, representative low molecular weight non-volatile thiols are *N-acetyl-cysteine* (NAC), L-homocysteine (HCys) and L-glutathione (γ -L-glutamyl-L-cysteinyl-glycine, GSH).^{160,161} The oxidation of 2 R-SH moieties can lead to the formation of a disulfide (RSSR), which are significantly contributing to the conformation, stability and functionality of proteins.^{162,163} The antioxidative effect of thiols is mainly related to their reaction with H₂O₂ (Reaction 12)¹⁶⁴ and their scavenging ability of hydroxyl radicals via H-abstraction and thiyl radical (RS[•]) formation (Reaction 13).¹⁶⁵



The dimerization of 2 thiyl radicals (Reaction 14) is generally considered as the radical termination reaction of thiols. Besides its capability of inactivating ROS, GSH has received high attention in medicine and cell physiological research, as it also appears to play a unique role in general homeostatic processes such as redox regulation and defense against oxidative stress.^{166–168} The chemical structures of Cys, GSH and its dipeptides cystine and glutathione disulfide (GSSG) are illustrated in Figure 12.

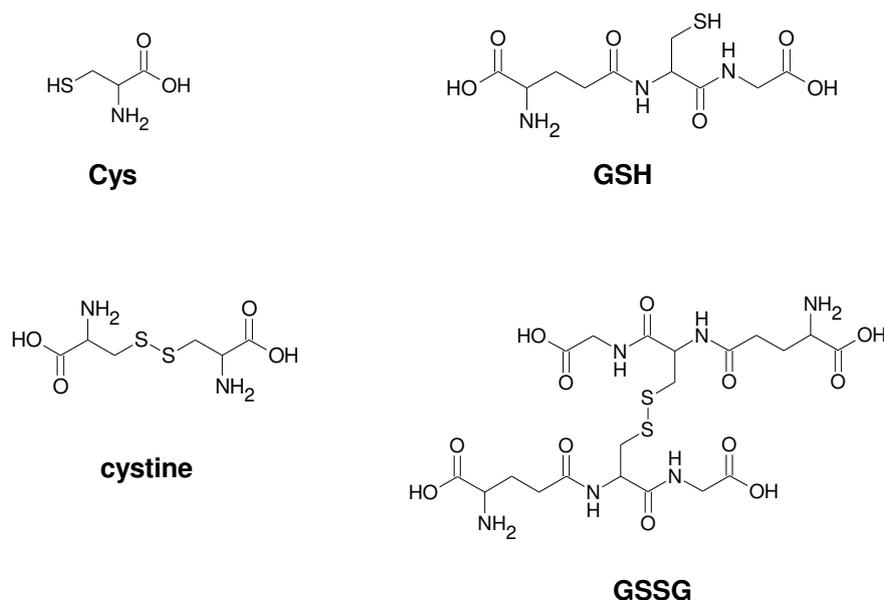


Figure 12: Chemical structures of cysteine (Cys), glutathione (GSH) and their oxidation products (cystine and glutathione disulfide (GSSG)).

However, there exists doubt on the significance of the dimerization reaction of thiyl radicals in natural systems (RS^{\bullet}) (Reaction 14) as they may also react with biomolecules like alcohols,¹⁶⁹ phenols,¹⁷⁰ or fatty acids.¹⁷¹ Thereby their redox potential is increased, which enhances their susceptibility to oxidation by O_2 .

Another prooxidative property of thiols is their capability of forming complexes with transition metal ions. In the presence of O_2 the reduced complexes were reported to generate H_2O_2 and $\bullet OH$.^{172–176} Also Muller¹⁷⁷ found that oxidation of thiol-rich proteins can form H_2O_2 during mashing.

However, in beer it was proposed by Rogers and Clarke¹⁷⁸ and later by Lund and Andersen¹⁷⁹ that protein-derived thiols play a significant role as antioxidants as they react with H_2O_2 to form a sulfenic acid intermediate (R-SOH) and the respective disulfide (RSSR) by a subsequent reaction with another protein-derived thiol. In wine model systems the antioxidative properties of thiols, like Cys or GSH were shown to originate from their reaction with 1-HER (as formed from $\bullet OH$ and EtOH) as demonstrated by electron resonance spectroscopy (ESR) after spin trapping with phenyl-*N-tert*-butylnitron (PBN).¹⁸⁰ Those findings were later confirmed in beer,¹¹⁰ whereas thiols were designated as secondary antioxidants by scavenging of 1-HER after sulfite depletion. Based on these findings an elaborate effort has been made to enrich the levels of thiols in wort and beer.^{181,182} Still, the role of thiols in oxidative reactions in malting and wort production, where oxygen is mostly present, remains poorly understood.

Sulfites

Sulfur dioxide (SO₂) is a non-metal oxide gas, which is emitted to the atmosphere mostly from fossil fuel power plants. The SO₂ pollution affects health problems, especially in organisms that lack sulfite oxidase. SO₂ also occurs naturally in all living organisms as intermediate of the synthesis of sulfur-containing amino acids (methionine, Cys). In aqueous systems SO₂ undergoes a pH-dependent ionization. Below pH ~2 it retains its molecular form (SO₂) whereas above pH ~3-6 its majority is present as its first ionization product, bisulfite anion (HSO₃⁻). At pH >7 it is mainly present as sulfite (SO₃²⁻) (Figure 13).¹⁸³



Figure 13: Protonation equilibria of sulfur dioxide in aqueous solution.

Besides its natural occurrence, especially in fermented foods, sulfites, such as sodium sulfite (Na₂SO₃), sodium metabisulfite (Na₂S₂O₅) or the respective potassium salts are widely applied in the food industry as antioxidants,^{184–188} anti-browning agents^{187,189,190} as well as antimicrobials.^{184,191} In the malting process sulfite is used as adjuvant during kilning to avoid nitrosamine formation.^{192,193} In beer, sulfite was proposed of being the primary, most effective antioxidant.^{86,106,193,194} Andersen et al.⁸⁶ proposed that the role of sulfite in the prevention oxidative deterioration of beer is primarily related to its reaction with H₂O₂ (Reaction 15).¹⁹⁵ Thereby sulfite suppresses “Fenton-like” reactions and 1-HER formation leading to a higher physical-chemical- as well flavor stability of beer.



Prooxidative behavior of sulfite in malt, beer or other foodstuffs has not been reported before.

Secondary antioxidants

Secondary antioxidants prevent ROS formation at early stages of oxygen activation. The combined action of enzymes, like superoxide dismutase and catalase, inactivate O₂⁻ and H₂O₂ before the more harmful •OH are formed. Those enzymes have also been characterized in barley and malt.¹⁹⁶ Other examples for antioxidant enzymes are ascorbate peroxidase¹¹¹ or MSRA,⁶⁰ whereas the latter repairs oxidative damage rather than preventing it from the first place. Other secondary antioxidants are substances that capture catalysts of O₂ reduction (e.g. Mⁿ⁺), for example chelators, like EDTA or humulones.¹⁹⁷

2 Problem and objectives of this study

The role of SMM as the primary, thermal precursor of DMS in malt, wort and beer is well established. However, as depicted in the introduction, there is a substantial lack of knowledge regarding the role of DMSO as DMS precursor and its contribution to the final DMS levels in beer. Currently, there is no established method available for the quantification of DMSO in malting and brewing. Hence, the levels of DMSO in different malt types, its extraction behavior during mashing as well as its fate in the entire brewing process are essentially unknown. Elevated DMS oxidation and DMSO formation would adversely increase the risk of DMS formation during fermentation. This would also crucially affect the levels of DMS in beer and consequently, beer flavor. Also, the extent to which different globally applied yeast strains are capable of DMSO reduction is mostly unknown. In packaged beer DMSO reduction may also affect beer flavor and needs to be investigated. Under consideration of the aforementioned aspects it is of indispensable importance to further characterize the role of DMSO as DMS precursor in beer. After DMS formation there are basically two potential chemical interconversion reactions between DMS and DMSO that might be of significant importance for the levels of DMS in beer. Firstly, the oxidation of DMS to DMSO (DMS oxidation) and secondly, the reduction of DMSO, which yields DMS (DMSO reduction). The holistic consideration of those reactions is further defined as the “redox behavior of DMS and DMSO”. The characterization of the redox-behavior of DMS under consideration of technological-, bio- and physical-chemical factors was the central goal of this dissertation.

Part one of this dissertation aimed to develop an analytical method for the quantification of DMSO in malt, wort and beer as prerequisite to assess the role of DMSO as a DMS precursor and to obtain knowledge on reactions that yield DMSO. The method should be integrable into commonly applied methodologies for DMS quantification with main focus on low sensitivity towards matrix effects, stability of other DMS precursor during sample preparation and moderate time of analysis.

In **part two** the method is aimed to be applied for the investigation of the behavior of DMSO in malt, throughout the brewing process and during beer storage. In this context the major objective was to elucidate the potential interconversion reactions, DMS oxidation during wort production and DMSO reduction during fermentation and to assess their contribution to the final levels of DMS in beer. Specifically, besides the extractability of DMS and its precursors (SMM and DMSO) during mashing, a central goal was set on the investigation of the yet-uncharacterized DMS oxidation in the brewing process as well as factors that influence the

reduction of DMSO during fermentation. Also, the impact of a variety of beer-derived potential antioxidants (ascorbic acid, cysteine, glutathione, gallic acid and sulfite) was aimed to be tested on DMSO reduction and DMS formation during beer storage.

The ambition of **part three** of this dissertation work was to characterize reaction mechanisms of DMS oxidation and DMSO formation. The understanding of such reactions is a basic requirement to develop approaches for the control of DMSO formation in malting and brewing as well as in and other foodstuffs. In context to the in part one investigated potential reducing capability of antioxidants, their high abundance in malt together with their potential involvement in oxidative reactions (cp. 1.5) in general, the same compounds were tested this time on their efficacy to minimize DMS oxidation and DMSO formation in buffered model solutions. As the abundance of transition metal ions is inevitable in the malting process, the investigations were aimed to be carried in presence of transition metal ions (e.g. Cu^{2+}) and molecular oxygen as a premise to also cover potential prooxidative effects of the antioxidants. To further include potential side reactions including metabolic-biochemical as well as physical-chemical factors, an *in vivo* malting experiment should elucidate the effect and relevance of the addition of antioxidants and copper to the germination process on the final levels of DMSO in malt. As it is unclear which oxidants are responsible for DMSO formation in malt different reactive oxygen species (O_2 , H_2O_2 , $\cdot\text{OH}$) were tested on their capability of DMS oxidation in buffered model solutions. The goal was to characterize reaction mechanisms to understand the redox behavior of DMS and DMSO in malting and brewing. Outcomes of this work should serve for approaches to control DMSO formation in malt to decrease the risk of DMS formation during fermentation and thus, to increasing beer quality. An overview about the structure of this dissertation work is illustrated in Figure 14.

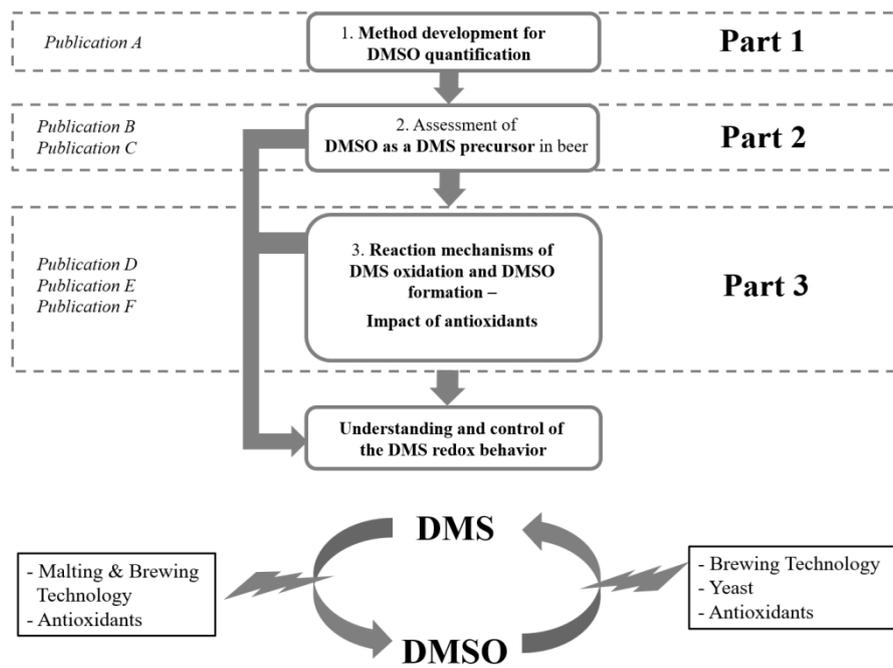


Figure 14: Schematic illustration of the structure and goals of the dissertation work.

3 Publications

The results and outcomes of the whole thesis were published via 6 manuscripts in 4 different international peer-reviewed journals. The manuscripts are constitutively connected to each other following the long term goal of elucidating the redox behavior of DMS and DMSO in malting and brewing to develop technological approaches for minimization DMS formation via DMSO. In publication A the development of the analytical method for the quantitation of DMSO in diverse malting and brewery samples was elucidated. The method was shown to be applicable to investigate the behavior of DMSO and its role as DMS precursor in the brewing process as well as during beer storage (publication B and C). While in publications C potential antioxidants were tested on their capability to reduce DMSO in beer under low oxygen environment, publications D and E address the impact of potential malt-derived antioxidants and transition metal on DMS oxidation and DMSO formation in the presence of oxygen. Publication F provides an overview of diverse (reactive) oxygen species that are capable of DMS oxidation and thus are sources for DMSO in malt, wort and beer. All experimental work of this thesis was conducted at the Technische Universität Berlin at the Lab of Brewing Science during the period of the years 2013-2018.

The own-share of all publications in sum is approximately 80-85 %. Ideas for all publications were initially created in cooperation with Prof. Dr.-Ing. Frank-Jürgen Methner. In publication B, the selection for the “screening of top-fermenting yeast on DMSO reduction” (~20 % of the total investigations) was planned together with Dr. Mathias Hutzler (Research Center Weihenstephan for Brewing and Food Quality, Technical University of Munich). For each other publication the planning, experimental design, execution, and data evaluation was carried out solely by the first author. The Lab of of Brewing Science at the Technische Universität Berlin provided analytical support in publication C for sulfite analysis. The experiments were mostly conducted with assistance from student co-workers, except for publication A and C, which were generated solely by the first author. Thomas Kunz provided technical assistance in the ESR measurements in publication F.

In the following chapters 3.1 - 3.3., the publications are presented in a constitutive order, not necessarily related to their dates of publication. The approaches as well as the outcomes are briefly summarized. In the summarizing discussion (cp. 4) the most significant findings are reflected and their individual relevance is discussed. Coherently, holistic considerations and their significance towards implementations in the malting and brewing industry are outlined.

3.1 Method development for DMSO quantification

Publication A

The first step of this dissertation was to develop a method for the quantification of DMSO to be used for the investigation of the redox behavior of DMS and DMSO. In biological systems, DMSO is often quantified via reduction and subsequent analysis of the generated DMS using gas chromatography (GC). Therefore several reducing agents, like stannous chloride or titanium chloride, are applied for acidic DMSO reduction at temperatures above 90°C.^{65,198,199} In general, these methodologies are costly in terms of time and technical infrastructure. Evolving acidic fumes are damaging towards a series of GC capillary columns. Also, the risk of thermal degradation of SMM to DMS and homoserine cannot be excluded. Sodium borohydride is a further potential reducing agent of DMSO,²⁰⁰ whereas it is not suitable for the brewing industry as the alkaline working conditions evoke significant SMM hydrolysis, which adversely impairs the DMSO assessment. The initial goal was to find a sufficient DMSO reducing agent that generates the equivalent amounts of DMS that can be detected by established gas chromatographic methodologies. The reduction step should be integrable into commonly applied DMS quantification methods^{54,55,201} on the premises of the stability of other potential DMS precursors, high recovery rates and low matrix effects.

The entire approach of the method development, results and discussion are manifested in the Journal *BrewingScience – Monatschrift für Brauwissenschaften*, in a paper entitled “*The Analysis of Dimethyl Sulfoxide in Malt, Wort and Beer using Headspace Gas Chromatography combined with Pulsed Flame Photometric Detection - Methodology and Applications*” (Publication A²⁰²).

Based on the screening of diverse reducing agents, sodium metabisulfite was found to be the most suitable substance for DMSO reduction and its following analysis as DMS equivalents using headspace GC combined with pulsed photometric detection (HS-GC-PFPD). The applicability of the developed method was further underlined by the DMSO recovery from wort and beer (>95 %), which is the fundament of this dissertation work.

Publication A

M. Baldus, T. Kunz and F.-J. Methner

The Analysis of Dimethyl Sulfoxide in Malt, Wort and Beer using Headspace Gas Chromatography combined with Pulsed Flame Photometric Detection (HS-GC-PFPD) – Methodology and Applications

Besides S-methyl methionine (SMM) as the thermal precursor for the unpleasant aroma compound dimethyl sulfide (DMS) in the brewhouse dimethyl sulfoxide (DMSO) is a further potent precursor during fermentation. In this paper a short and sensitive method for its analysis is introduced. In the first step DMSO is reduced to DMS by sodium metabisulfite and the generated free DMS is subsequently analysed employing HS-GC-PFPD. In comparison to other substances sodium metabisulfite emerged as an efficient reducing agent whereby SMM and other potential precursors of volatile sulfuric compounds are not contributing to DMS under the applied conditions. In saturation of sodium metabisulfite the reduction of DMSO is exposed to be independent of its initial concentration and the recovery rate is constantly above 95 % in its usual wort- and beer concentration range. In first applications of the developed method DMSO and DMS levels are tracked during fermentation. The DMSO reduction was about 11 %, which is consistent with the accordant literature. Additionally, a non enzymatic pattern of DMS formation by reduction of DMSO with sulfite is proofed and discussed. The described method is easy to integrate to standard DMS analyses. Thereby breweries and related sectors have the possibility to upgrade their existing system without high financial and technical effort.

Descriptors: DMSO, DMSO analysis, DMS, S-methyl methionine, DMS precursor, sulfite, wine, SO₂, thiols, fermentation, oxidation, reduction

1 Introduction

Off-flavor in beer induced by dimethyl sulfide (DMS) is often considered as avoidable by efficient wort boiling or heating step and evaporation prior fermentation. Furthermore DMS decreases generally during fermentation owing to the stripping effect of the exhausting CO₂. However, several yeast strains are able to produce DMS by enzymatic reduction of DMSO [2, 3, 11, 12, 13, 14]. Unfortunately many breweries don't have the possibility to judge the potential risk of this source because a quick and exact method for the analysis of DMSO is still missing. In general DMS is generated from two precursors, S-methyl methionine (SMM) and DMSO. Primarily DMS originates from the thermal decomposition of the malt non protein amino acid SMM. This pattern plays a superior role in DMS production during malt kilning and in the brewhouse

where operation temperatures of more than 70 °C are achieved [2, 18, 31]. Besides the temperature the pH value is the next limiting factor of the SMM degradation. In solution for example, at 98 °C (pH 6) the first order rate constant k of the SMM degradation is $2.43 \cdot 10^{-4} \cdot \text{s}^{-1}$, whereas at 60 °C (pH 4), k is $0 \cdot \text{s}^{-1}$ [18]. The oxidation products of DMS are DMSO and DMSO₂ which are additionally formed during malt kilning [2, 4, 29]. A new method for the SMM determination according to *Scherb et al.* [25] employs stable isotope dilution and LC tandem MS for highly sensitive measurements of SMM. This method is hardly to engage for daily brewery routine analysis due to the high acquisition and operation costs. In consequence the method of *White and Wainwright* [26] keeps the widest accepted method for SMM determination in the brewing industry up to now. The analytical procedure is done by heat-alkaline boiling of the sample and the measurement of DMS before and after treatment. The method was critical discussed a few times owing to the affirmed potential of DMS oxidation in alkaline solution during the measurement and the accompanied falsification of the SMM content [9, 27, 28]. In consequence *De Rouck et al.* [9] developed a non alkaline thermal degradation procedure, in which a SMM recovery of about 98 % was achieved.

In the brewing process after wort cooling the amount of SMM can be assumed as constant, when no further heat treatment is applied. From this point DMSO remains as potent precursor owing

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to the yeast specific enzymatic reduction mentioned before. In this context DMSO₂ is regarded as resistant and does not produce DMS via this pattern [4]. By reason of the high boiling point of DMSO (189 °C) and its thermal stability its concentration is assumed as being constant over the conventional brewhouse process, when an efficient removal of DMS after its evolution is practiced [2]. During fermentation it is known that DMSO reductase activity of several yeasts strains is able to reduce DMSO up to 21 % [3]. In dependence on fermentation parameters like the DMS desorption rate of the CO₂, fermentation temperature and especially the DMSO content in the pitching wort, this pattern may be responsible for relative higher DMS levels in the finished product compared to the pitching wort. According to the literature malt as the main source for DMSO in the pitching wort contains between 0–9.3 µg/g [4, 13, 29]. Relatively high DMSO levels up to 1230 ppb were also found in wine. In this connection DMSO was pronounced as one of the most abundant natural occurring sulfuric compound in wine [8]. A much higher – not necessarily natural – abundant sulfuric substance in wine is sulfur dioxide (SO₂), which is commonly added to wine as antioxidant up to 400 ppm (specific wine) [17]. *De Mora et al.* [8] added DMSO and Cysteine to young wine and found an enhanced DMS formation during storage and pointed out that DMSO remains as potent precursor even after fermentation.

For these reasons it is helpful to determine the DMSO content in the malt and pitching wort or yet in must to assess the potential of DMS formation caused by DMSO reduction. In general DMSO is analysed and quantified by its initial reduction and subsequent analysis of the generated free DMS by gas chromatography (GC). Thereby several reducing agents, like stannous (II) chloride or titanium (III) chloride, are applied for DMSO reduction by acidic boiling [1, 5, 15, 29]. These types of methodologies require a high time and cost intensive operation expense. Whereby evolving acidic fumes are harmful to a series of GC capillary columns. Moreover the risk of thermal degradation of SMM to DMS and Homoserine cannot be excluded. Sodium borohydrid, as a further reducing agent, is only applicable under a high mechanistic build-up procedure using inert gas stripping for the removal and analysis of DMS [1]. In solution the substance is highly alkaline and might cause an extended breakdown of SMM in wort at higher temperatures. In consequence this method is unsuitable for the brewing industry. *Dickenson* [10] describes the reduction of DMSO in wort and beer in saturated sodium metabisulfite and the analysis by GC with flame ionization detection (FID) of free DMS before and after treatment. He achieved a reduction rate of about 90 % but further details of the procedure and GC settings are lacking in this paper. *Dickenson* [14] also reports that reduction of DMSO with L-Cysteine represents an alternative for sodium metabisulfite. The reducing activity of sulfite is known to depend on the pH value [16]. The main reducing activity of sulphite in solution is referred to the content of available sulfuric acid (HSO₃⁻), which is the quantitative present form at pH values from around 3.5–5.5, similar to beer and wort pH. The FID used by *Dickenson* [10] was found to be less sensitive for sulfuric compounds and is generally not applied anymore for the detection of DMS. In contrast the Flame Photometric Detector (FPD) has been proved to be highly sensitive for the detection of sulfur compounds. *Annes* [5] reduced DMSO with stannous (II) chloride and hydrochloric acid (HCl) by boiling treatment and detected DMS before and after treatment

using GC-FPD. In this context the stannous chloride contained impurities of DMS when it was treated with HCl. Furthermore harmful acidic fumes emerge to the GC column as described previously. Another disadvantage was that the thermal degradation of SMM influenced the measurement noticeably when it was not removed prior DMSO reduction. *Yang and Schwarz* [28] extended the latter method and availed this circumstance to measure SMM and DMSO in malt extracts back-to-back by GC-FPD. Free DMS was purged out of the sample with nitrogen stripping after its analysis. SMM was then determined according to *White and Wainwright* [26]. A second step of nitrogen purging removed the newly built free DMS. DMSO was then reduced by stannous chloride as described before [5]. Before injecting an equilibrated headspace volume into the GC port, the acidic fumes had to be neutralized by a further step where sodium hydroxide is transferred through the vials crimp sealed teflon septa. The SMM determination is obligatory for the further DMSO determination. Summarised this method needs at least 2h plus sample preparation to gain the DMSO concentration of a single sample. Additionally the authors declare that sodium metabisulfite as used according to *Dickenson* [10, 13] could not be applied under their described conditions because an unidentified peak covered the DMS peak. The purpose of this study was the development of a more efficient and sensitive analytical method for DMSO based on suitable DMSO reducing agents, which can be used at lower temperatures to exclude SMM breakdown and to reduce oxidative reactions. Especially the method of *Dickenson* [10] is focussed to be optimized and extended by pulsed flame photometric detection (PFPD) for the DMSO determination in malt, wort and beer. The new method should acquire low technical effort, less operation time and has to be easily adopted by breweries and related sectors.

2 Materials and methods

2.1 Chemicals

Materials including dimethyl sulfoxide (DMSO; C₂H₆OS; >99 %), dimethyl sulphide (DMS; (CH₃)₂S; >98 %), dimethyl sulfone (DMSO₂; C₂H₆O₂S; 99 %), DL-methionine sulfonium chloride (SMM; C₆H₁₄CINO₂; >99 %), L-cysteine (L-Cys; C₂H₁₂N₂O₄S₂; ≥99 %), L-cystine (C₆H₁₂N₂O₄S₂; 98 %), trans-ferulic acid (C₁₀H₁₀O₄; 99 %), gallic acid (C₇H₆O₅; 97.5–102.5 %) and sodium sulfide (Na₂S; –) were purchased from Sigma Aldrich (St. Louis, USA). Ethyl methyl sulphide (EMS; C₂H₈S; >97 %) and L-glutathione (L-GSH; 97 %) were purchased from Alfa Aesar (Karlsruhe, Germany). Sodium metabisulfite (Na₂S₂O₅; >97 %) and L-ascorbic acid (C₆H₈O₆; 99 %) were obtained from Merck Chemicals (Darmstadt, Germany).

2.2 Screening for reducing substances

To figure out the most suitable reducing substance for the DMSO determination, 2 g of the sulfuric compounds sodium metabisulfite, L-Cys, L-GSH, the reductone ascorbic acid and the phenolic compounds gallic and ferulic acid were added to 5 ml of 0.1 M sodium acetate buffer (pH 4.5), respectively. The buffer was spiked with DMSO to reach concentrations of 76, 134, 190 and 288 ppb. The reduction and determination of the evolved DMS was carried out as depicted in sample preparation.

2.3 Impact of potential precursor substances on the DMSO results

To test the potential of potent precursors of volatile sulfuric compounds the following substances were added to a 0.1 M sodium acetate buffer as DMS equivalent amounts: SMM (1500 ppb), DMSO₂ (500 ppb), L-cystine (3000 ppb), GSH (3000 ppb), DL-methionine sulfoxide (2000 ppb). H₂S was generated by adding sodium sulfide to a nitrogen purged beer with a pH value of 4.3 (1000 ppb). All samples were then analysed as described in sample preparation and GC section.

2.4 Malt extraction

The malt extraction was carried out according to MEBAK [22] with exception of some deviations. 5 g instead of 10 g fine ground malts were extracted in 100 ml bidistilled water in closed round flasks for 30 min. The mixture was centrifuged afterwards at 7000 rpm for 10 min. The supernatant was then further processed as described in sample preparation.

2.5 Fermentation trial

Fermentation was carried out in 1 l Schott flasks at 12 °C. The yeast strain RH was used as representative bottom fermenting strain with a pitching rate of 2.5×10^7 cells/ml. The pitching wort (pw) was produced from 100 % pilsner malt with a final gravity of 11.2 °P in the pitching wort. Gentle stirring of the media was applied in a range just enough to prevent extended yeast settling and to compensate the missing convection in the flasks.

2.6 Test on DMSO reduction by sulfite in lower concentration ranges

To test the impact of DMSO reduction by sulfite sodium in lower concentrations acetic buffer solution (pH 4.5) was spiked with DMSO to gain 2 ppm, sodium metabisulfite to 50 ppm (ratio 1:25). The study was carried out by the addition of 5 mL buffer/DMSO/sodium metabisulfite into a 25 mL headspace vial. The buffer and the headspace vial were purged with nitrogen before to reduce the oxygen content. DMS evolution was tested according to MEBAK [22] after 2, 14, 23 and 28 days of storage in the dark at room temperature.

2.7 Gaschromatographic Analysis

2.7.1 Calibration

In the first step 0.1 M sodium acetate/ acetic acid buffer solution was adjusted to a pH value of 5.5. Besides this bidistilled water was boiled and purged with nitrogen to reduce the oxygen content. Afterwards the buffer solution was spiked with a DMSO/bid.-water solution ($1.104 \cdot 10^4$ ppb) to adjust DMSO concentrations of 38, 76, 134, 190 and 288 ppb. For beer the calibration is treated the same way, but the pH was adjusted to 4.5 and EtOH was added to 5 vol/vol%. The measurement was carried out as described in the sample preparation and GC section. To transform the exponential response of the PFPD into linear response, the calibration graphs of the detected DMS were constructed by plotting the log

(DMS/EMS) peak area ratios against the log (DMS/EMS) concentration ratios. The calculation of the DMS concentration was carried out according to MEBAK guidelines [22].

2.7.2 Sample preparation

Free DMS was purged out of the sample using nitrogen stripping as previously described by Yang and Schwarz [28]. After treatment the sample was cooled down to 1 °C. 3.85 g of sodium metabisulfite, (20 % over saturation) was added into a headspace crimp top vial (Supelco, Sigma-Aldrich, St. Louis), which was purged with nitrogen before to reduce the oxygen content. Afterwards the vial was sealed and immediately cooled down to 20 °C before a quantity of 5 g of each ice-cold sample (1 °C) was added. Subsequently 50 µL of an ethanol-ethyl methyl sulfide solution ($2.53 \cdot 10^5$ ppb) was injected and directly hermetically closed using an aluminum crimp cap with a PTFE/silicone septum (Supelco, Sigma-Aldrich, St. Louis). The accelerated reduction of DMSO was carried out for 10 min in a water bath at 60 °C under shaking (95 rpm). Thereafter the sample was cooled down to 4 °C before the headspace vial was incubated at 50 °C in a water bath for a period of 30 min. The sample preparation for free DMS was done according to the MEBAK guidelines [22] with exception that the headspace vials were purged with nitrogen before to reduce the oxygen content.

2.7.3 Gas chromatography (GC)

200 µL of the headspace gas was injected into a HP 5890 Series II GC equipped with a pulsed flame photometric detector (PFPD 5380, OI Analytical, Texas, USA). The column used was a HP-5 model (39.0 m x 0.32 mm) from Agilent Technologies. Nitrogen was used as carrier gas with a flow rate of 2.1 ml/min. Injection was done in split mode (split ratio 1:37) at 230 °C with a split flow of 78.5 ml/min. The applied temperature program started with 1 min at 35 °C and was followed by a 7 °C/min temperature gradient to reach 55 °C and a subsequent 60 °C/min gradient to 150 °C. The PFPD was set at 250 °C, 500 V, 20 ms gate width, 6 msec delay and a trigger level of 300 mV. The detector gas flow rates were 16.0 ml hydrogen and 22.0 ml synthetic air. The obtained data were sent to a HP ChemStation (Agilent Technologies, Germany), where they were integrated and processed.

3 Results and Discussion

3.1 Optimization of the DMSO reduction with sodium metabisulfite

In the first step of the method development sodium metabisulfite was used for the reduction of DMSO. The chromatograms of the reduced solutions showed up overlapped signals of DMS and a further component, which was reported previously by Yang and Schwarz [28] using the GC oven program for DMS according to MEBAK [22]. This program was optimized in a series of trials leading to the program described in the material and methods section. Applying the optimized temperature gradient and the determined flow rates an exact separation of the two peaks could be achieved under the described GC conditions as illustrated in figure 1.

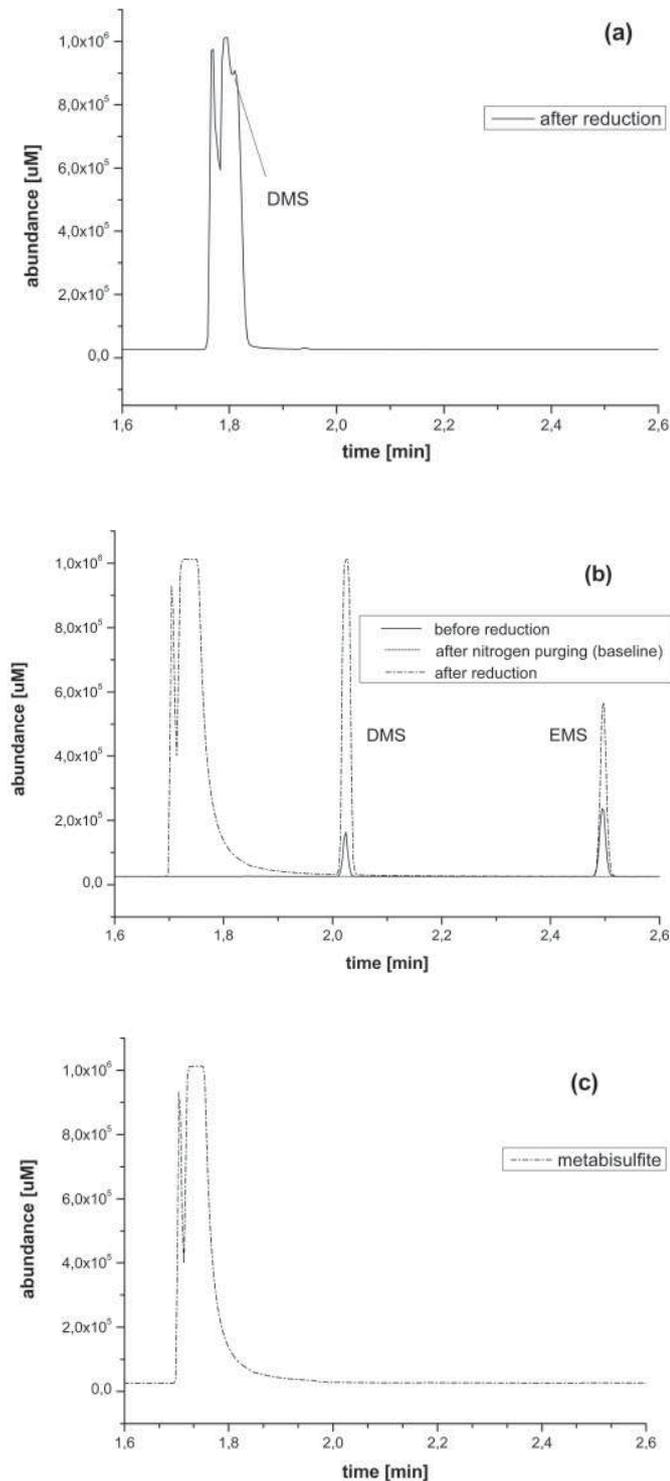


Fig. 1 chromatograms of sulfite, DMS and EMS signals:
(a) conventional method,
(b) optimized method,
(c) conventional with sodium metabisulfite exclusively

For the identification of the first peak which was declared as unknown before [28] aqueous solutions of sodium metabisulfite were investigated under the described optimized analytical conditions. Heating up aqueous HSO_3^- it dissociates to free SO_2 . In case of the used calibration buffer solution (pH 4.5) only containing sodium metabisulfite the first peak was detected exclusively (Fig. 1 (c)), which verifies the assumption that the first peak originates from the generated SO_2 during the analyses. The double signal nature of

this peak might be lead back to a mixture of SO_2 and HSO_3^- . This assumption remains to be investigated.

3.2 Screening for DMSO reducing agents

Since SO_2 is a natural reducing substance of beer it was of interest to investigate the DMSO reducing ability of other 'beer-own' reducing substances like ascorbic acid, phenols, and other sulfuric compounds like L-cysteine (L-Cys) and L-glutathione (L-GSH). For the qualitative comparison of their reducing behaviour against DMSO equivalent mass fractions of all substances were used, as described in the methods section. In figure 2 the added DMSO concentration is plotted against the resulting DMS peak area caused by DMSO reduction.

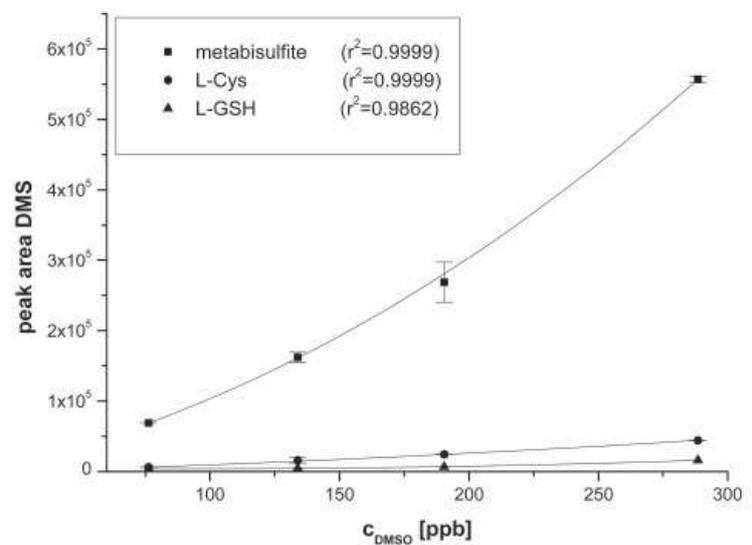


Fig. 2 DMSO calibration with different reducing agents (mean of duplicate trials, error bars: \pm standard deviation(s.d.))

Figure 2 demonstrates that all tested sulfuric substances (sodium metabisulfite, L-Cys, L-GSH) are able to reduce DMSO resulting in a high correlating component specific increase of the DMS peak area. Outstandingly, sodium metabisulfite exhibits the highest capacity for the DMSO reduction in the tested DMSO concentration range. Ascorbic acid, ferulic acid and gallic acid did not produce any signal at the retention time of DMS, indicating that no reduction of DMSO occurred at the chosen measuring conditions (not shown). Moreover the solubilities of the substances were different at sample preparation conditions in order of sodium metabisulfite, L-GSH, L-Cys. Simultaneously, reduced solubility diminishes the functionality. Thereby the unproportional minor difference between L-Cys and L-GSH might be explained.

L-Cys represents a limited suitability for the DMSO reduction under the used conditions due to its considerable small peak area increases. The peak areas of DMS produced by the reduction with sodium metabisulfite were in average around 10 times higher than of L-Cys, for example.

Owing to this background the sample preparation and dosage for sodium metabisulfite was further optimized resulting in the procedure described in material and methods. The calibration was

carried out in triplicate every week. The correlation coefficient was $r^2 = 0.9996 \pm 0.0004$ ($n = 10$; \pm s.d.). In comparison to the conventional DMS calibration, the sample preparation and calibration of DMSO is easier to handle due to its high water solubility and its non volatile nature.

3.3 Impact of potential precursor substances on the DMSO results

As described in the material and methods section the reduction of DMSO is done at 60 °C. After addition of sodium metabisulfite to wort or beer a pH value of 3.9 is achieved. To make sure that SMM does not contribute to the DMSO results, the thermal stability of SMM was proofed by the treatment of aqueous SMM solutions under the reduction conditions. Besides SMM also other potential precursors for volatile sulfuric compounds were tested. Table 1 gives an overview about the impact of chosen sulfuric compounds (DMSO₂, DL-methionine sulfoxide, H₂S, L-Cys, L-cystine and L-GSH) on the DMS formation during the DMSO measurement.

Table 1 Impact of potential precursors of volatile sulfuric compounds on the DMSO measurement (SMM check was carried out six times, all other substance checks three times)

compound	signal
SMM	n.d.
DMSO ₂	d.
L-Cystine	n.d.
L-Cys	n.d.
L-GSH	n.d.
DL-methionine sulfoxide	d.
H ₂ S*	n.d.

n.d. no signal detectable
s.d. signal detectable / not interfering with DMS signal
d. signal detectable / interfering with DMS signal

*prepared by sodium sulfide addition to beer of pH 4.4

In case of SMM no DMS signal was detected, which is in line with the results of Kovatscheva [18]. Hence SMM does not contribute to the DMSO levels at the used pH-value (3.8–3.9) and temperature (60 °C) conditions it is to conclude that SMM is stable during the DMSO determination. In the case of DMSO₂ and DL-methionine sulfoxide minor peaks were observed at the retention time of DMS but in both cases the areas were below the detection limit. Processing of aqueous solutions of both substances without sodium metabisulfite addition minor DMS peaks occurred likewise. Behind this observation the DMS signals might be lead back to impurities of containing DMSO or DMS in the commercially obtained substances. Additionally, it has to be mentioned that the samples for DMSO determination are generally diluted 1 : 5. In this concentration range, only the base line was detected for DMSO₂ and DL-methionine sulfoxide. All other tested substances like H₂S, L-Cys and L-Cystin were found to not produce any DMS signal under the applied conditions.

3.4 DMSO and DMS levels in malt, wort and beer

In this step malts, wort and beer were screened for DMSO levels. Table 2 gives an overview about the detected DMSO levels in

commercially used malt types, a pitching wort and different german beers. All determinations were carried out in triplicate except of the pitching wort and the pilsner type 1 beer. The measurements of DMSO in these cases were done 8 times to increase the accuracy of the reference value for the determination of the recovery rates in the next step.

Tab. 2 DMS and DMSO concentrations of malt, wort and beer types

		DMS	DMSO
malt ^{a, c}	pilsner malt	2.63 ± 0.14	4.44 ± 0.09
	munich malt type I	3.19 ± 0.09	7.16 ± 0.04
	munich malt type II	1.64 ± 0.04	10.31 ± 0.15
	roasted barley malt	n.d.	n.d.
	wheat malt	1.20 ± 0.02	2.1 ± 0.1
wort ^{b, **}	all malt pitching wort ^b	44.8 ± 2.8	588 ± 3.6 ^b
beer ^{**}	pb 1	28.3 ± 0.5 ^c	542 ± 0.6 ^b
	pb 2 ^c	21.1 ± 0.14 ^c	515 ± 0.4 ^c
	pb 3 ^c	25.0 ± 0.6 ^c	501 ± 1.7 ^c
	wb 1 ^c	26.3 ± 1.9 ^c	388 ± 1.7 ^c
	wb 2 ^c	27.5 ± 2.0 ^c	435.7 ± 3.2 ^c

** – concentration in µg per kg DMS equivalents

a – concentration in µg per g

b – mean value ($n=8$) ± standard deviation (s.d.)

c – mean value ($n=3$) ± standard deviation (s.d.)

pb – pilsner beer

wb – wheat beer

The DMSO distribution especially in several types of malt shows noticeable differences. The wheat malt contained the lowest amount of DMSO followed by pilsner malt and a remarkable increase in munich malt type I and type II. In this connection it is to point out that the levels of DMS and DMSO in roasted barley malt were too low to detect. Owing to missing details on the malting conditions a direct comparison between the DMSO levels of the samples cannot be accomplished reasonable. For the roasted barley malt it can be suggested that a decelerated germination produced less amount of SMM resulting in a reduced DMS formation and its oxidation to DMSO during kilning. On the other hand the levels of DMSO₂ have not been analyzed in these trials, thus the further oxidation of DMSO caused by the high roasting temperatures of roasted barley malt cannot be excluded. Corresponding to the wheat malt also lower DMSO levels were detected in the analyzed wheat beer types, which were around 390 ppb. The pilsner type beers contained DMSO levels between 501 and 542 ppb. In comparison to the all pilsner malt pitching wort, the levels in beer were all lower, which might be explained by the yeast reducing activity [2, 3, 4, 13, 14].

3.5 Test on DMSO recovery in wort and beer

The pitching wort and the pilsner beer type 1 were taken further for the determination of the recovery rates as illustrated in figure 3.

In both cases the recovery rates were generally above 95 %. For wort the recovery rate was 96.5 ± 5.0 %, for beer 99.0 ± 4.9 % over the shown range of concentration steps ($n = 12$, \pm s.d.). The

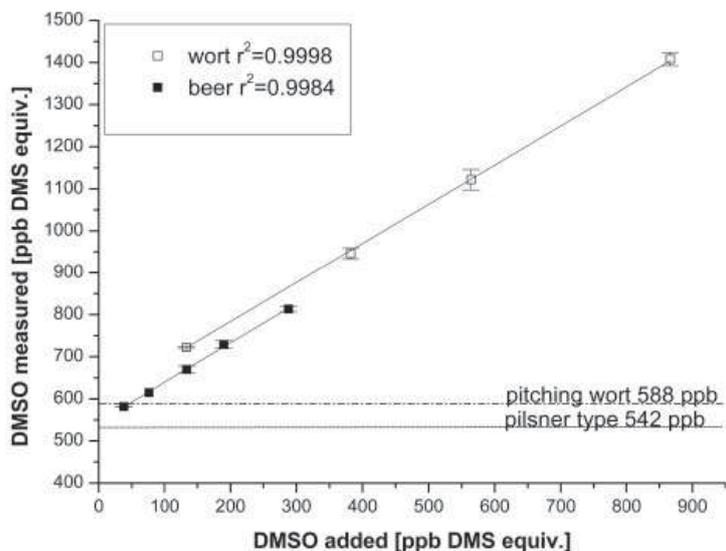


Fig. 3 recovery rates of DMSO in wort and beer (means of triplicate trial, error bars: \pm s.d.)

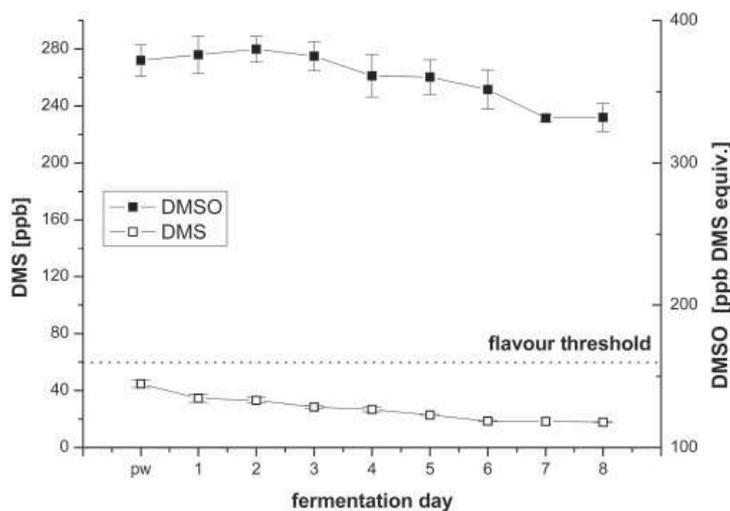


Fig. 4 course of DMSO and DMS during fermentation (means of duplicate trial, error bars: \pm s.d.)

results demonstrate that the method is able to deliver exact DMSO measurements and proves to be applicable for routine analysis.

3.6 DMSO and DMS during fermentation

In an additional application the levels of DMS and DMSO were tracked during fermentation. Figure 4 demonstrates the course of DMS and DMSO during fermentation.

In the first 3 days of fermentation the level of DMSO remained approximately constant, whereas DMS was steadily reduced up to about 40 % on the third day. Followed by a considerable DMSO concentration drop of about 4 % during the fourth fermentation day, in correlation the decline of DMS was slightly reduced. A similar behaviour with a DMSO reduction of about 6 % could be observed from sixth to the seventh fermentation day. In this case the DMS decline was almost stopped and remained constant until the end of fermentation. The DMSO content did not change in the last day of fermentation. The observation from the last day might be explained by the reduced yeast activity in that period because the attenuation degree of 78 % was already achieved after seven days.

In general no direct correlation between the net DMSO reduction of about 11 % and the overall DMS reduction of about 60 % could be observed. For the explanation two important aspects have to be considered. As described in literature [2, 3, 4, 13, 14, 23] the interplay between DMSO and DMS during fermentation is mainly attributed by DMS desorption caused by evolving CO₂ bubbles and newly generated DMS by DMSO reductase.

3.7 Test on DMSO reduction by sulfite in lower concentration ranges

Since the reduction of DMSO to free DMS is done by high concentration of sodium metabisulfite in the introduced analytical method, it was of further interest to evaluate a potential contribution of the endogenous sulfite in beer or wine to rising DMS levels during storage. In this connection additional model trials with reduced amounts of DMSO and sulfite in buffer solutions (pH 4.5) were carried out. The DMS formation in the used sodium acetate buffer containing around 2 ppm DMSO and 50 ppm sulfite over a storage time of 28 days is presented in table 3.

Tab. 3 DMS evolution in a buffer solution (pH 4.5) containing 2 ppm DMSO and 50 ppm sulfite (means of duplicate trial \pm s.d.)

d	DMS [ppb]
2	n.d.
14	30.8 \pm 0.04
23	36.5 \pm 0.32
28	43.2 \pm 1.3

After two days at room temperature in the dark no significant DMS formation was observed. Whereas after 14 days of incubation the solution contained already around 31 ppb DMS followed by a further increase to 36.5 ppb after 23 days and 43.2 ppb after 28 days which represents a total DMSO reduction of around 2.2 %. At every day of measurement the reference buffer solution which only contained DMSO showed no signal at the retention time of DMS. The results give a clear advice that the reduction of DMSO by sulfite can also take place in low concentration ranges.

4 General discussion

The results have clearly shown that specific beer own reducing agents like ascorbic acid, ferulic acid and gallic acid are unsuitable for DMSO analyses owing to their missing DMSO reducing ability under the applied conditions. Additional investigated thiols like L-Cys and L-GSH exhibit only slightly linear DMSO reducing properties with increasing DMSO concentration. With a big distance sodium metabisulfite demonstrated the remarkable highest sensitivities for DMSO reduction and best solubility during the applied sample processing. In this connection it has to be noted, that the reducing ability of the single substances were compared only at constant parameters (pH, temperature, time) and the conducted reduction procedure was not suitable for the determination of the quantitative substances endogenous DMSO reducing ability, therefore the substances were just qualitatively compared via the generated DMS signals. Additionally, it is not to exclude that

the investigated substances can change their reducing properties by deviating conditions. In this context the slightly linear reducing properties of L-Cys under the applied conditions could not verify L-Cys as alternative reducing agent for sodium metabisulfite as declared according to Dickenson [14].

Behind all these observations the focus was set on sodium metabisulfite owing to its demonstrated remarkable highest sensitivity for DMSO reduction and better solubility during the applied sample processing and a reliable method for DMSO determination was generated. Moreover, sodium metabisulfite offers further benefits like easy handling, comparable high solubility in water and low costs.

In the application of the optimized method the main precursor SMM was stable against thermal decomposition and did not generate DMS during the sample preparation at a comparative low temperature of 60 °C and a pH of 3.8. Also the higher DMS oxidation product DMSO₂ and other precursors for further volatile sulfuric compounds were not contributing to the DMSO results. DMSO₂ is much more stable against reduction than DMSO and might thereby not be reduced by the reducing agent sodium metabisulfite. Apart from that the levels of DMSO₂ in malt and beer are negligible low and most yeast strains are not able to reduce this substance leading to DMS [4]. Moreover the further tested precursors like DL-methionine sulfoxide, L-Cys and L-Cystine are not influencing the DMSO results.

Additionally, compared to the method of Yang and Schwarz [28] the introduced method requires less operation time and expense. Furthermore it is possible to eliminate the influences of the obligatory determination of SMM and the retention time for DMSO reduction at 100 °C in highly acidic solutions on the redox behavior of DMS and DMSO. Supplementary the practical application of the new method involves the minimization of oxidative reactions by comparative low temperatures and nitrogen purging of the sample vials and the samples themselves. On this way a potential DMSO generation during the sample preparation was minimized or inhibited. The tested recovery rates of DMSO in wort and beer were above 95 % indicating the accuracy of the developed method in the solution matrix of wort and of beer.

In the application of the method different malt types, wort and beers were screened for DMS and DMSO levels. Although remarkable differences especially in the malt types were observed, direct conclusions could not be carried out owing to the missing production information like germination and kilning conditions. Nevertheless the malt DMSO levels of this work lie in a range of 0–10.3 µg/g, which is in line with the accordant literature [4, 13, 29].

In an additional application DMSO and DMS were tracked over the course of fermentation. It was shown that the DMSO reduction had a slightly undesirable influence on the DMS decline during fermentation. Nevertheless the DMS desorption caused by evolving CO₂ bubbles had the predominant influence and is responsible for the decreased DMS content in the beer in compared to the pitching wort. Apart from that the conducted stirring mechanism could have enhanced the DMS reduction even it was very gently just enough to keep the yeast in despende. The average DMSO reduction of 11 % in this study is significant but compared to the literature [3, 2, 13]

where DMSO reductions up to 21 % are reported it is rather low. The low impact of DMSO reduction to DMS increases was reported previously by Dickenson and Anderson [13]. They concluded in their investigations on DMS formation during fermentation, that the DMS formation is not significantly contributing to the DMS levels in beer when the pitching wort contains less than 500 ppb of DMSO. In line with these findings, it has to be pointed out the detected DMSO decrease was from around 380 ppb in the pitching wort to around 330 ppb after fermentation.

Regarding the non enzymatic DMSO reduction at lower concentrations compared to the applied reduction procedure for the DMSO analysis further investigations showed that this pattern also deserves some attention. The applied concentrations were not as low as average beer values for these substances, nevertheless not as far away (both around 5–6 times higher). The overall DMSO reduction after 28 days of incubation time of around 2.2 % seems to be low at first side. Considering the DMS flavour threshold of 50–60 ppb [2, 24] and average DMSO contents in beer of about 500 ppb a reduction of around 2.2 % after 4 weeks would increase the DMS level significantly and might be responsible for DMS off-flavors in the finished beer and would be dependent on the initial DMSO concentration after bottling, for example. In case of wine, this beverage contains much higher levels of sulfite and conditionally also of DMSO [8, 17]. The DMS increase by the addition of DMSO and Cysteine to wine detected by De Mora et al. [8] could be corresponding to DMSO reduction by sulfite. In their study data of the sulfite content in the wines were not available. On the other hand L-Cysteine and other thiols might have had an impact on DMS formation in these wines which would be in line with the results of the present work. Further investigations on the impact of the non enzymatic reduction of DMSO by sulfite have to be carried out owing to the bulk of reactions in which sulfite is involved in beer or in wine, for example consumption by oxygen [19, 20, 21] or complexation reactions with aldehydes [6, 7].

5 Conclusion

Taking all results of this study into account it can be concluded that the developed method proofed to be a sufficient tool for the determination of DMSO in the brewing industry and related sectors. Moreover new insights were generated indicating that the reduction of DMSO by specific beer own reducing substances like sulphite may contribute to DMS formation during fermentation and storage of beer and wine. Current investigations at the Technische Universität Berlin, Institute of Biotechnology, Chair of Brewing Science are dealing with the kinetics of DMSO reduction by sulphites and thiols to evaluate these pathways on their influence on off flavour induced by DMS.

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3.2 Relevance of DMSO as a precursor of DMS in beer

Publication B:

The successfully implemented method for DMSO quantification was now applied to monitor the levels of DMSO together with DMS and SMM throughout the brewing process. The main intention was to obtain insights into the yet unknown potential interconversion reactions among DMS and DMSO. As a consequence of rising oil prices and ecological aspects, the brewing industry intends to reduce the amount of primary energy by partial substitution of the wort boiling process, mostly by wort heat holding and separated evaporation of DMS and other undesired volatiles. Compared to an open, atmospheric wort boiling process, DMS accumulates in the hot wort before an up-following desorption process is applied for its removal. Therefore particular attention was dedicated to the investigation of DMS oxidation during wort heat holding. Also the DMSO reducing- and DMS formation capability of genetic-diverse yeast strains was aimed to be investigated.

All procedures, results and outcomes were published in the Journal *BrewingScience – Monatsschrift für Brauwissenschaften*, in a paper entitled “*On the Behaviour of Dimethyl Sulfoxide in the Brewing Process and its Role as Dimethyl Sulfide Precursor in Beer*” (Publication B²⁰³).

In this publication it could be demonstrated that DMSO and SMM were quickly extracted during mashing, whereas DMS was mostly lost by evaporation and was not subject to significant oxidation. However, DMS was oxidized during wort heat holding, whereas the contribution of wort production to the levels of DMSO found in pitching wort was shown to be negligible compared to the DMSO levels present in malt. During fermentation it could be evidently shown, that DMSO reduction significantly contributed to the final levels of DMS in beer, whereas top-fermenting yeast strains generated significantly more DMS than the bottom-fermenting yeast strain TUM 34/70. New insights on the uptake of DMSO by yeast and its reduction to DMS were generated. The maximum DMSO reduction was determined as ~26%, implying that its majority remains in the beer.

Publication B

M. Baldus, R. Klie, M. Biermann, P. Kreuzschner, M. Hutzler and F.-J. Methner

On the Behaviour of Dimethyl Sulfoxide in the Brewing Process and its Role as Dimethyl Sulfide Precursor in Beer

Dimethyl sulfide (DMS) has a considerable impact on the aroma of beer and may lead to undesirable flavour impressions. The undoubted role of S-methyl methionine (SMM), as the thermal precursor of DMS has been elaborately elucidated in the literature. DMS can also be generated via reduction of dimethyl sulfoxide (DMSO) during fermentation. However, there are uncertainties regarding the role of DMSO as DMS precursor and its contribution to final DMS levels in beer. The behaviour of DMSO in the brewing process has not been investigated in detail. Also, the extent to which different yeast strains reduce DMSO is mostly unknown. In this work the behaviour of DMS and its precursor SMM and DMSO was investigated throughout the brewing process. The main focus was to ascertain DMSO reduction during fermentation by lager and top-fermenting yeast. During mashing, SMM and DMSO were extracted rapidly owing to their high water solubility, whereas SMM was extracted faster. In the further course of mashing SMM and DMSO levels remained approximately constant. DMS was found to be evaporated steadily in open mashing systems and was not subject of significant oxidation to DMSO, even in a closed mashing system. During wort boiling SMM was degraded in a 1st order mechanism ($k = 0.021 \text{ min}^{-1}$) whereas the generated DMS was evaporated subsequently. The levels of DMSO increased linearly with increasing evaporation of water but were not affected when boiling was conducted with a rectification column. During wort heat holding in hermetically closed systems ~15 % of the accumulated DMS was oxidized to DMSO. During fermentation significant DMS formation was observed. DMSO reduction was higher in top fermenting *Saccharomyces cerevisiae* yeast (TUM 149) than in *Saccharomyces pastorianus* lager yeast (TUM 34/70) but was not correlated with genetic yeast diversity (domestication clusters). This work demonstrates that DMSO reduction during fermentation significantly contributed to the levels of DMS in beer. Therefore we suggest that DMSO should be recognized and assessed as DMS precursor by maltsters and brewers.

Descriptors: dimethyl sulfide, dimethyl sulfoxide, S-methyl methionine, DMS precursor, fermentation, *Saccharomyces pastorianus/cerevisiae*

1 Introduction

Dimethyl sulfide (DMS) is probably the most investigated volatile sulfuric aroma compound in beer.

At concentrations above its flavour threshold (30–100 µg/L), DMS can induce specific off-flavours, which are often ascribed as 'cooked-vegetable'-like [1, 2]. However, subthreshold-levels of DMS were reported to have a positive effect on beer flavour [1]. The impact of DMS on beer flavour is highly affected by the beer type [2] and masking effects of other beer aroma compounds [3].

DMS primarily originates from the non-protein amino acid S-methyl methionine (SMM), which is synthesized during germination of brewing cereals [4, 5]. SMM is heat-labile and decomposes during malt kilning to DMS and L-homoserine [6–8]. Relatively high amounts of the generated DMS diffuse out of the grain into the ventilation air. In dependence of the kilning technology, DMS is also oxidized to DMSO and minor amounts of dimethyl sulfone (DMSO₂) [8, 9]. In the final malt SMM levels of 0–14.5 µg/g [5, 8, 10] as well as DMSO levels of 0–10 µg/g [8, 9, 11] were reported.

Besides DMS itself, SMM and DMSO are introduced into the brewing process acting as DMS precursors. SMM generates DMS mostly during wort boiling and hot trub separation whereas DMSO can be reduced to DMS during fermentation. A main reason for the expensive wort boiling process is to hydrolyse SMM and subsequently evaporate the generated DMS and other undesirable volatile aroma compounds. There is a general agreement about the reaction mechanism of SMM decomposition and its contribution to DMS formation in the literature. Thermal SMM degradation follows a 1st order mechanism and is favoured at high temperatures and high pH values. Rate constants were reported to vary between 0.018–0.03 min⁻¹ (pH 5.3–5.5, T = 98.5 °C–100 °C) [6, 7, 12, 13].

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In contrast to SMM, DMSO is heat stable. It reveals a high water solubility owing to its polar sulfoxide group. Its high entropy of vaporisation makes DMSO unlikely to be evaporated during wort production and its entirety probably remains in the pitching wort [2]. However, the actual pattern and the behaviour of DMSO during wort production has not been investigated in detail.

DMSO itself has no impact on beer flavour but it acts as a secondary DMS precursor during fermentation as it can be reduced by yeast [6, 9, 14–17]. Thioredoxin dependent methionine sulfoxide reductases (MSRA) reduce the sulfoxide group thereby forming DMS as a side reaction of the methionine sulfoxide reduction [18, 19]. SMM cannot be metabolized to DMS by the yeast [14, 20].

The relationship of the DMSO concentration in the pitching wort (400–800 µg/L) and the flavour threshold of DMS (30–100 µg/L), indicate that even minor DMSO reduction during fermentation may contribute to significant DMS formation, thus potentially affecting beer flavour.

DMSO reduction during fermentation was reported to be elevated at low fermentation temperatures [14, 20]. Low fermentation temperatures likewise diminish the diffusion rate of CO₂ bubbles, by which DMS is partly removed from the green beer [21].

It is also known that the species *Saccharomyces cerevisiae* reduces more DMSO than *Saccharomyces uvarum* [14, 20]. It should be mentioned that the taxonomic name of the lager yeast is now *Saccharomyces pastorianus* var. *carlsbergensis* instead of *Saccharomyces uvarum*, when the cited publications were released. Other factors promoting DMSO reduction are high pitching wort pH [2, 22], low free amino nitrogen levels [23] and high original gravity [14, 20].

Still, there are discrepancies concerning the role of DMSO as DMS precursor in beer. *Annes* [14] reported that lager yeasts were able to reduce DMSO up to 21 % and that the reduction was accompanied by significant DMS formation. By the addition of labelled DMSO to pitching wort it was shown that substantial amounts of DMS in the finished beers were originating from DMSO reduction [16, 17]. *Leemans* et al. [17] even stated that 80 percent of the total DMS present in beer was generated from spiked deuterated DMSO. On the contrary, *Dickenson* [20] reported that yeast is able of DMSO reduction, whereas the reduction has no big influence on final DMS levels in beer.

Bacterial contaminations of the family *Enterobacteriaceae* can also be a source of DMS in beer [2]. Some species, like *Escherichia coli* or *Rhodobacter sphaeroides* possess DMSO reductases using DMSO as electron acceptors in anaerobic growth [24], which may lead to a much higher DMS formation than by yeast. However, these species are rather unlikely to occur as fermentation contaminants.

This work will solely focus on DMSO reduction by beer yeast, which is controversially discussed regarding its relevance to final DMS levels in beer. The fate and behaviour of DMSO in the brewing process has not been investigated in detail. Regarding rising oil prices and ecological aspects, the brewing industry intends to

reduce the amount of primary energy by partial substitution of the wort boiling process, mostly by wort heat holding and separated evaporation of DMS and other undesired volatiles [25, 26].

In comparison to the wort boiling process, DMS accumulates in the hot wort before an upfollowing desorption process is applied for its removal. There is no information available if DMS may be subject to oxidation in wort. DMS oxidation and accompanied DMSO formation would increase the risk of DMS formation during fermentation. Also, the extent to which different globally applied yeast strains are capable of DMSO reduction is mostly unknown.

The aim of this work was to track the levels of DMS and its precursors SMM and DMSO throughout the brewing process to obtain insights into potential interconversion reactions among these substances. Specifically, the effect of wort composition as well as wort boiling and wort heat holding techniques were investigated. As mentioned before, top-fermenting yeast reduces more DMSO than bottom-fermenting yeast. It is likely that diverse genetic evolution during domestication of the yeast is a possible explanation. Recently, *Gonçalves* et al. [27] showed that top-fermenting yeast strains possess distinctive genomic signatures related to their domestication and that most top-fermenting *Saccharomyces cerevisiae* brewing strains belong to a major brewing strain cluster. Interestingly, some matched to other genetic clusters like the bread-, wine- or sake strain cluster. In this study we specifically selected top-fermenting strains heterogeneously spread over different clusters to investigate their capability of DMSO reduction and DMS formation. The results of this work should serve for a better understanding of the role of DMSO on DMS formation and its potential contribution to the levels of DMS in beer.

2 Material and methods

2.1 Fate of DMS, SMM and DMSO during wort production

2.1.1 Closed mashing system

Since it is not clear if interconversion reactions of DMS and its precursors, for example DMS oxidation to DMSO, take place during mashing, the procedure was conducted in closed 2 L Duran® bottles (Schott AG, Mainz, Germany) to avoid DMS evaporation. The bottles were closed with open screw caps containing silicone/PTFE seals. The seals were perforated with a closable hole (diameter 7 mm) for sampling. 1800 g of double distilled ultrapure water (Milli-Q®) was introduced into the bottle. The bottle was heated to 63 °C in a water bath, which was mounted on a magnetic stirring device (3581200 C-MAG HS 7, IKA®-Werke GmbH & CO. KG, Staufen, Germany). 514.2 g of fine grist from Pilsner malt (Ireks GmbH, Kulmbach, Germany) were introduced into the bottle under magnetic stirring. The grist:water ratio was 1:3.5 and the headspace volume was minimized in order to prevent extensive DMS desorption. After dispersion, the mash was heated (1.5 °C/min) to 66 °C where it was incubated for 30 minutes. Afterwards, the temperature was increased to 72 °C, where a holding period of 20 minutes was carried out. For mashing-out, the temperature was increased to 78 °C. After mashing-in (~5 minutes) as well as

at the end of mashing samples were taken for the quantification of DMS, SMM and DMSO. ~ 10 g of mash were withdrawn using a 10 mL pipette, to which a tip with enlarged diameter (4 mm) was attached. The content was added into 40 mL of ice-cold ultrapure water in a Falcon® tube (50 mL), which was subsequently closed. The screwing thread of the tube was covered with 2 layers of PARAFILM®. The sample weight was recorded and the tubes were centrifuged at 1 °C and 7340 × g for 10 minutes. DMS, SMM and DMSO were analysed from the supernatant.

2.1.2 Open mashing system

Mashing in the brewing industry can be regarded as an open system as DMS is able to diffuse out of the mash into the atmosphere. Therefore we conducted mashing in a mashing bath (Bender & Hobein GmbH, Munich, Germany) with stainless steel beakers on which stainless steel caps were attached. The caps contained a hole (diameter ~ 10 mm) through which the stirrer (diameter ~ 4 mm) was connected to the stirring motor of the mashing bath. 350 g of double distilled ultrapure water were filled into the beakers, which were heated to 63 °C. 100 g of fine grist (100 % Pilsner malt, Ireks GmbH, Kulmbach, Germany) were introduced under stirring. The mashing regime used was the same as described for the closed mashing system. Samples were taken as described before.

2.1.3 Wort boiling, rectification and wort heat holding

Wort boiling was carried out in an open 2L Duran® bottle. The bottle was filled with 1800 g of wort (original gravity of 12 °P), which was produced via the open mashing regime as described before. The bottle was covered with 2 layers of aluminium foil before it was placed on a combined heating / magnetic stirring device (3581200 C-MAG HS 7, IKA®-Werke GmbH & CO. KG, Staufen, Germany). At the beginning of boiling crushed hop pellets (Bravo, 15.3 % α -acids, Simon H. Steiner Hopfen GmbH, Mainburg, Germany) were added to achieve 25 bitter units in the resulting cast-out wort. Samples were taken at the beginning of boiling, after 30-, 60-, 90- and 120 minutes. For sampling, 20 mL of wort were injected into 30 mL of ice-cold double-distilled ultrapure water. Evaporation was determined gravimetrically and via assessment of the original gravity at each sampling step.

2.1.4 Rectification

To minimize the effect of water evaporation, the same boiling procedure was conducted with a 450 mm long DURAN® Vigreux column (VWR GmbH, Darmstadt, Germany), which was mounted onto the neck of the bottle. Samples were taken at the beginning and after 90 minutes of boiling as described under wort boiling.

2.1.5 Wort heat holding

To exclude water as well as DMS evaporation, 50 g of kettle-full wort (produced as described under wort boiling) were separated into 50 mL DURAN® bottles (Schott AG, Mainz, Germany) and were tightly closed using screw caps with PTFE coated silicone seals. The bottles were placed in a water bath, which was adjusted to 99.9 °C. After reaching of 98 °C inside the bottles, the heat holding time was started. Samples were taken after 0-, 30-, 90- and 120

minutes. The bottles were cooled down stepwise with tap water (2 minutes) and subsequently ice-cold water before they were analysed for DMS, SMM and DMSO.

2.1.6 Fermentation

Wort was produced in an open mashing system. After mashing, the wort was cooled down to 20 °C and was filtered over cellulose filters (Whatman, grade 597 ½, GE healthcare, Buckinghamshire, Great Britain). The original gravity of the wort was adjusted to 12 °P with double distilled ultrapure water. Boiling was conducted for 60 minutes as described before. After boiling, the wort was stirred gently and the bottles were placed in a water bath (95 °C) for 20 minutes. After sedimentation of the hot trub the wort was decanted into sterile 2 L Duran® bottles and was cooled down to 20 °C, were the extract was adjusted again to 12 °P. 3 drops of autoclaved, diluted (8 % (v/v)) antifoam reagent (Antifoam A Concentrate, Sigma Aldrich, St. Louis, USA) were added to the wort. The wort was then stripped with sterile air for 30 minutes in order to achieve a dissolved O₂ concentration of ~8 mg/L. Furthermore the air stripping quantitatively removed the DMS from the pitching wort. We used this procedure to ascertain the yeast-related formation of DMS during fermentation. After stripping, 40 g of pitching wort were filled into 50 mL Falcon® tubes. The screwing thread of the tubes were covered with 2 layers of PARAFILM®. The respective yeast suspension was added to achieve a final cell count of 2.5 × 10⁷ cells/mL. Fermentation locks containing 15 mL of ethanol solution (70 % (v/v)) were installed onto the tubes. Fermentation was carried in the Falcon® tubes at whether 12 °C for lager yeast or 18 °C for top-fermenting yeast in an incubator. Fermentation was stopped after achieving the yeast specific final degree of attenuation. The fermentation locks were removed and the tubes were tightly closed with screw caps before they were centrifuged as described before. The supernatant was used for beer analysis (extract, pH, ethanol concentration) as well as for the determination of the DMS- and DMSO concentration.

2.1.7 Ultrasonic treatment of yeast

After fermentation and centrifugation the yeast pellet was resuspended in 10 g of sterile double-distilled ultrapure water. The mixture was incubated for 20 minutes in an ultrasonic bath (Sonorex Digital DK 1028 P, Bandelin electronic GmbH und Co. KG, Berlin, Germany) maintaining a temperature of 4 °C. After the treatment, the suspension was centrifuged and the concentration of DMSO was analysed in the supernatant.

2.1.8 Impact of wort composition on DMSO reduction during fermentation

The impact of different wort composition was investigated by varying the grist composition with different malt types. The grist compositions were as follows: 100 % Pilsner Malt (A), 80 % Pilsner malt + 20 % Melanoidin malt (Weyermann® Malzfabrik, Bamberg, Germany) (B), 80 % Pilsner malt + 20 % CARAFA® Type II malt (C) (Weyermann® Malzfabrik, Bamberg, Germany). Furthermore 70 % Pilsner malt was combined with 30 % of unmalted barley (Quench) (D). Wort production and fermentation was carried out as described under fermentation. Fermentation temperature was 12 °C.

2.1.9 Screening of top-fermenting yeast strains on DMSO reduction during fermentation

In this section the extent to which top fermenting yeast strains are capable of DMSO reduction and DMS formation was aimed to be investigated. Yeast strains were selected based on their genetic diversity as shown by whole genome sequencing by *Gonçalves* et al. [27]. The strains chosen from the main beer cluster were TUM 149 (German wheat beer yeast), TUM 177 (Altbier / Kölsch yeast) and TUM 210 (English ale yeast). Furthermore TUM 511 (American ale yeast from the wine cluster), TUM 480 (African beer yeast from the bread cluster) and TUM 68 (German wheat beer yeast from the sake cluster) were selected. Wort production and fermentation were carried out as described before, whereas the fermentation temperature was set to 18 °C in this trial.

2.1.10 Time course of DMSO and DMS during fermentation

The fermentation was carried out as described in the screening trial. The yeast strains selected for the demonstration of DMSO reduction and DMS formation were TUM 149 (top-fermenting, 18 °C) and TUM 34/70 (bottom-fermenting, 12 °C). On each fermentation day, sample tubes were analysed for Extract, DMS and DMSO.

2.1.11 Quantification of DMS, SMM and DMSO

Quantification of DMS and DMSO was conducted using headspace gas chromatography combined with pulsed flame photometric detection (HS-GC-PFPD) according to a previously described method [11]. The detection limit for DMS of the applied system was 6 µg/L. SMM was quantified according to *White* and *Wainwright* [10]. The data of SMM and DMSO are presented in DMS equivalents (SMM*, DMSO*).

2.1.12 Wort and beer analysis

Extract (2.19.3), color (3.1.2), pH (3.1.3), apparent degree of attenuation (2.9.5), free amino nitrogen (2.8.4.1.1) and ethanol (2.10.5) were analysed according to MEBAK guidelines [28].

2.1.13 Quantification of amino acids

Amino acids (alanine, glycine, valine, leucine, isoleucine, threonine, serine, proline, aspartic acid, glutamic acid, methionine, phenyl alanine, lysine, histidine, tyrosine) were analysed in the pitching worts as described by *Wietstock* et al. [29].

2.1.14 Microbiological analyses

To exclude that microbial contaminations are responsible for DMSO reduction and DMS formation during fermentation, we analysed yeast and beer after fermentation on contaminations by bacteria and wild yeast according to MEBAK guidelines (10.12.2) [30]. After centrifugation of the green beer, approximately 2–3 g of yeast remained in the tube, which was resuspended in 10 mL autoclaved, double distilled, ultrapure water. Abundance of *Lactobacilli* and *Pediococci* was tested using VLB-S7S-Agar. Lysin-Agar was used to test the presence of non-*Saccharomyces* yeast. VRBD-Agar was used to assure that *Enterobacteriaceae* were not present in wort (anaerobic incubation for 24 h at 28 °C).

2.2.15 Statistics and data analysis

All experiments of this work have been carried out in triplicate. The mean values ($n = 3$) of the DMSO concentrations were compared using the Tukey Honest Significance Difference (HSD) test at a confidence level of 95 %. The analyses were conducted with Microsoft XLstat (version 2014.5.03, Addinsoft, USA).

3 Results and discussion

During malt kilning, SMM is largely degraded to L-homoserine and DMS, whereas the latter is partially oxidized to DMSO. Substantial amounts of DMS and its precursors remain in the malt and are introduced into the brewing process via mashing. Yet, it is not entirely clear to which extent interconversion reactions of DMS and its precursors, especially DMSO, take place during wort production. We initially investigated the behaviour of DMS, SMM and DMSO in a closed mashing system in order to prevent DMS desorption. We used this approach to more precisely balance the amounts of DMS and its precursors before and after mashing (Fig. 1).

In the open mashing system (Fig. 1 a) the DMS concentration was

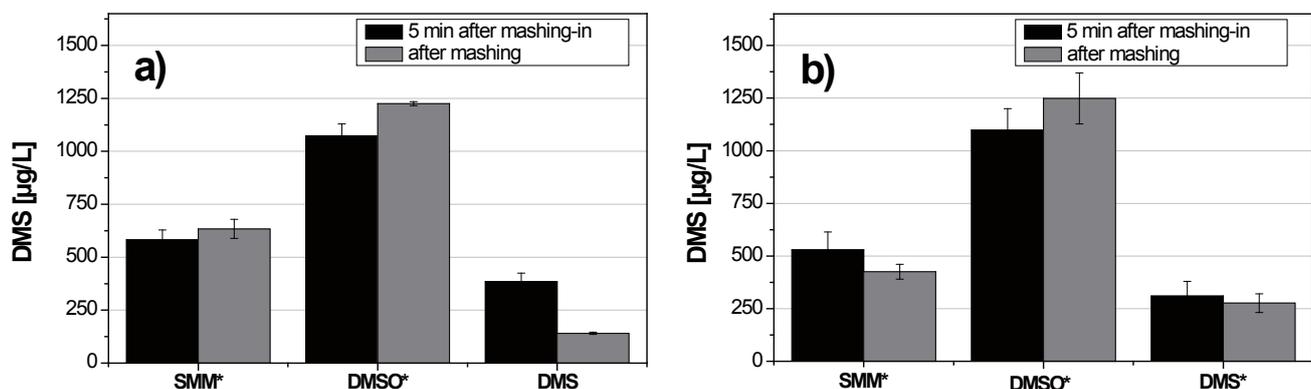


Fig. 1 Levels of SMM, DMSO and DMS before and after mashing in an open mashing system (a) and in a closed mashing system (b). Results are presented as mean values ($n = 3$) \pm standard deviation

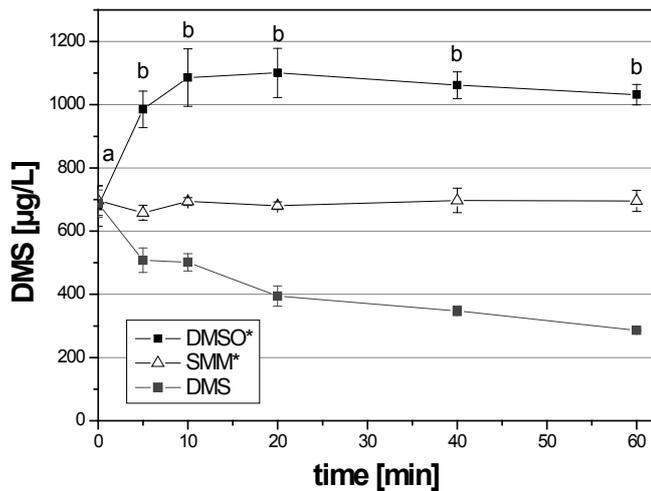


Fig. 2 Levels of SMM, DMSO and DMS during isothermal mashing at 62 °C in an open mashing system. Results are presented as mean values ($n = 3$) \pm standard deviation. Different letters above the DMSO data indicate significant differences according to the Tukey HSD test

significantly reduced (~63%), probably as a result of evaporation. In the closed mashing system (Fig. 1 b) no significant change of the DMS concentration was observed. A similar observation was made by *Scheuren et al.* [31].

Even though higher levels of DMS were accumulated in the mash of the closed system, the DMSO increase was similar to the open mashing system. As the concentration of SMM remained unchanged during mashing, we propose that DMS was not subject to significant oxidation to DMSO in the course of the mashing process. The abundance of reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radicals or fatty acid hydroperoxides during mashing is well known [32]. Recently, it was reported, that hydrogen peroxide and hydroxyl radicals are capable of DMS oxidation and DMSO formation [33]. However, DMS seemed to be not affected by potentially abundant ROS in this trial. Mash consists of miscellaneous biomolecules, which are susceptible to oxidation. Therefore, it is likely that for example fatty acids, polyphenols or proteins may have been preferably oxidized over DMS, probably also related to their higher water

solubility and accompanied higher availability for ROS. Still, from the present results, we cannot exclude that any kind of red-ox reactions of DMS-DMSO did occur, even though they have not been observable within the experimental data. In the next experiment, we monitored the course of DMS and its precursors in an open mashing system at 62 °C.

Unsurprisingly, DMS continuously decreased in the course of isothermal mashing at 62 °C, which is most likely a result of the high relative volatility of DMS [34] and its low boiling point (37 °C). In the first 5 minutes the biggest drop of the DMS concentration was detected. This observation was probably evoked by the DMS concentration gradient and the rapid diffusion of DMS out of the mash into the headspace of the mashing beaker. This process is supported by the evaporation of water. The headspace volume accounted for ~20 % of the total beaker volume. The further smaller decline of DMS during mashing may be explained by water saturation and decreasing DMS concentration gradient in the headspace. Diffusion of DMS into the atmosphere was probably decelerated by the small hole diameter of the beaker cap, through which also the stirring staff was inserted. However, at the end of mashing ~58 % of the total DMS had vanished from the system. The DMSO concentration was significantly increased after mashing-in (1 min) and reached its maximum concentration after a mashing time of 10 minutes. In the further course of mashing the DMSO concentration remained approximately constant. Referring to the aforementioned diffusion behaviour of DMS, the increase of DMSO during mashing is most likely related to an extraction process from the grist particles into the aqueous mash phase. The observed slower extraction behaviour, compared to SMM, can potentially be explained by the amphiphilicity of DMSO, which makes it capable of interacting with proteins [35] or amylose helices [36]. It is also likely that DMSO interacts with hemicelluloses. In such case DMSO extraction behaviour would probably correlate with malt modification.

3.1 Wort boiling, rectification and heat holding

The behaviour of DMS, SMM and DMSO in wort boiling and -heat holding is illustrated in figure 3. During atmospheric wort boiling, DMS was evaporated below the detection limit within 30 minutes, most certainly because of its high relative volatility [34]. SMM was thermally degraded following an exponential decline (dotted line,

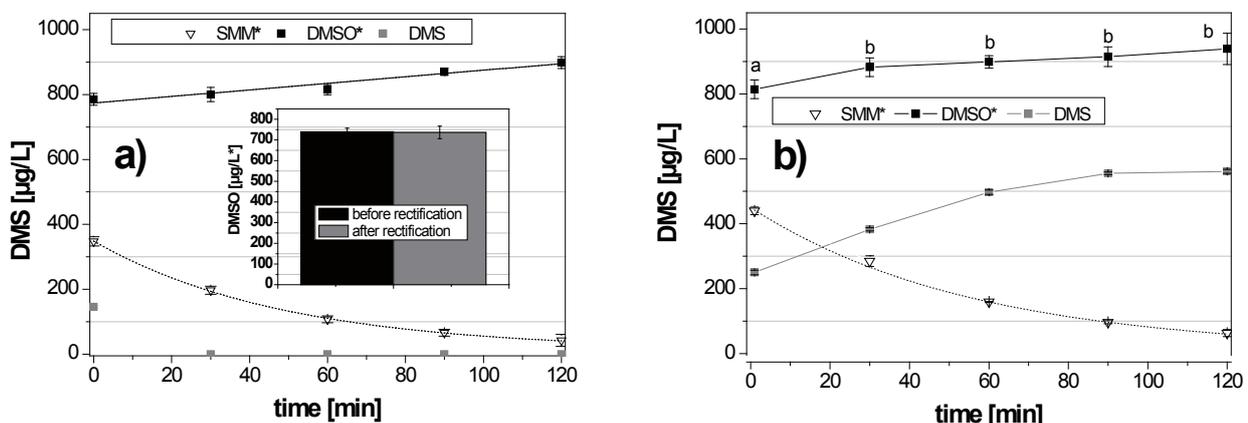


Fig. 3 Behaviour of DMS, SMM and DMSO during atmospheric wort boiling (a) inserted graphic DMSO concentration before- and after boiling with a rectification column) and wort heat holding (b). Results are presented as mean values ($n = 3$) \pm standard deviation. Different letters in b) indicate significant differences according to the Tukey HSD test

R^2 : 0.999). From the slope of the exponential fit, the 1st order rate constant was determined to be $k = 0.021 \text{ min}^{-1}$, which is consistent with the half-life of SMM under similar conditions determined by Dickenson [6]. The DMSO concentration increased linearly with boiling time. As the DMSO levels were still rising while no DMS was detectable anymore, DMS oxidation is very unlikely to be the source of the DMSO increase. The total evaporation of water was ~16 %. As the boiling point of DMSO (189 °C) is relatively high, it is supposed that DMSO was concentrated in the progression of wort boiling as a result of the loss of water. To test this assumption, a further wort boiling experiment was conducted in which the evaporation was minimised to 0.2 % by the installation of a Vigreux-column (rectification boiling). The inserted diagram in figure 3 a shows that the concentration of DMSO before and after rectification boiling did not change, which verifies the previous assumption. From the wort boiling trials we conclude that DMSO was not subject of significant biochemical alteration, whether in an oxidative nor reductive way.

During wort heat holding, the residence time of DMS in the hot wort is elevated as a result of the missing evaporation of water. To test the extent to which DMS oxidation occurs during heat holding, wort was separated into closed Duran® bottles which were incubated at 99.9 °C (Fig. 3 b). Again, an exponential decline of SMM was observed ($R^2 = 0.998$). The rate constant was determined to 0.017 min^{-1} , which is smaller than the rate constant in the atmospheric boiling trial. This was probably related to the fact, that the wort temperature in the bottles did not exceed 98 °C. As expected, the DMS concentration increased during heat holding as a result of the missing evaporation. In this context the DMSO concentration increased significantly within 30 minutes, whereas no significant increase was observed in the further progress of wort heat holding. It is supposed that the DMSO increase was evoked by DMS oxidation. Though, after 120 minutes the DMSO increase was solely ~15 %, which suggest a certain stability of DMS against oxidation under the applied conditions. According to Henry's law, the solubility of oxygen above 95 °C is below 1

mg/L. The fact that significant DMS oxidation only took place in the first period of heat holding may have been related to the higher solubility of oxygen at lower temperatures. During heating of the wort to ~98 °C the dissolved oxygen may have been reduced to ROS, such as hydrogen peroxide or hydroxyl radicals, for example. Recently, it was reported that molecular oxygen was not capable of DMS oxidation [33], therefore we proposed that ROS formation was responsible for DMS oxidation. Again, the relative low amount of DMSO formation can probably explained by the fact that ROS likewise reacts with miscellaneous wort components. As discussed before, these reactions would have competed with DMS for ROS and eventually proceed at much higher reaction rates. Again, the low water solubility of DMS may additionally have diminished its availability for ROS and concomitant DMSO formation.

3.2 DMSO reduction during fermentation

In all fermentation trials of this work, we did not find any bacteria or wild yeast contaminations relevant for DMS formation, which was the premise for the analyses of DMS and DMSO. Also, as a consequence of the wort stripping described in the material and methods section, there was no DMS detectable in the pitching worts. This was also crucial to evaluate the contribution of the yeast to DMS formation. To assess the DMSO reduction by yeast during fermentation and to test if this pathway influences the DMS levels in beer, a 100 % Pilsner malt wort was fermented with lager strain TUM 34/70 at 12 °C. Additionally, worts with different grist compositions (A: 100 % Pilsner malt; B: 80 % Pilsner malt, 20 % Melanoidin malt; C: 80 % Pilsner malt, 20 % Carafa II malt; D: 70 % Pilsner malt, 30 % barley) were fermented under the same conditions to examine the impact of wort composition on DMS formation during fermentation.

The levels of FAN and amino acids, especially methionine, were analysed in the respective pitching worts as they are known to have a decisive impact on the reduction of DMSO by yeast [22, 23]. The data are presented in table 1.

Table 1 Analytical data of pitching worts (A: 100 % Pilsner malt; B: 80 % Pilsner malt, 20 % Melanoidin malt; C: 80 % Pilsner malt, 20 % Carafa II malt; 70 %; D: Pilsner malt, 30 % barley)

wort	A	B	C	D
FAN [mg/L]	166	116	129	184
Amino acids [mg/L]	954	817	474	665
Methionine [mg/L]	21	15	8	11

Table 2 Analytical data of beer produced from different pitching worts (12 °P) (A: 100 % Pilsner malt; B: 80 % Pilsner malt, 20 % Melanoidin malt; C: 80 % Pilsner malt, 20 % Carafa II malt; 70 %; D: Pilsner malt, 30 % barley)

	Apparent degree of Attenuation	pH	Ethanol (v/v)
	[%]	–	[%]
A	77.4	4.27	5.7
B	74.8	4.24	5.5
C	80.2	4.31	6.0
D	74.8	4.21	5.5

Fermentation was stopped after 8 days of fermentation as the apparent final degree of attenuation of TUM 34/70 (>74 %) was reached for all fermentations. Some parameters of the respective beer analysis are presented in table 2. In figure 4 a, the levels of DMSO before (after pitching) and after fermentation are illustrated. Noticable is the significant difference of the DMSO concentration among the pitching worts. These differences can be explained by the different DMSO concentrations in the malt types, from which the worts were produced from.

Though, no significant reduction of the DMSO concentration was found for each of the fermented worts, implying that there was also no direct correlation between DMSO reduction and the levels of FAN and methionine in the pitching wort. The levels of DMS after fermentation are shown in Figure 4 b. As described in the material & methods section, we stripped the pitching wort with sterile air for the quantitative removal of DMS, therefore the data depict the formation of DMS during fermentation. In all fermentations, significant formation of DMS took place. The increase of DMS varied between 8–14 µg/L whereas no significant difference was found among the wort compositions. In this study, we did not find

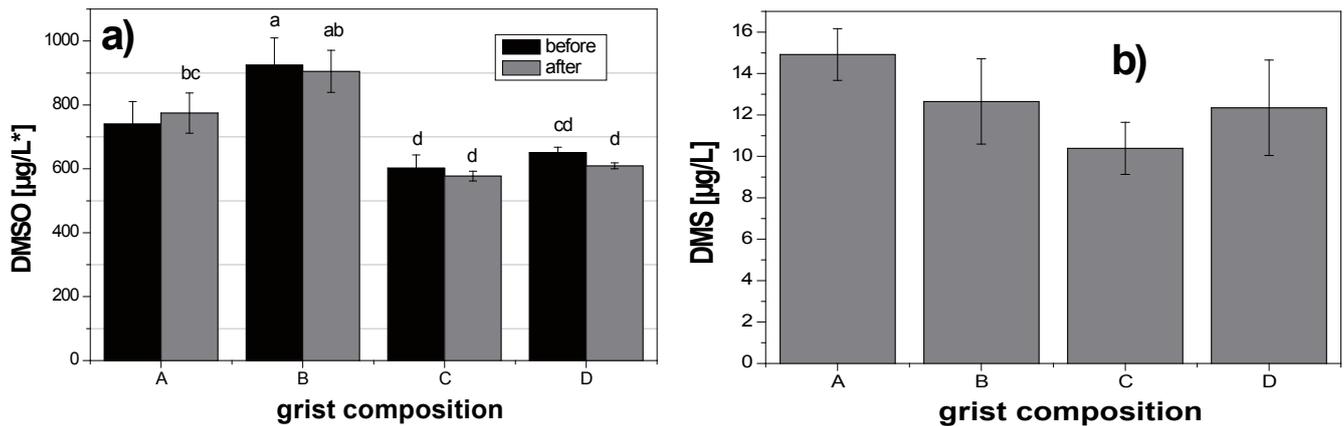


Fig. 4 Levels of DMSO before and after fermentation (a) and DMS increase during fermentation (b) with TUM 34/70 at 12 °C. (A: 100 % Pilsner malt; B: 80 % Pilsner malt, 20 % Melanoidin malt; C: 80 % Pilsner malt, 20 % Carafa II malt; 70 %; D: Pilsner malt, 30 % barley). Results are presented as mean values ($n = 3$) \pm standard deviation. Different letters in a) indicate significant differences according to the Tukey HSD test.

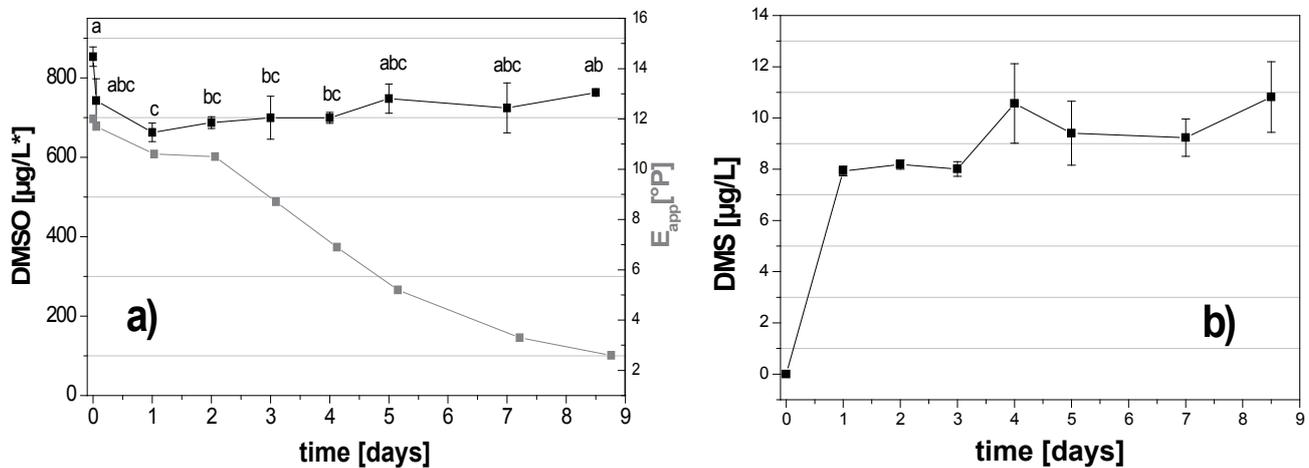


Fig. 5 Behaviour DMSO and apparent extract (a) and DMS increase during fermentation (b) with TUM 34/70 at 12 °C. Results are presented as mean values ($n = 3$) \pm standard deviation. Different letters in a) indicate significant differences according to the Tukey HSD test

correlations between FAN, DMSO reduction and DMS formation. Gibson et al. [23] reported that DMSO reduction by lager yeast was declined from ~70 % to ~20–30 %, when FAN was increased from 70 mg/L to 3.5 g/L. In our study the levels of FAN varied between 116–184 mg/L, which is representative for pitching worts. Though, the relative small differences compared to the investigations of Gibson et al. [23] were probably insufficient to account for different rates of DMSO reduction.

The worts with 20 % Carafa malt proportion (wort C) and 30 % barley proportion (wort D) produced similar levels of DMS even though the initial DMSO concentrations in these worts were lower. In this case, a possible explanation may be found in the lower levels of amino acids, especially methionine (Table 1). It is also likely that other inhibitors, especially methionine sulfoxide (MetSO) [23] have affected the DMS formation in this investigation. It was reported that the inhibitory effect of MetSO is even higher than the effect of methionine [37]. Unfortunately, whether from this study or others, there are no data available on the concentration of MetSO in different malt types and the corresponding pitching worts. From the results of figure 4 DMSO can only be assumed to be the source of DMS in the resulting beers.

To ascertain the period of DMS formation, the fate of DMSO (a) and DMS (b) were recorded daily during fermentation of wort A with TUM 34/70 at 12 °C (Fig. 5). The first data point in the diagram presents the concentration of DMSO in the pitching wort. After yeast addition and centrifugation, the DMSO concentration was significantly decreased (~13 %). The processing time until DMSO analysis was approximately 30 minutes. These data suggest that a part of DMSO was quickly absorbed by the yeast cells after pitching. In the further course of fermentation the DMSO concentration tended to increase slightly until the end of fermentation. At the end of fermentation, the apparent degree of attenuation was 78.5 % and ~11 % of the DMSO content was reduced.

A significant DMS increase was observed in the first period of fermentation, whereas the main DMS formation of ~8 µg/L took place during the 1st day of fermentation. The DMS concentration remained approximately constant for another 2 days, before it was slightly increased to 9–12 µg/L, which ended up to be final DMS concentration in the green beer. On fermentation day 4, we removed all of the supernatant from the centrifuged sample and resuspended the remaining yeast pellet in sterile, ultrapure water. The suspension was exposed to an ultrasonic treatment for cell

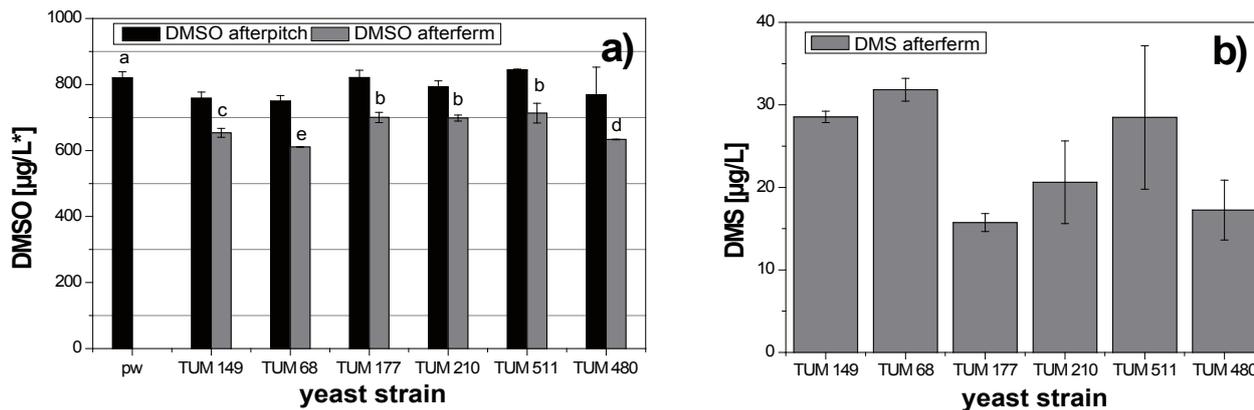


Fig. 6 Reduction of DMSO (a) and DMS increaseduring fermentation (b) by top-fermenting yeast strains. pw: pitching wort; afterpitch: after pitching; after ferm: after fermentation. Results are presented as mean values ($n = 3$) \pm standard deviation. Different letters above DMSO data of pitching wort and after fermentation indicate significant differences according to the Tukey HSD test

lysis. After the treatment the dispersion was centrifuged again. The supernatant was then analysed for DMSO. The analysis revealed that 104 ± 10 µg/L of DMSO were released from the yeast by this treatment.

Leekumjorn and *Sum* [38] reported that DMSO can accumulate in the intermembrane space of phospholipid bilayers as a result of amphiphilic interactions. It is therefore likely that DMSO had accumulated in the yeast plasma membrane. The data further indicate that the small increase of DMSO in the course of fermentation may be related to yeast autolysis and concomitant release of DMSO into the green beer. The difference between the sum of DMSO in the supernatant (~ 700 µg/L) and the release after ultrasonic treatment to the levels of DMSO in the pitching wort (~ 854 µg/L) is ~ 50 µg/L, suggesting that also 50 µg/L of DMS have been generated. The discrepancy to the levels detected in the green beer (~ 10 µL) may have been removed from the green beer via CO₂ desorption [21]. Still, the results demonstrate that the amount of DMSO taken up by the yeast in the 1st period of fermentation is not strictly related to its biochemical reduction to DMS. It is also likely that yeast accumulates DMSO in the course of propagation and therefore also yeast management (propagation, re-pitching) seems to play a role in DMS formation during fermentation. In summary of this experiment we propose therefore, that a minor part DMSO was reduced to DMS by TUM 34/70, which was responsible for DMS formation. The observations may also explain the slight increases of DMSO in the previous trial (Fig. 4 a, e.g. grist composition A), where significant DMS formation was observed likewise.

As described in the introduction, top-fermenting yeast was declared to produce more DMS during fermentation than bottom-fermenting yeast. The extent to which genetically diverse top-fermenting yeasts are capable of DMSO reduction and DMS formation during fermentation of wort A is shown in figure 6.

The degree of attenuation of all fermentations was $>74\%$ and the pH values of the resulting beers varied between 4.2–4.3.

All tested top fermenting yeast strains were able to significantly reduce the DMSO content in the course of fermentation. Highest DMSO reductions were found for TUM 68 from the sake cluster ($\sim 26\%$), the African beer strain TUM 480 from the bread cluster

($\sim 23\%$) and the German wheat beer strain TUM 149 from the main beer cluster ($\sim 20\%$), which were significantly different from each other. The Kölsch/Alt strain TUM 177, the English ale strain TUM 210 (both $\sim 15\%$) and the American ale strain from the wine cluster TUM 511 ($\sim 13\%$) reduced less DMSO, but there were no significant differences among the latter strains. Interestingly, TUM 149 and TUM 68, which are widely applied for the production of wheat beer reduced more DMSO after pitching compared to the other strains. TUM 68 reduced more DMSO than TUM 149, which is in accordance to the respective increase in the DMS concentration. Also, TUM 177, used for the production of “Alt” and “Kölsch” reduced less DMSO and consequently generated less DMS. Unfortunately, the relatively high standard deviations in the DMS concentrations, especially in the fermentations with TUM 210 and TUM 511 impeded a statistical comparison of DMS formation among the yeast strains. However, in each fermentation significant DMSO reduction was accompanied by a significant increase of DMS (Fig. 6 b). Highest levels were found for TUM 68 (~ 32 µg/L) and TUM 149 (~ 29 µg/L). TUM 210 ($\sim 21\%$), TUM 177 (~ 16 µg/L) and TUM 480 (~ 17 µg/L) formed less DMS.

The DMS levels found in the beers were much lower than the overall DMSO reduction, which may, again, have been evoked by CO₂ desorption. As it was shown for the lager strain TUM 34/70 another explanation may be that the DMSO uptake by the yeast is not necessarily followed by its reduction to DMS.

The screening of the top-fermenting yeast strains shows that there was no huge difference in DMSO reduction among the tested top-fermenting strains. It is likely that MSRA is highly expressed in each strain as it is an ubiquitously abundant enzyme in pro- and eukaryotic cells, which is essential for the recovery of methionine.

The higher DMSO reduction and DMS formation by the top-fermenting strains is in agreement with the literature [2, 9]. TUM 149 was further applied to monitor the DMSO reduction and DMS formation over time (Fig. 7).

As observed by fermentation with TUM 34/70 a fast absorption of DMSO into the yeast was observed, whereas it was less pronounced in TUM 149. Significant DMSO reduction only took place in the first period of fermentation, while DMS formation reached its

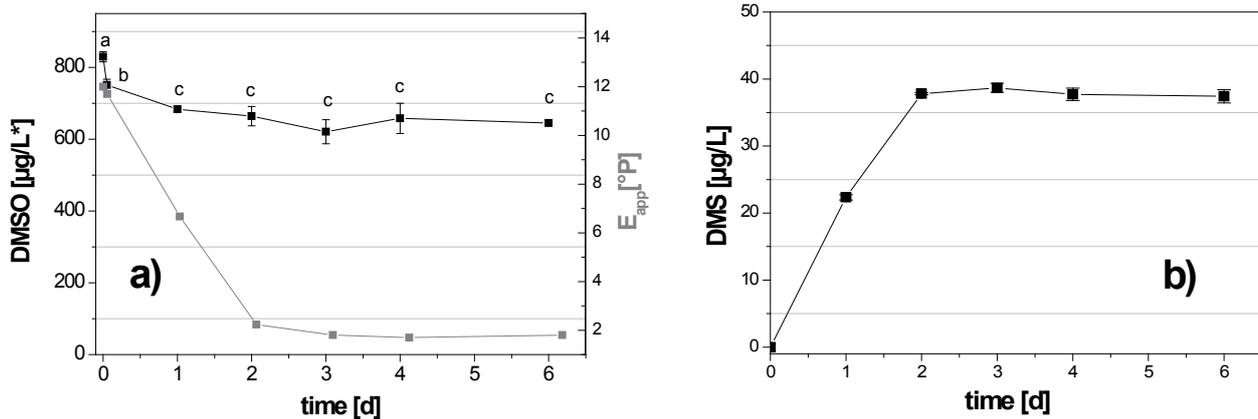


Fig. 7 Fate of DMSO and apparent extract (a) and DMS (b) during fermentation with TUM 149 at 18 °C. Results are presented as mean values ($n = 3$) \pm standard deviation. Different letters in a) indicate significant differences according to the Tukey HSD test

maximum (~ 38 $\mu\text{g/L}$) after the 2nd day of fermentation. At the end of fermentation the apparent degree of attenuation was 85.4 % and ~ 19 % of the DMSO content was reduced. The experiment confirms that the top-fermenting yeast strain TUM 149 was capable of significant DMSO reduction and elevated DMS formation during fermentation.

4 Conclusion/Summary

In this work the behaviour of DMS and its precursors SMM and DMSO was investigated in the brewing process.

During mashing SMM and DMSO were extracted quickly but were not subject of significant biochemical changes thereafter. ~ 60 % of the DMS was lost by evaporation. As expected, SMM was thermally degraded during wort boiling and wort heat holding following a first order mechanism. DMSO was concentrated during wort boiling owing to the loss of water, whereas it was not subject to significant biochemical alterations. However, during wort heat holding, when evaporation was excluded, significant amounts of DMS were oxidized to form DMSO. Though, the extent was below 20 % and the contribution of DMS oxidation to final DMSO levels in the pitching wort is rather small. These findings indicate that the wort production has only a minor influence on the levels of DMSO in the pitching wort and that more auspicious approaches for DMSO minimization are rather to be found in the malting process. However, DMSO concentrations of ~ 800 $\mu\text{g/L}$ were quantified in the pitching worts (100 % Pilsner malt), which is around 15 times higher than the DMS flavour threshold in lager beers, for example. During fermentation TUM 34/70 generated significant amounts of DMS even though DMSO reduction was insignificant. The yeast quickly absorbed a part of the DMSO whereas it was not recovered entirely as DMS. Besides CO_2 desorption, accumulation of DMSO in the plasma membrane may further elucidate this observation.

In biology, DMSO is widely applied as cryoprotectant for enzymes and cell cultures [38, 39]. In this context Leekumjorn and Sum [38] showed that DMSO diffuses fastly into phospholipid bilayers and that significant amounts accumulate in the intermembrane space. These findings are probably transferable to the observations of the present study and may explain the DMSO release from yeast

evoked by the ultrasonic treatment. The enzymes responsible for DMSO reduction (MSRA) are located in the cytosol and in the mitochondria of the yeast cell, whereas they are not present in the yeast plasma membrane [40]. It is therefore supposed that higher levels of DMSO were recovered from the intermembrane space of the yeast plasma membrane than from the cytosol or intracellular compartments. This would also explain the relative low enzymatic formation of DMS by yeast.

In in *E. coli* for example, the DMSO reductase (DMSORA) is bound to the plasma membrane [39], meaning that the bioavailability of DMSO for the enzyme is higher. This seems to be further explanation why such bacteria are capable of a much higher DMSO reduction and DMS formation than yeast.

The increase of DMS during fermentation evoked by the bottom-fermenting yeast TUM 34/70 was ~ 8 -15 $\mu\text{g/L}$, whereas the top-fermenting yeasts produced much more DMS (~ 15 -38 $\mu\text{g/L}$). It is also very likely that another part of the generated DMS was removed from the green beer by CO_2 desorption [21]. The DMS concentration found in the beers were below the flavour threshold, especially regarding bottom-fermented beers. The DMS increase during fermentation by the bottom fermenting TUM 34/70 does certainly not evoke an off-flavour impression. However, the contribution of DMSO reduction to the levels of DMS can be estimated as significant and thus, potentially affects beer flavour.

Even though much higher levels of DMS were found in the top-fermented beers, it is important to mention that these beers are generally less sensitive towards DMS off-flavours as a result of masking effects by other aroma compounds, like esters or phenolic aroma compounds.

However, in this study, there was no DMS detectable in the pitching worts, which is generally not the case in the brewing industry. Pitching wort DMS levels can be increased by improper wort management, for example insufficient degradation of SMM during wort boiling followed by extensive re-formation of DMS in the whirlpool. In such cases, a formation of DMS during fermentation may be of high relevance to beer flavour.

In this work DMS formation during fermentation was investigated in

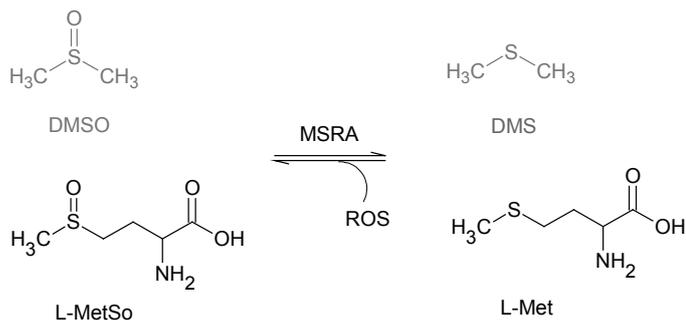


Fig. 8 Proposed mechanism of DMSO reduction and DMS formation by yeast based on literature findings [18,19]

cylindro-conical tubes in small scale fermentations. Fermentation tanks applied by the brewing industry reach heights of up to ~20 m. The upscale factor would be ~200. The biggest difference is most certainly the higher hydrostatic pressure, which leads to a higher CO_2 dissolution. This consequently diminishes DMS desorption. Therefore, DMS formation may eventually be of higher relevance in bigger tanks or even under application of pressure during fermentation. However, more investigations have to be carried out in industrial scales combined with sensory analyses to further evaluate the contribution of fermentation derived DMS formation on the beer flavour.

In this work, the differences in DMSO reduction among the top-fermenting yeasts (~13–26%) were rather small and did not directly correlate with genetic diversity and domestication clusters. The enzyme, responsible for DMSO reduction is actually a methionine sulfoxide reductase (MSRA). Such enzymes can be found ubiquitously in nature. MSRA are present among mammals, yeast and bacteria serving as a defense system against oxidative stress [41]. They can repair oxidative damage (e.g. by ROS) via reduction of the sulfoxide group. To some extent all aerobic cells are exposed to oxidative stress in their environment. We believe that MSRA is equally distributed and expressed among the different yeast strains investigated in this work as the DMSO reduction in yeast is a side-effect of the action of MSRA (Fig. 8).

The fact that DMSO reduction by yeast is controlled by FAN- and especially methionine and MetSO concentration and that MSRA are located in the yeast mitochondria indicate a correlation to mitochondrial activity, which was also shown by *Samp* et al. [37]. Mitochondrial activity as well as FAN utilization proceeds under aerobic growth conditions early during fermentation. This would explain why DMSO reduction and DMS formation in this work was also observed in the early stages of fermentation. The maximum of DMSO reduction in this work was ~26%, meaning that relatively high amounts of DMSO are recovered in the final beer. To our knowledge, the behaviour of DMSO during beer storage is unknown and needs further research. As DMSO contributed significantly to the final DMS concentration in beer, we suggest that maltsters and brewers should consider DMSO as an additional DMS precursor.

Acknowledgement

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Additional data – Publication B

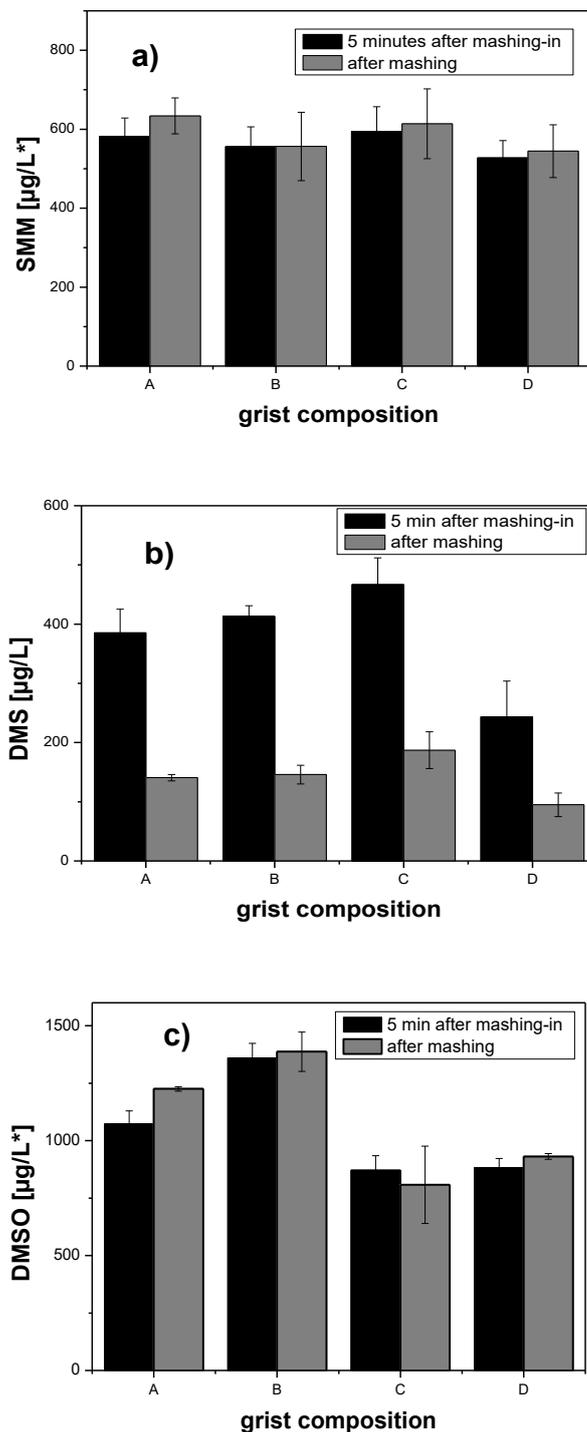


Figure 15: Concentration of SMM (a), DMS (b) and DMSO (c) 5 minutes after mashing-in (black bars) and after mashing (gray bars).

Results are shown as mean values (n=3) ± standard deviation.

Publication C:

The majority of DMSO, as mostly derived from malt, was found to survive wort production and fermentation and therefore, is transferred into the beer. Considering the relative high levels of DMSO in relation to the DMS flavor threshold, even minor DMSO reduction during beer storage may be significant to beer flavor.

As it was shown in publication A,²⁰² the antioxidants that can also be found in beer, notably sulfite, Cys and GSH were capable of DMSO reduction and DMS formation. The following publication aimed to test the feasibility of a selected variety of antioxidants to reduce DMSO and generate DMS in buffered model solution as well as in a Pilsner type beer.

The concept of these investigations, results and deductions were published in the *Journal of the Institute of Brewing*, in a paper entitled “*Effect of antioxidants on dimethyl sulfoxide reduction and impact on dimethyl sulphide formation during beer storage*” (Publication C²⁰⁴).

Summarizing the findings of publication C,²⁰⁴ it could be stated that sulfite as well as the thiols Cys and GSH are capable of significant DMSO reduction, which is accompanied by significant DMS formation in beer model solution as well as in a Pilsner type beer. However, levels of sulfite and thiols higher than normally found in beer were necessary to evoke an increase of DMS in a range potentially relevant for off-flavor formation. However, based on these findings, potential reaction mechanism could be proposed elucidating a yet undiscovered potential source of DMS in beer.

Publication C



Effect of antioxidants on dimethyl sulphoxide reduction and impact on dimethyl sulphide formation during beer storage

Matthias Baldus* and Frank-Jürgen Methner

Dimethyl sulphide (DMS) can be important in beer aroma. Although primarily formed during wort boiling, it can evolve during fermentation by reduction of its oxidation product dimethyl sulphoxide (DMSO). In this study, a number of antioxidants – sulphite, thiols [L-cysteine, L-glutathione (GSH)], gallic acid and ascorbic acid – were tested on DMSO reduction and DMS formation in a buffered model solution [pH 4.3, 5% (v/v) ethanol] together with a Pilsner type beer. In the model solution, significant DMSO reduction and DMS formation were observed for sulphite and the thiols L-cysteine and GSH. Ascorbic acid and gallic acid were not capable of reducing DMSO. During beer storage at 28°C, the highest DMS formation was found with the combined addition of sulphite, GSH and DMSO followed by sulphite/DMSO and GSH/DMSO. In conclusion, the reaction mechanisms of DMSO reduction and their relevance to DMS formation during beer storage are discussed. © 2018 The Institute of Brewing & Distilling

Keywords: DMSO; DMS; antioxidants; sulphite; thiols

Introduction

Dimethyl sulphide (DMS) is an aroma intensive thioether that can be found in diverse foodstuffs. It contributes significantly to beer flavour, and lager beers are especially sensitive towards DMS off flavours ('boiled vegetable') above concentrations of ~30–100 µg/L (1,2). The role of S-methyl methionine (SMM) as the primary DMS precursor has been extensively characterised (2–6). Another precursor of DMS is its oxidation product, dimethyl sulphoxide (DMSO), as it can be reduced to DMS by yeast (2,7,8). Both precursors derive mainly from malt (9), whereas SMM is largely thermally degraded to DMS and L-homoserine during malt kilning (4,9) and wort boiling (5,6). Consequently, the pitching wort should contain less than 100 µg/L of SMM (10). In contrast to SMM, DMSO is thermally stable and remains constant throughout the brewing process, leading to DMSO concentrations of up to 800 µg/L (100% Pilsner malt) in wort (11).

Approximately 11–21% of the DMSO content is reduced during fermentation (7,11), whereas the majority can be recovered in the final beer. In relation to the DMS flavour threshold, minor DMSO reduction may contribute to DMS formation. The formation of DMS during beer storage was reported previously (12,13), whereas information regarding its origin is limited. Beer possesses a variety of natural antioxidants such as sulphite (14–16), polyphenols (17,18) and thiols (19,20). Further, antioxidants such as ascorbic acid, can be exogenously added to increase the oxidative stability of beer (21). The common property of these antioxidants is their capability to donate electrons and by definition, they can also be regarded as reducing substances.

The aim of the present study was to test a select variety of beer derived antioxidants for their capability of DMSO reduction. DMS formation via DMSO reduction was already reported for sulphite (22,23) as well as for the thiols L-cysteine (CYS) (22–24) and L-glutathione (GSH) (23). However, most investigations were not related to beer storage conditions.

Therefore DMSO reduction was compared by combining equimolar amounts of sulphite, thiols (CYS, GSH), ascorbic acid and gallic acid with DMSO in buffered model solutions. Furthermore, DMSO reduction and DMS formation by antioxidants was monitored during beer storage. The outcomes of this study are discussed regarding the potential of DMSO as a DMS precursor in beer in relation to the abundance of antioxidants and storage conditions.

Materials and methods

Chemicals

Dimethyl sulphoxide (DMSO, >99%) was purchased from Sigma Aldrich (St Louis, MO, USA). L-Glutathione (>98%) was purchased from Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany). Ethanol (EtOH, 99.9%) and sodium metabisulphite (Na₂S₂O₅, >98%) were obtained from Merck Chemicals Darmstadt, Germany. Gallic acid monohydrate (GA, ≥99%), L-(+)-ascorbic acid (ASCO, ≥ 99%) and L-cysteine (CYS, ≥99%) were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Nitrogen (99.999%) was obtained from Air Liquide GmbH (Berlin, Germany).

Preparation and spiking of model solutions and beer

In order to avoid early oxidative consumption of the respective reducing substances, all samples were prepared under a nitrogen atmosphere in a hermetically closed glove box. The constituents

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of the model solution were placed inside the box and the inner atmosphere as well as the solutions were purged with nitrogen overnight. The oxygen concentrations in the gas phase as well as in the buffer solutions were $<60 \mu\text{g/L}$ as measured continuously using a Fibox 3/trace V3 optical oxygen sensor (PreSens Precision Sensing GmbH, Regensburg, Germany). In addition, all samples were closed with crown corks containing oxygen scavengers (sodium sulphite based, Rauh GmbH & Co. KG, K ups, Germany). These crown corks were used for the minimisation of oxidative side reactions of the applied antioxidants to more precisely characterise their potential reactions with DMSO.

DMSO reduction by sulphite in buffered model solutions

Kinetic investigations were all carried out in 10 mM sodium acetate–acetic acid buffer containing 5% (v/v) ethanol (buffered model solution). The solution was adjusted to a pH of 4.3, before adding to 330 mL long-neck bottles. The buffered model solution was spiked with aqueous stock solution of DMSO to reach 1.5 mg/L DMS equivalents (DMS eq.) and with an aqueous solution of sodium bisulphite to reach 50 mg/L of sulphite. After spiking, the bottles were closed with crown corks. The bottles were incubated at 40°C in the dark. Periodically, the bottles were removed and cooled down to 1°C before they were analysed for DMS and sulphite. The trial was conducted in duplicate ($n = 2$).

Comparison of DMSO reducing capability of different antioxidants

DMSO reducing capability by equimolar concentrations (1.56 mM) of sulphite, cysteine, glutathione, gallic acid and ascorbic acid was compared in buffered model solution containing 1.5 mg/L DMSO (DMS eq.). The chemical structures of the investigated substances are illustrated in Figure 1.

Incubation was carried out at 40°C for 10 days in the dark before DMSO reduction and DMS formation were investigated. The trials were carried out in triplicate ($n = 3$).

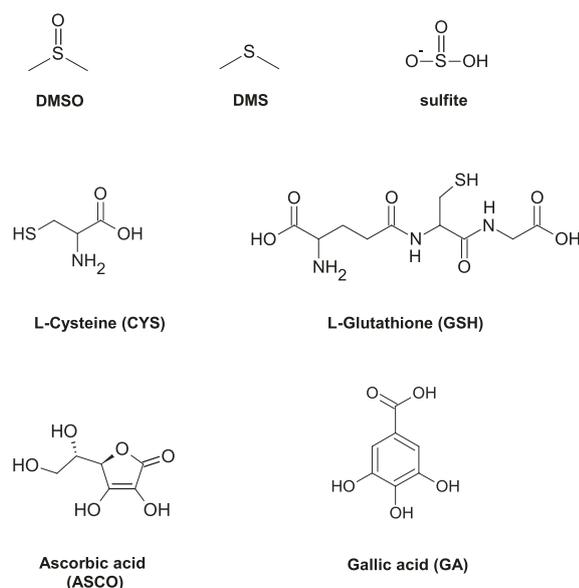


Figure 1. Chemical structures of the antioxidants.

DMSO reduction during beer storage

To evaluate DMSO reduction in beer, a commercially obtained Pilsner type beer [5% (v/v) ethanol, pH 4.3] was spiked with different concentrations of aqueous stock solutions of DMSO, sulphite and GSH under an atmosphere of nitrogen. In Table 1 an overview of the concentrations of the added substances is presented. The bottles were stored in the dark at 28°C for 120 days. Periodically, bottles were removed, cooled down to 1°C and analysed for DMS, DMSO and sulphite. The trials were carried out in duplicate ($n = 2$).

Quantification of SMM, DMSO and DMS

DMS and DMSO were analysed according to a previously described method (14). DMSO was reduced by sodium metabisulphite and subsequently analysed as DMS equivalents by headspace gas chromatography and pulsed flame photometric detection. SMM was quantified according to White and Wainwright (25). The data for SMM and DMSO are presented in DMS equivalents (SMM*, DMSO*). The DMS detection limit of the applied methodologies was 6 $\mu\text{g/L}$.

Quantification of sulphite

Sulphite was quantified using continuous flow analysis combined with UV–vis detection according to MEBAK guidelines (2.21.8.3) (10). The sulphite concentration is presented as sulphur dioxide equivalents in mg/L.

Statistics and data analysis

Comparison of DMSO reduction and DMS formation by antioxidants in buffered model solutions was carried out in triplicate. The mean values ($n = 3$) were compared with each other using the Tukey honest significance difference test at a confidence level of 95% after analysis of variance (ANOVA). The data of the investigation of DMSO reduction during beer storage were compared before and after storage using a two-tailed *t*-test at a confidence level of 95%. All statistical analyses were performed with Microsoft XLstat (version 2014.5.03, Addinsoft, USA).

Results and discussion

During storage, oxygen diffuses through the packaging material into the beer, where it is reduced into forms of higher reactivity. These reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide and hydroxyl radicals, significantly contribute to beer flavour instability (26,27). In this context

Table 1. Addition of dimethyl sulphoxide (DMSO) and antioxidants to beer

Trial	Addition(s)
1	No additions (reference)
2	+ 50 mg/L sulphite +1.0 mg/L DMSO (DMS eq.)
3	+ 25 mg/L sulphite +0.5 mg/L DMSO (DMS eq.)
4	+ 25 mg/L sulphite +50 mg/L GSH + 0.5 mg/L DMSO (DMS eq.)
5	+ 50 mg/L GSH+ 0.5 mg/L DMSO (DMS eq.)

antioxidants can be regarded as potential antagonists towards damage caused by ROS in beer.

For example, sulphite predominantly reacts with hydrogen peroxide, by which the formation of higher reducing states of oxygen is diminished as long as sulphite is present (28,29). Afterwards, hydrogen peroxide undergoes the Fenton reaction, thereby forming highly reactive hydroxyl radicals. These react primarily with ethanol, forming hydroxyethyl radicals, which are often designated as the most harmful organic radicals in beer as they degrade hop bitter substances (27,30) or amino acids (31), leading to deterioration in beer quality. Thiols are reported to act as secondary antioxidants by inactivating hydroxyethyl radicals at rate constants close to their respective diffusion limits (30). Also, polyphenols are representative antioxidants in beer (17,18), whereas their effectiveness regarding oxygen radical scavenging is debateable (16,31). However, in this study we used gallic acid as representative phenol in beer (32) and also investigated ascorbic acid as one of the most representative supplementary antioxidants to beer and food in general (E 300).

DMSO reduction by sulphite in buffered model solution As noted in the introduction, the reduction of DMSO in excess of sulfite was reported previously (22,23). To test if sulphite is also capable of DMSO reduction at a lower concentration ratio, DMS formation was investigated in a buffered model solution containing ~50 mg/L sulphite and ~1.5 mg/L DMSO (DMS eq.). The DMS and sulphite concentrations were monitored over 25 days at 40°C (Figure 2).

Within this period, the sulphite concentration remained broadly constant. It is assumed that sample preparation under nitrogen in combination with oxygen scavenging closures was sufficient to prevent sulphite from oxidation. Also, compared with beer or other foodstuffs, the buffered model solution used can be anticipated to contain significantly lower amounts of transition metal ions or any other catalysts of oxidative reactions. Under these conditions oxygen activation may have been less pronounced, leaving the sulphite concentration unaffected. Within the storage period, a linear DMS formation ($R^2 = 0.9888$) of up to 125 µg/L was observed. This may be explained by DMSO reduction in molar overshoot of sulphite (sulphite–DMSO ~33). Of the added DMSO, only ~6% was recovered as DMS. The reaction mechanism of the DMSO reduction by sulphite is unknown. However, considering the

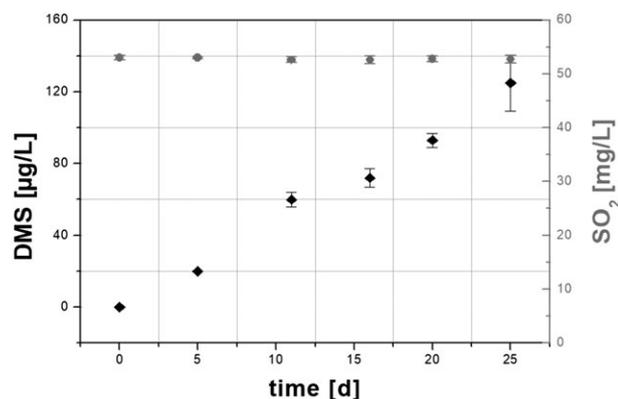


Figure 2. Formation of dimethyl sulphide (DMS) in buffered model solution containing 50 mg/L sulphite and 1.5 mg/L dimethyl sulphoxide (DMSO)* at 40°C. (Grey squares, right axis) Sulphite concentration; (black diamond, left axis) DMS concentration. Results are presented as mean values of duplicate trials ($n = 2$) ± standard deviation (SD).

equimolar stoichiometry of the reaction, only ~0.24% of the sulphite would have been necessary for the formation of the observed DMS levels. This small difference was not measurable by the method used for sulphite quantification. However, considering the DMS flavour threshold in beer (30–100 µg/L), the observed increase in the DMS concentration may be of significance.

From these results it can be assumed that the velocity of DMSO reduction by sulphite in the applied concentration range is relatively low.

Comparison of DMSO reducing capability of different antioxidants After verification of the sulphite mediated reduction of DMSO, further representative antioxidants from beer were tested for their capability in DMSO reduction and DMS formation (Table 2).

As well as sulphite, the thiols CYS and GSH were able to significantly reduce DMSO and concomitantly generate significant amounts of DMS. Regarding DMSO reduction, no significant differences were found between the thiols and sulphite, whereas the thiols produced significantly less DMS than sulphite. Also, CYS and GSH did not differ in their capability of DMSO reduction, whereas GSH produced significantly less DMS than CYS. However, the difference was relatively small (23 µg/L). The combination of sulphite and GSH resulted in the highest DMSO reduction and concomitantly the highest formation of DMS. However, the effect of sulphite and GSH was not cumulative, which suggests the interaction of sulphite and GSH that limits their capability of DMSO reduction. A potential reaction would be the addition of sulphite to the keto group of C-5 of the glutamic acid moiety of GSH (Figure 1). Also, part of the sulphite may have been consumed by the reduction of oxidised glutathione (33). However, these aspects were not further investigated in this study. A small reduction in the DMSO content was found for ASCO, whereas it was not accompanied by DMS formation. No effect was observed with gallic acid in DMSO reduction and DMS formation.

Impact of sulphite and L-GSH on DMSO reduction during beer storage The above trials were carried out in buffered model solution to avoid side reactions of the added substances with other beer constituents. To test if DMSO reduction by sulphite and thiols was also relevant for DMS formation in beer, a Pilsner type beer

Table 2. Concentration of dimethyl sulphide (DMS) and DMSO after incubation of 1.5 mg/L DMSO* and 1.156 mM antioxidant in deoxygenated buffered model solutions for 10 days at 40°C

Antioxidant	DMSO*	DMS
—	1350 ± 27 ^a	n.d.
sulphite	1142 ± 58 ^d	174 ± 1 ^a
CYS	1165 ± 75 ^d	155 ± 4 ^b
GSH	1193 ± 48 ^d	132 ± 11 ^c
ASCO	1291 ± 11 ^b	n.d.
GA	1323 ± 18 ^a	n.d.
Sulphite + GSH	1074 ± 6 ^c	258 ± 14 ^d
Sulphite + GSH + ASCO	1051 ± 30 ^c	240 ± 6 ^d

Results are presented as mean value of triplicate trials ($n = 3$) ± standard deviation (SD). Different letters indicate significant differences according to the Tukey honest significant difference test at a confidence level of 95%

was spiked to achieve different concentrations of DMSO, sulphite and GSH (Table 1). The concentrations of sulphite, DMSO and DMS were monitored over 120 days at 28°C.

Sulphite consumption. In comparison with the investigations in the buffered model solutions (Figure 2), the sulphite concentration was consumed by ~50% in each trial until the end of storage. In the first period (after 14 days), the sulphite consumption was lower compared with the further course of storage, especially in trials 1, 2 and 5. As the samples were prepared under nitrogen it can be assumed that the absence (or low concentration) of oxygen prevented the sulphite consumption. However, during further storage, the diffusion of oxygen through the crown cork sealing material into the bottle was inevitable when using common sealing compounds, such as polyethylene (34). After entering the gaseous headspace of the bottle, the oxygen diffuses into the beer where it can be reduced to ROS involving transition metal ions. During this period, the oxygen scavenging material (sulphite based) of the crown corks may also have suppressed the sulphite consumption (35) in the beer via reacting with ROS, most likely with hydrogen peroxide. The reaction mechanism of the protective effect of sulphite enriched compound material is unknown. It is supposed that the faster decline of the sulphite concentration after day 14 of storage was a result of the limited capacity of the scavenging material.

It was reported by Andersen *et al.*, (29) that sulphite acts as a primary antioxidant by reacting with hydrogen peroxide, whereas the antioxidative functionality of thiols in beer is related to the reaction with hydroxyethyl radicals after sulphite depletion. However, in this work, the sulphite consumption was diminished when the beer was additionally spiked with GSH (Figure 2, trials 1 and 5). At the end of storage the sulphite concentration in trial 5 (GSH addition) was ~30% higher than in the reference (trial 1). The molar GSH concentration (addition of ~163 μM) was at least 1.7 times higher than the molar sulphite concentration (~94 μM). This ratio is representative for the later stages of beer ageing, when most of the sulphite has already been consumed (29).

Under these circumstances GSH may have reacted with ROS, thereby leaving more sulphite in the beer. The effect was less pronounced when a combination of sulphite and GSH was added to the beer. At the end of storage, the sulphite concentration in trial 4 (GSH and sulphite addition) was ~10% higher than in the beer, which was exclusively spiked with sulphite (trial 3).

SMM, DMSO and DMS. The concentration of SMM in the reference beer was 13 $\mu\text{g/L}$, whereas SMM could not be detected in all beers after storage. The DMSO concentration in the reference (no additions, trial 1) was not significantly changed throughout the storage time ($p = 0.053$), whereas the DMS concentration increased from ~35 to ~43 $\mu\text{g/L}$ ($p = 0.046$). DMSO was significantly reduced when sulphite (50 mg/L) and DMSO (1 mg/L) were added to the beer (trial 2, $p < 0.0001$). The observed DMSO reduction was accompanied by significant formation of DMS ($p = 0.0001$) from ~35 to ~100 $\mu\text{g/L}$. When only half the amount of sulphite (25 mg/L) and DMSO (0.5 mg/L) were spiked to the beer, DMSO reduction was not significant (trial 3, $p = 0.91$), whereas the DMS concentration was increased significantly from ~35 to ~51 $\mu\text{g/L}$ ($p = 0.015$) and was higher than in the reference. When GSH (50 mg/L) was added in combination with sulphite (25 mg/L) and DMSO (0.5 mg/L), the DMSO reduction was significant (trial 4, $p = 0.032$). The DMS concentration at the end of storage (~68 $\mu\text{g/L}$) was significantly higher in this case ($p = 0.001$). The addition of GSH (50 mg/L) and DMSO (0.5 mg/L) did not lead to a significant change in the DMSO concentration ($p = 0.41$). However,

again, the DMS concentration was significantly increased to ~51 $\mu\text{g/L}$ ($p = 0.004$), which was also higher than the reference. As noted previously, 13 $\mu\text{g/L}$ of SMM were degraded below the detection limit and could potentially account for an equivalent increase in DMS. This was reported before by Hysert *et al.*, (12). In their study the degraded SMM was not fully recovered as DMS. As a result of the high relative volatility of DMS it is likely that DMS partly diffused into the headspace of the bottle and potentially out of the bottle through the crown cork liner.

In this study, SMM degradation may have contributed to the increase of DMS in the reference beer (~8 $\mu\text{g/L}$), whereas it cannot explain DMS formation in the other experiments. It is therefore proposed that DMS formation in these beers was the result of DMSO reduction by sulphite and/or thiols. However, it becomes apparent that significant DMS formation was not strictly related to significant changes in the DMSO concentration, particularly in trials 1, 3 and 5. These phenomena can probably be explained by the relative quantities of DMS and DMSO in the beers. For example, the increase in DMS in trial 5 was ~16 $\mu\text{g/L}$ and the initial DMSO concentration was ~1090 $\mu\text{g/L}$ (DMS equivalents). The required reduction of DMSO to explain an increase of 16 $\mu\text{g/L}$ DMS would have been ~1.4%, which is higher than the applied confidence level of 95%. Consequently, these relatively small changes are not detectable. The data also suggest that DMSO is relatively stable in beer. However, considering the flavour threshold of DMS in beer (30–100 $\mu\text{g/L}$), even small reductions in DMSO may lead to significant DMS formation.

Taking into consideration the above mentioned aspects it can be stated that sulphite as well as the thiols CYS and GSH are capable of reducing DMSO, which results in DMS formation in buffered model solutions. The reactions were also shown to take place in a Pilsner type beer, which was spiked with different combinations of DMSO, sulphite and/or GSH.

The reduction of a sulphoxide group to the corresponding thioether by sulphite was first reported in 1939 by Micheel and Schmitz (36). Johnson *et al.*, (37) proposed a mechanism in which the sulphur atom of the sulphoxide is subject to a nucleophilic attack by bisulphite anion forming sulphate and the corresponding thioether. These findings could be verified for the reduction of methionine sulphoxide by Snow *et al.*, (38). To our knowledge, no information regarding the mechanism of DMSO reduction by sulphite has been reported before. In beer (pH ~4.3), sulphite is mainly present as bisulphite anion. Based on the aforementioned literature (34–36) a simplified reaction mechanism of the DMSO reduction by sulphite is presented in Figure 3. After protonation of DMSO, (a) a nucleophilic addition of the bisulphite anion takes place whereupon a DMSO–bisulphite adduct (b) is formed. The latter compound is then decomposed forming DMS and sulphate as final products.

The formation of DMS by the combined addition of DMSO and CYS has been reported before (5,23,24). The mechanism by which DMSO is reduced by thiols was elucidated by Madesclaire (39). This mechanism was adapted to illustrate the potential process of DMSO reduction by thiols in beer (Figure 4).

Similar to the reduction by sulphite, the reaction requires protonation of DMSO followed by a nucleophilic addition of the thiol forming compound 'b'. The latter compound is further reduced by another thiol, leading to the formation of DMS, the corresponding disulphide and water as final products. The aforementioned reactions may be relevant to the levels of DMS in beer, whereas the concentrations of DMSO, sulphite and thiols seem to play a significant role. Also, according to the reaction mechanisms (Figures 4 and 5), the reaction is favoured by protonation of DMSO.

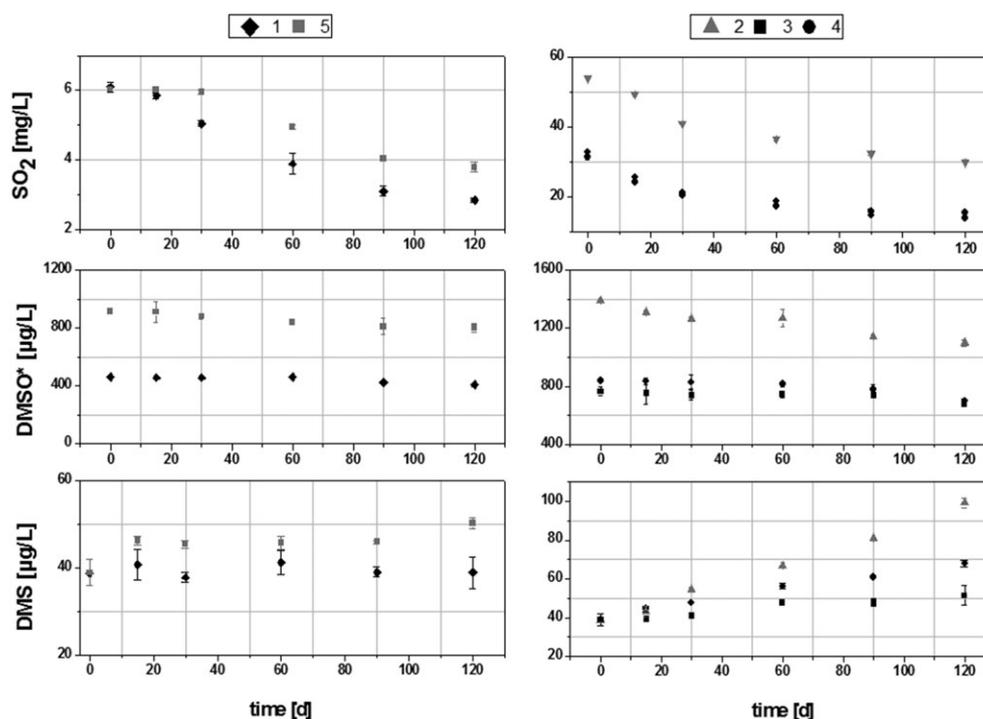


Figure 3. Behaviour of sulphite, DMSO and DMS during beer storage at 28°C. 1, Reference (no additions); 2, addition of sulphite (50 mg/L) and DMSO* (1 mg/L); 3, addition of sulphite (25 mg/L) and DMSO* (0.5 mg/L); 4, addition of sulphite (25 mg/L), GSH (50 mg/L) and DMSO* (0.5 mg/L); 5, addition of GSH (50 mg/L) and DMSO* (0.5 mg/L). Results are presented as mean values of duplicate trials ($n = 2$) \pm standard deviation (SD).

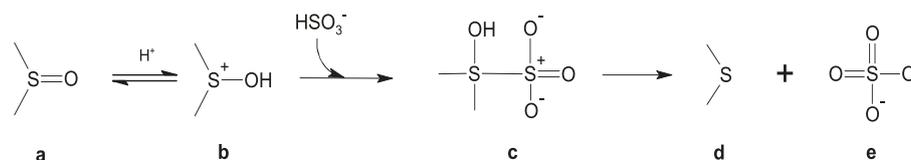


Figure 4. Proposed mechanism of the reduction of DMSO by sulphite in beer based on the literature mentioned in the text (32–34). (a) DMSO; (b) protonated DMSO; (c) DMSO–sulphite adduct; (d) DMS; and (e) sulphate.

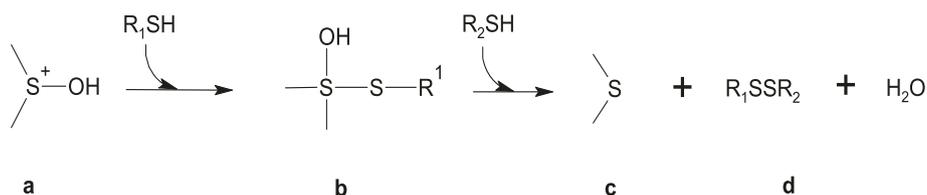


Figure 5. Proposed mechanism of the reduction of DMSO by thiols in beer adapted from Madesclaire (39). (a) Protonated DMSO; (b) DMSO–thiol adduct; (c) DMS; and (d) disulphide.

As protonation initiates the reaction, it may be rate limiting. Unfortunately, to our knowledge, there are no data available on DMSO protonation equilibria in aqueous solution. Therefore precise deductions on the effect of DMSO protonation on its reduction in beer cannot be drawn. Gijs *et al.*, (40) reported that the addition of 50 mg/L of GSH to beer led to strong off-flavours (e.g. sulphurous) after storage for 5 days at 40°C.

The DMS content in the present study was significantly increased in beer by the addition of addition of 50 mg/L GSH and 0.5 mg/L DMSO. However, the levels of DMS were in the lower flavour threshold range and therefore it is unlikely that an off-flavour

was formed. However, based on the study of Gijs *et al.*, (40), DMSO and DMS data were not available for their beers. In the present study the levels of DMSO in the Pilsner beer were ~ 470 $\mu\text{g/L}$. Recently, much higher DMSO concentrations of ~ 700 – 800 $\mu\text{g/L}$ of DMSO were found in green lager beer (11). As DMSO mainly originates from the malt (3,9), its quantities in beer are also influenced by the germination and kilning technology (9). Consequently, highly modified and high temperature cured malt contain higher levels of DMSO (11,23), which are also transferred into the beer.

Besides DMS formation during fermentation, higher DMSO concentrations in malt and wort would also increase the probability of

DMS formation during beer storage. In this context, the quantities of sulphite and thiols can be regarded as further factors influencing DMSO reduction and DMS formation in beer. The levels applied of sulphite in this study (25 and 50 mg/L) were generally higher than those naturally found in beer (≤ 10 mg/L). However, the sulphite concentration of beer can be increased by the application of sulphiting agents (E 220–224), when declared on the label as 'contains sulphite'. Certainly, an increase of DMS during beer storage and its flavour impact can be masked by typical stale flavour aroma compounds, especially aldehydes (41–43). However, under 'reductive' conditions, like innovative packaging material, that avoids oxygen ingress and/or exogenous addition of sulphite and/or thiols, DMS formation may be more pronounced and thus, potentially, affect beer flavour.

Especially in beers whose DMS concentration is close to the flavour threshold at racking, a surplus formation of DMS may be critical. Therefore, further investigations of different beer types are necessary to further evaluate the relevance of the described reactions to the final levels of DMS in beer.

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3.3 Reaction mechanisms of DMS oxidation and DMSO formation – Impact of potential malt-derived antioxidants

In Publication B²⁰³ it was shown that the impact of the brewing process on the levels of DMSO that can potentially be reduced to fermentation is rather marginal and that most DMSO formed during malting. The second part of this dissertation comprises the investigations into reaction mechanisms of DMS oxidation to obtain information on the origin of DMSO in malt (Publications D-F). The first experiments intended to test if DMS is subject to oxidation in oxygenated model solutions containing Mⁿ⁺. The DMSO reducing capability as detected for the sulfuric antioxidants sulfite, Cys and GSH, suggests a potential antioxidative behavior via shifting the redox equilibrium towards DMS (Figure 16).

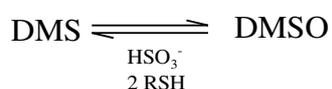


Figure 16: Potential impact of thiols and sulfite on the redox behavior of DMS and DMSO.

Those antioxidants may also serve as antagonists towards ROS (cp. 1.5) which may oxidize DMS and may therefore eventually be applicable in the malting process to minimize DMSO formation. Therefore it was of particular interest to include potential malt-derived antioxidants into the investigation of DMS oxidation in model systems.

Publication D:

Cys is one of the most abundant antioxidants in malt, therefore its effect on DMS oxidation in combination with Mⁿ⁺ was investigated in the first step. The entire approach, methodologies and outcomes were published in the *Journal of Agricultural and Food Chemistry*, in a paper entitled “*Effect of L-Cysteine and Transition Metal Ions on Dimethyl Sulfide Oxidation*” (Publication D²⁰⁵).

It could be evidently demonstrated by response surface modeling and product analysis that, in the presence of transition metal ions, Cys catalyzed the oxidation of DMS throughout a wide concentration range. The prooxidative behavior in the presence of Cu²⁺ was retraced to the formation of a bis-Cys-Cu⁺ complex, which catalyzed the bivalent reduction of O₂ thereby forming H₂O₂. A reaction mechanism could be postulated declaring H₂O₂ as primary source of DMS oxidation and DMSO formation in the investigated systems.

Publication D

Effect of L-Cysteine and Transition Metal Ions on Dimethyl Sulfide Oxidation

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S Supporting Information

ABSTRACT: During malt kilning, significant amounts of dimethyl sulfide (DMS) oxidize leading to the formation of dimethyl sulfoxide (DMSO), a precursor of DMS during fermentation. Yet, knowledge regarding reaction mechanisms of DMSO formation during malt production is limited. The role of thiols in sulfide oxidation is unclear as they possess sulfoxide reducing ability as well as pro- and antioxidative properties. This study investigated the effects of the thiol L-cysteine (Cys), molecular oxygen, transition metal ions, and EDTA on DMS oxidation in aqueous model solutions. Highest oxidative DMS consumption was observed when Cys was combined with iron(II) (~12%) and copper(II) (~40%). Response surface modeling (RSM) revealed that Cys together with copper(II) had a strictly prooxidative effect and no antioxidative behavior was found. Hydrogen peroxide, as generated via autoxidation of Cys–Cu(I)–Cys complexes, was supposed to be the primary DMS oxidant in this work. Based on redox kinetics, potential reaction mechanisms, and their impact on oxidative processes in thermal food processing, such as malt and beer production, are discussed.

KEYWORDS: dimethyl sulfide, oxidation, cysteine, metal ions, malting and brewing

■ INTRODUCTION

Dimethyl sulfide (DMS) is the most abundant volatile thioether in food. It contributes significantly to the overall flavor impression of many thermally treated vegetables, such as tomatoes,^{1,5} asparagus,^{2–5} or cabbages.^{2,5}

In beer, DMS can lead to specific off-flavors at concentrations above its flavor threshold of 30–100 µg/L.⁶ Those flavor impressions are often ascribed as “cooked-vegetable”-like.^{6,7} Though, at subthreshold-levels, DMS was reported to contribute positively to beer flavor.⁶ The origin of DMS in beer can be primarily traced back to S-methyl methionine (SMM), a nonprotein amino acid which is synthesized during the germination of brewing cereals.^{8,9} During malt kilning, SMM decomposes hydrolytically thereby forming DMS and L-homoserine.^{10,11} The formed DMS either remains in the grain, evaporates into the ventilation air, and/or is oxidized thereby forming dimethyl sulfoxide (DMSO), and minor amounts of dimethyl sulfone (DMSO₂).^{10,12,13} Up to 14.5 µg/g^{10,11,15,16} of SMM remains in the malt and enters the brewhouse as a so-called thermal DMS precursor, which is generally degraded and removed by wort boiling. The malt contains DMSO levels of up to 10 µg/g.^{10,12,14} The high polarity of the sulfoxide group as well as the pyramidal structure of DMSO in aqueous solution is accountable for dipole–dipole interactions within DMSO molecules leading to a high water solubility and a high entropy of vaporization. Consequently, DMSO, in contrast to DMS, cannot be removed by commonly used evaporation techniques and “survives” wort production thus acting as a DMS precursor during fermentation. Thioredoxin dependent methionine sulfoxide reductases from the yeast deoxygenate DMSO as a functional analog of methionine sulfoxide, which results in DMS formation.^{17–21}

Leemans et al.¹³ declared DMSO as the most potential source of DMS in beer as they found 80% of the total DMS in beer being originated from spiked DMSO-*d*₆. Even though, this assumption implies efficient combination of SMM degradation and DMS evaporation during wort production. The enzymatic reduction of DMSO by lager yeast was shown to account for about 21%.²¹ Considering DMSO levels in pitching wort of about 400–800 µg/L and the DMS flavor threshold of 30–100 µg/L, even minor DMSO reduction during fermentation may contribute to significant DMS formation, thus adversely affecting beer quality.

The levels of DMSO should therefore be minimized in malt and wort to prevent extensive DMS formation in the later stages of beer production, where conventional DMS removal cannot be realized anymore. Only a substantial understanding of the reactions which yield DMSO allows taking steps to minimize DMSO and DMS levels in finished beer. To our knowledge, reactions affecting DMSO formation have not been investigated in the field of thermal food processing so far. Though, oxidative reactions have been the subject of a multitude of investigations in malting and brewing, predominantly in relation to beer flavor stability. Transition metal ions, such as Cu(I), Fe(II), and Mn(II), were suggested as being electron donors for the activation of oxygen, yielding reactive oxygen species (ROS), such as superoxide radicals (O₂^{•-}), hydrogen peroxide (H₂O₂), and hydroxyl radicals (OH[•]).²²

With respect to oxidative reactions, thiols are known to possess ambiguous properties. They can act as antioxidants^{23,24}

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as well as catalysts in oxidative reactions.^{25–29} Antioxidative properties are mainly related to the reaction of the sulfhydryl group (SH) with peroxides in a bimolecular mechanism, generating the corresponding disulfide and water.²³ Furthermore, thiols were declared to enhance the flavor stability of beer by scavenging of hydroxy ethyl radicals.²⁴

Another property of thiols is their ability to reduce sulfoxides to the corresponding thioether.^{30,31} Recently, the specific reduction of DMSO to DMS by Cys was addressed,^{14,32} which suggests a rather antioxidative functionality via thioether preservation. The prooxidative character of thiols is explained by their capability to donate electrons to transition metal ions, thus lowering their valence state, and ultimately leading to oxygen activation and hydrogen peroxide formation via complexation reactions.^{25,26,29,33}

The objective of this work was to investigate DMS oxidation and concomitant DMSO formation in oxygenated aqueous model solutions containing transition metal ions at temperatures representative for thermal food processing, such as the malt kilning process. The main intention was to study the effect of Cys and its relation and interplay with oxygen, Fe(II), Cu(II), or EDTA on DMS oxidation and DMSO formation. To evaluate the scope of potential antioxidative and/or prooxidative effects, response surface modeling (RSM) was applied in malting- and brewing-related concentration ranges of Cys and copper(II). Outcomes of the RSM should serve to depict potential interactions and “crossovers” between antioxidative and prooxidative behavior of Cys and to understand its potential involvement in DMS oxidation and DMSO formation in thermal food processing.

MATERIALS AND METHODS

Chemicals. Dimethyl sulfide (DMS; $\geq 99\%$), dimethyl sulfoxide (DMSO; $> 99\%$), iron(II) sulfate heptahydrate (Fe(II), $> 99\%$), acetic acid ($\geq 99\%$), and hydrochloric acid (HCl, 37%) were purchased from Sigma-Aldrich (St. Louis, USA). Sodium acetate anhydrous ($\geq 99\%$) and horseradish peroxidase (HRP, grade I) were obtained from Applichem (Darmstadt, Germany). Hydrogen peroxide (H_2O_2 , 30%), L-cysteine (Cys; $> 99\%$), leuco crystal violet (LCV, $\geq 98\%$), 5,5'-Dithiobis-2-benzoic acid (DTNB, $\geq 98\%$), and copper(II) sulfate pentahydrate (Cu(II), $\geq 99\%$) were purchased from Carl Roth GmbH & Co KG (Karlsruhe, Germany). Nitrogen (99,999%) and synthetic air ($20.5 \pm 0.5\% \text{ O}_2$ in N_2) were obtained from Air Liquide GmbH (Berlin, Germany). Ethanol ($>99.5\%$), ethylenedinitrilotetraacetic acid disodium salt dehydrate (EDTA), disodium phosphate ($\geq 99\%$), and sodium dihydrogen phosphate (99.0–102%) were acquired from Merck Millipore (Darmstadt, Germany).

Incubation of DMS in Model Solutions. The incubation of DMS combined with Cu(II), Fe(II), and Cys was conducted in 0.1 M sodium acetate/acetic acid buffer solution (model solution) (pH 5.5). All aqueous solutions were prepared with ultrapure water (Milli-Q). The final model solutions were purged with synthetic air for 20 min at 20 °C to reach oxygen concentrations of 8 mg/L as measured optically by a Visiferm DO 120 oxygen sensor (Hamilton GmbH, Planegg, Germany). The model solution (62 g) was filled in 50 mL Duran bottles (Schott AG, Mainz, Germany), which minimized the bottle headspace to diminish DMS diffusion out of the solution. The bottles were tightly closed with screw caps containing PTFE coated silicone seals and covered with aluminum foil to avoid light exposure. Afterward, the bottles were cooled to 1 °C. In

the next step, specific combinations of aliquots of aqueous reactant solutions (stock solution concentrations: Cu(II): 0.04M; Cu(II)EDTA: 0.02 M; Fe(II): 0.036 M or Fe(II)-EDTA: 0.018 M; EDTA: 0.036 M; Cys: 0.163 M) were spiked into the bottles to reach the desired concentration as described in the separate trials. All spiking solutions were prepared volumetrically shortly before spiking at 20 °C. For Cu(II)-EDTA additions, equimolar Cu(II) and EDTA solutions were dissolved separately before they were combined. In the last step, 78 μL of ice-cold DMS-ethanol mixture (DMS concentration: 0.01347 M) was spiked into the bottles to achieve initial DMS concentrations of 16.95 μM . The DMS stock solution was kept in a tightly closed vial with minimized headspace volume, which was placed in an ice block to reduce volatilization of DMS. Immediately after spiking, the bottles were tightly closed and placed in a water bath, which was heated to 95 °C. The temperature increase was tracked using a HI935002 Dual Channel K-Type Thermocouple Thermometer (Hanna Instruments, Voehringen, Germany) in a separate bottle containing model solution only. When 95 °C was reached, the respective incubation time of 0–180 min was started. After incubation, the bottles were cooled down stepwise with tap water and ice cold water prior to analyzing DMS, DMSO, Cys, or H_2O_2 .

Experimental Design of the Effect of Cu(II) and Cys on DMS Oxidation. To further ascertain the impact of Cu(II)-EDTA and Cys as well as potential interactions between the factors, a wider concentration range was investigated using a rotatable central composite design and response surface modeling (RSM). Each factor is varied over five levels including two axial points ($\pm\alpha$), six factorial points (± 1), and five center points (0) (Table 1) thus resulting in 13 test runs. Center point

Table 1. Experimental Set-Up for the Response Surface Design (RSM) of the Effect of Cys and Cu(II)EDTA on DMS Oxidation and DMSO Formation

factor	$-\alpha$	-1	0	$+1$	$+\alpha$
Cys [μM]	0	36.61	125	213.39	250
Cu(II)EDTA [μM]	0	2.62	8.95	15.28	17.91

trials were repeated five times, axial and factorial points were carried out in triplicate. All combinations were incubated with 16.95 μM DMS at 95 °C for 180 min.

Determination of DMS and DMSO. DMS and DMSO were analyzed according to a previously described method.¹⁴ DMSO was reduced by sodium metabisulfite and subsequently analyzed as DMS equivalents by headspace gas chromatography and pulsed flame photometric detection.

Spectrophotometric Determination of Copper Complexes. The formation of Cu complexes were investigated using a Lambda 25 spectrophotometer from PerkinElmer, Waltham, USA. Cys (100 μM) was combined with 50 μM of Cu(II) or Cu(II)EDTA, respectively. Spectra of the mixtures were recorded in nitrogen purged model solution at 200–700 nm after 10 min incubation of the respective sample at 20 and 95 °C.

Determination of Cys. Cys was determined using DTNB as described elsewhere.²⁵ Following modifications were done: 300 μL of sample were combined with 210 μL of aqueous DTNB solution (5 mM). The mixture was diluted with 2760 μL of phosphate buffer solution (0.5 M, pH 7). After 10 min,

the absorption of the TNB^{2-} anion was measured at 412 nm. A six-point calibration was carried out ($R^2_{\text{lin}} = 0.9963$).

Determination of H_2O_2 . The determination of H_2O_2 was carried out according to the method of Boatright³⁴ with some modifications: 50 mg of leuco crystal violet (LCV) were dissolved in 100 mL of 0.5% (v/v) aqueous HCl (LCV solution). Horseradish peroxidase (HRP) (20 mg) was filled up to 20 mL with ultrapure water, which was boiled before (HRP solution). LCV solution (1 mL), 0.5 mL of HRP solution, 1 mL of sample, and 7.5 mL of 0.1 M sodium acetate/acetic acid buffer (pH 4.0) containing 1 mM EDTA were combined. The absorption of the leuco crystal violet ion was measured at 590 nm after 1 min. A six-point calibration was carried out in the respective buffer solution ($R^2_{\text{lin}} = 0.9975$).

Determination of Cu, Fe, Mn, and Zn. Inductively coupled plasma optic emission spectroscopy (ICP-OES) was applied to quantitate transition metal ions in the model solution exclusively as well as the combination with 300 μM of Cys. Measurements were carried out as described elsewhere.³⁵

Reduction of DMSO by Cys. Investigations of DMSO reduction by Cys were carried out in model solution containing 1 mM EDTA, 250 μM Cys, and 15 μM DMSO, which were adjusted by spiking aqueous stock solutions to 62 g of model solution in 50 mL Duran bottles. Ultrapure water was boiled and cooled before buffer preparation. Before spiking, the buffer was purged with nitrogen for 20 min reaching final oxygen concentrations of <0.08 mg/L. The bottles were tightly closed using screw caps with PTFE coated silicone seals and were incubated for 24 h at 95 °C. Afterward the bottles were cooled down stepwise to 1 °C before DMS as well as DMSO were analyzed.

Statistics and Data Analyses. All experiments in this work were carried out in triplicate. Analysis of variance (ANOVA) was carried out at a confidence-level of 95% using Microsoft XLstat (version 2014.5.03, Addinsoft, USA), after verification of normal distribution of the data. Classification of significant different groups was then performed by a Tukey Kramer honest significant difference test (HSD) at a confidence level of 95%. The design of experiments as well as the multivariate data analysis was constructed using Design-Expert software (Ver. 7.0, Stat-Ease, Inc., MN, USA). A backward elimination of model terms was carried out at $\alpha > 0.1$.

RESULTS AND DISCUSSION

During malting, the brewing cereal is modified in a forced germination process under aerobic conditions to achieve high rates of cytolysis and proteolysis. Significant amounts of free amino acids including thiols, mostly Cys, are released. Additionally, glutathione and other higher molecular weight thiols are present in a protein- or peptide bound state. Malt contains a series of transition metal ions which are well-known to catalyze oxidative reactions.²² Moreover, steeping water as well as brewing water introduces significant amounts of transition metal ions into the respective process, where oxygen is also available. In the present study, oxidation of DMS was explored in relation to thermal food processing conditions in general. The main focus was to elucidate the effect of the thiol Cys combined with transition metal ions and molecular oxygen on DMS oxidation to estimate its potential role related to malting, e.g., where significant amounts of the DMS oxidation product DMSO can be found. DMSO formation should be avoided to minimize the risk of DMS formation during fermentation.

Effect of Molecular Oxygen, Fe(II), Cu(II), and Cys on DMS Oxidation. In a first approach, varying compositions of the mentioned substances were incubated with DMS in air-saturated model solutions for 180 min at 95 °C in hermetically closed systems. As shown in Figure 1, the exclusive incubation

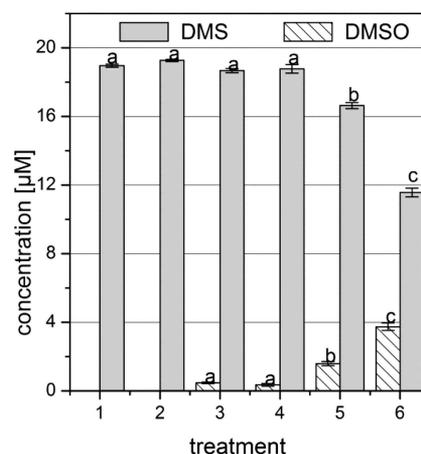


Figure 1. Effect of 18 μM Fe(II), 18 μM Cu(II), and 250 μM Cys on DMS oxidation in air-saturated model solutions at 95 °C. Control (model solution only containing DMS) after reaching of 95 °C, 0 min (1); the following compositions were incubated for 180 min: control (2); Fe(II) (3); Cu(II) (4); Fe(II)+Cys (5); Cu(II)+Cys (6). Results are presented as mean values ($n = 3$) \pm standard deviation. Letters indicate classes of significant different groups according to Tukey-Kramer's HSD test.

of DMS in air-saturated model solution for 180 min (column 2) did not change compared to the amount of DMS at the beginning of incubation (column 1). DMSO formation was not observed, either. The addition of Fe(II) (column 3) or Cu(II) (column 4) led to a slight DMS decline as well as significant DMSO formation compared to the control (column 2). Though, the oxidative consumption of DMS was below 2% and no significant differences between the transition metal ions used were found. This result indicates that molecular oxygen was not involved in the oxidation of DMS. An interpretation of this result might be more comprehensible when considering the respective redox potentials. For example, the standard redox potential difference between Fe(II)/Fe(III) and O_2/O_2^- is merely 0.1 V, which contradicts the contribution of such reactions to elevated H_2O_2 formation and successive DMS oxidation. Transferable deductions were recently drawn by Boatright³⁴ in relation to ascorbic acid autoxidation.

A major effect on DMS oxidation and DMSO formation was observed when Cys was added in combination with the respective transition metal ions, though (column 4 and 5). Significant differences in oxidative DMS-consumption (Fe(II): ~13%, Cu(II): ~40%) and DMSO formation (Fe(II): ~8.3%, Cu(II): ~20%) were found. These data suggest a redox cycling process of the transition metal ions by Cys acting as reducing agent. In molar excess of Cys, the lower valence state of the transition metal ions, i.e., Fe(II) or Cu(I), will therefore be maintained, in which electron donation to oxygen and formation of reactive oxygen species takes place. It is known that Cys exhibits a higher affinity to Cu compared to Fe ions.³⁹ The formed complexes, in which Cu is covalently bound to the sulfur atom of Cys as a result of d-d orbital interactions reveal a relatively high stability (stability constant $K = 19.2$).⁴⁰ Consequently, the transfer of electrons during the autoxidation

of Cys–Cu complexes is favored compared to the rather instable Cys–Fe complex ($K = 6.2$).³⁹ In further experiments the focus was set on the effect and interaction between Cys and Cu(II).

It should be noted that it took 20 min to reach the target temperature of 95 °C in the reaction bottles which may have affected the outcome of the trial. In the malting and brewing process, DMS is formed via hydrolysis of SMM at temperatures mainly above 70 °C. Also, proteolysis of malt proteins and peptides starts essentially above 50 °C. To investigate the effects of Cys and Cu(II) on DMS oxidation at malting-related temperatures, it was necessary to minimize their reactivity during heating from 1 to 95 °C. Therefore, Cu(II) was chelated with EDTA in a stoichiometric ratio of 1:1 (Cu(II)EDTA) before spiking to test, if Cys–Cu complex formation can be postponed through transformation of Cu(II) from EDTA to Cys.

To test interactions of Cu(II)EDTA with Cys during heating of the model solutions, spectrophotometric measurements were applied to monitor complex formation at 20 °C as well as 95 °C under nitrogen (Figure 2). The combination of Cys and Cu(II)

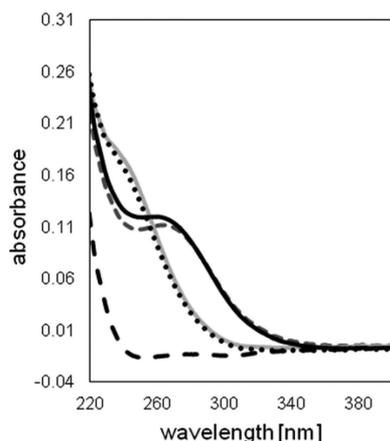


Figure 2. Absorption spectra of Cu complexes in nitrogen-purged model solution after 10 min of spiking at 20 or 95 °C. Reference model solution at 95 °C (black, dashed); 50 μM Cu(II)EDTA at 95 °C (dark gray, dashed); 50 μM Cu(II)EDTA and 100 μM Cys at 20 °C (black); 50 μM Cu(II)EDTA and 100 μM Cys at 95 °C (black, dotted). 100 μM Cys and 50 μM Cu(II) at 20 °C (gray).

at 20 °C led to a distinctive absorption pattern between 230 and 280 nm (gray line). Such complexes, with the general structure Cys–Cu(I)–Cys have been reported before.^{25,36,38} However, in our applied concentration ranges, we did not observe a specific absorption maximum of ~ 260 nm as described in the literature.^{25,38} The absence of a specific absorption pattern was also reported before.^{25,33} When Cu(II) was chelated with EDTA before Cys was introduced, only a slight tendency into the direction of the Cys–Cu signal (230–280 nm) from the absorption pattern of Cu(II)EDTA could be observed after 10 min (black line). However, at 95 °C, the Cu(II)EDTA spectrum was completely transformed into the Cys–Cu spectrum (black, dotted line).

We believe that this observation shows the exchange of Cu(II) from EDTA to Cys, which delays the reaction between Cys and Cu(II) to start at higher temperatures. For this reason, initial chelation of Cu(II) with EDTA (1:1) was further applied in successive experiments. The results in Figure 3 show that the combination of transition metal ions with EDTA had a similar

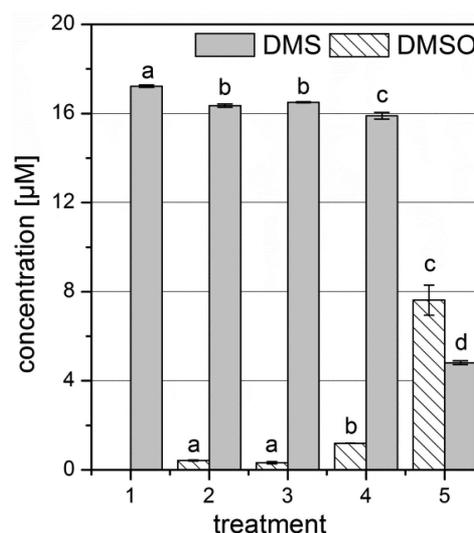


Figure 3. Effect of 18 μM Fe(II)EDTA, 18 μM Cu(II)EDTA, and 250 μM Cys on DMS oxidation in air-saturated model solutions at 95 °C for 180 min. Control (1); Fe(II)EDTA (2); Cu(II)EDTA (3); Fe(II)EDTA + Cys (4); Cu(II)EDTA + Cys (5). Results are presented as mean values ($n = 3$) \pm standard deviation. Letters indicate classes of significant different groups according to Tukey-Kramer's HSD test.

effect on DMS oxidation compared to the results, where solely Fe(II) and Cu(II) were tested. However, much higher DMS oxidation and accompanying DMSO formation was found, especially when Cu(II)EDTA was combined with Cys (DMS oxidation: Fe(II)EDTA: $\sim 7.7\%$, Cu(II)EDTA: $\sim 72\%$; DMSO formation: Fe(II)EDTA: $\sim 7\%$, Cu(II)EDTA: 44%). It is obvious from the results that the sum of DMS and DMSO after incubation is lower compared to the initial DMS concentration, especially in the case of Cu(II)–Cys combinations and that formation of further oxidation products may have occurred.

Response Surface Modeling (RSM). Cys is known to reduce DMSO and react with H_2O_2 as well as hydroxyl radicals, which represents its main antioxidative mode. To further clarify the effect of Cys and Cu(II) on DMS oxidation, RSM was applied in different concentration ranges of Cu(II)EDTA and Cys. Within the 13 selected combinations of Cu(II)EDTA and Cys, the mean experimental DMS decline varied from 16.1 to 4.9 μM (70%) and DMSO formation ranged between 0 and 8.7 μM (54% of total DMS) (Supporting Information, Table S1). As the molar sum between DMS consumption and DMSO formation was lowered as well, the results again indicate that further DMS oxidation products may have been generated. ANOVA was applied to estimate significant impact of Cu(II)EDTA and Cys as well as potential interactions. The ANOVA results (F - and p -values) of each model term are summarized in Table 2. Regarding DMS oxidation, a linear model represented the data best ($p < 0.0001$) and resulted in a high correlation coefficient ($R^2 = 0.9471$) and a good agreement with the adjusted R^2 ($R^2_{\text{adj}} = 0.9423$) as well as the predicted R^2 ($R^2_{\text{pred}} = 0.9142$). These data together with an absence of a significant lack of fit (LOF, $p_{\text{LOF}} = 0.5341$) point to the applicability of the model within the design space. For DMSO, an interaction model was chosen, which was shown to be reliable on the basis of the coefficients of determination and the LOF ($R^2 = 0.9538$, $R^2_{\text{adj}} = 0.9384$, $R^2_{\text{pred}} = 0.8607$; $p_{\text{LOF}} = 0.2311$).

Table 2. ANOVA Results of Model Terms (a) Linear Model Terms, (b) Interaction Model Terms, (c) Probability that *F*-value of this Size Is Determined if Model Terms Are Insignificant^d

factor	DMS ^a		DMSO ^b	
	<i>F</i> -value	<i>p</i> -value ^c	<i>F</i> -value	<i>p</i> -value ^c
Cys	197.12	<0.0001	172.24	<0.0001
Cu(II)EDTA	0.7952	0.3935	8.19	0.0187
Cys*Cu(II)EDTA			5.4973	0.0437

^dInsignificant model terms exceeding the confidence level of $\alpha=0.1$ were removed via backwards elimination.

For DMS oxidation, the effect of Cu was found to be insignificant and the amount of Cys determines the extent of DMS oxidation. Regarding DMSO, Cu(II)EDTA, Cys as well as their interaction were found to be significant model terms. However, the factor *F*-values (Table 2) suggest that Cys is the predominant factor in DMS oxidation and concomitant DMSO formation. Within the experimental setup, the oxidative consumption of DMS and the resulting DMSO formation can be predicted by the following models:

$$\text{DMS} = 14.99295 - 0.043752 * \text{L-Cys} \quad (1)$$

$$\text{DMSO} = 4.21 - 0.57 * \text{Cu(II)EDTA} + 2.60 * \text{Cys} - 0.66 * \text{Cu(II)EDTA} * \text{Cys} \quad (2)$$

When comparing the 3-dimensional plots of DMS oxidation and DMSO formation (Figure 4 and 5), the data of the

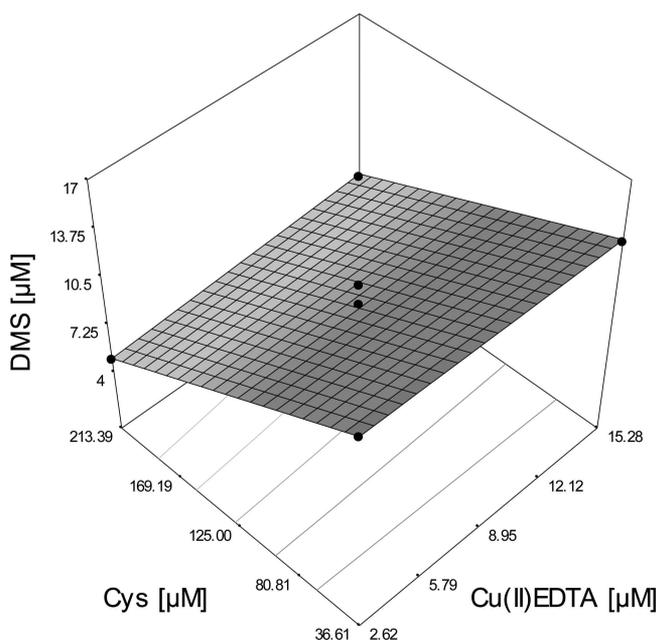


Figure 4. Response surface linear model of DMS oxidation.

ANOVA become more obvious. DMS is linearly consumed with increasing amounts of Cys, independent of the amount of Cu(II)EDTA. DMSO formation is mostly determined by Cys as well. However, as also observed in Figure 1 and Figure 3, when higher concentrations of Cu(II)EDTA and Cys were applied, a slight decline of DMSO can be observed. A possible explanation of this observation may be that DMSO was further oxidized to DMSO₂, which will be addressed later on.

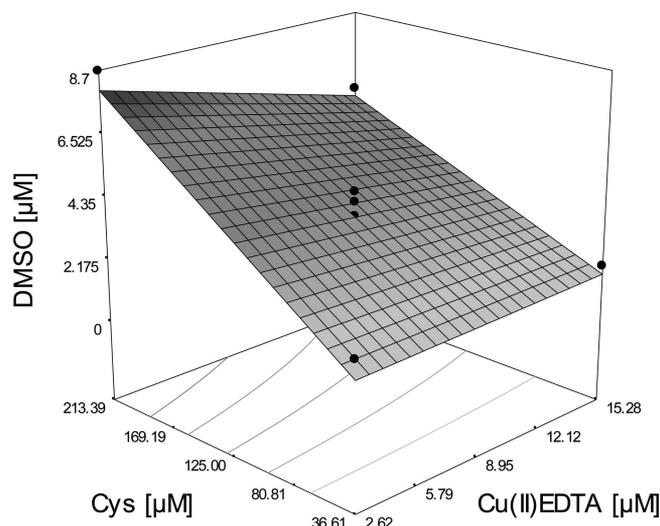


Figure 5. Response surface interaction model of DMSO formation.

Effect of EDTA and Cys on DMS Oxidation. Owing to the surprising insignificant effect of Cu(II)EDTA levels on DMS oxidation, questions regarding its contribution to DMS oxidation arose. Therefore, we tested Cys (13 μM) exclusively as well as combined with molar excess of EDTA (40 μM) on DMS oxidation and DMSO formation (Table 3). The data

Table 3. Effect of EDTA and Cys Concentration on DMS Oxidation and DMSO Formation^c

	DMS [μM]	DMSO [μM]
-	16.73 ^a ± 0.11	n.d.
Cys	15.32 ^b ± 0.15	0.72 ± 0.02
Cys + EDTA	16.80 ^a ± 0.07	n.d.

^cConcentrations were 13 μM Cys and 40 μM EDTA. Results are presented as mean values ($n = 3$) ± standard deviation. Letters indicate classes of significant different groups according to Tukey-Kramer's HSD test.

show that the exclusive addition of Cys resulted in low but significant DMS oxidation and accompanied DMSO formation. This prooxidative effect was eliminated by molar excess of EDTA. This indicates, that certain amounts of transition metal ions were already present in the model solution, which were then chelatively inactivated by the molar excess of EDTA.

To test the presence of transition metal ions in the model solution from other sources than the metal stock solutions, ICP-OES was applied for their quantitation. In the buffer solution, all tested transition metal ions were below the detection limit of 0.08 μM. However, the addition of 300 μM Cys led to an increase of Cu to 0.26 μM, whereas all other metal ions were still below the detection limit. On basis of these results, we propose that DMS oxidation in the presence of Cys is evoked by the circumstance that trace levels of Cu were already introduced to the model solution via impurities of the Cys product used, even though highest purity grade was applied. Such trace levels of Cu or other transition metal ions seem to be sufficient to evoke a prooxidative behavior of Cys as shown by DMS oxidation. DMS oxidation could only be eliminated when EDTA was added in relative molar excess to potentially abundant transition metal ions and Cys in the model solution (Table 3). In this case EDTA removed Cu from the redox system. The close proximity of the stability constants

indicates that the substance, which is present in relative molar excess to Cu, dominates its complexation.

Even though technically, the minus α -level of Cu(II)EDTA in the RSM was $\neq 0$, the trace amounts of copper, which were introduced by the Cys addition into the model solution did not significantly affect the chosen limits (Table 1) and the RSM was therefore regarded applicable to study the behavior of the reactants.

Reaction Mechanism of H₂O₂ Formation via Cys–Cu(I)–Cys Autoxidation. To further elucidate the pro-oxidative behavior of Cys–Cu interactions and to obtain insights into the reaction mechanism, levels of Cys and H₂O₂ as well as complex formation were recorded during heating and residence time of model solutions containing $\sim 228 \mu\text{M}$ Cys and $\sim 18 \mu\text{M}$ Cu(II)EDTA at 95 °C (Figure 6). After

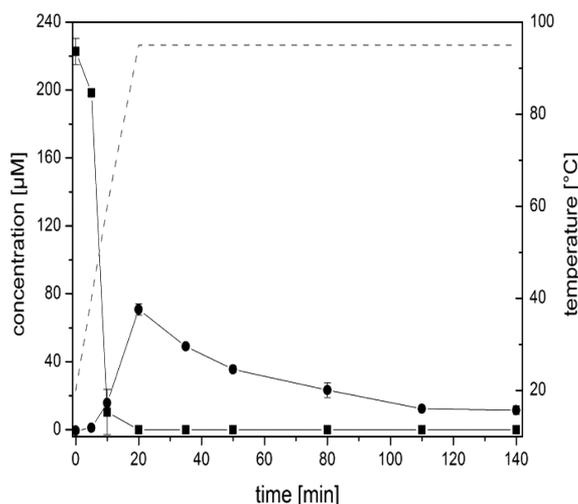


Figure 6. Cys consumption and H₂O₂ formation during incubation of 18 μM Cu(II)EDTA and 250 μM Cys. Cys (squares); H₂O₂ (circles); temperature (gray line, dashed). Results are presented as mean values ($n = 3$) \pm standard deviation.

combining Cys and Cu(II)EDTA, 220 μM of Cys were found. Between 40 and 60 °C, Cys consumption was quick and completed after reaching 95 °C. Simultaneously to the Cys consumption, the spectrophotometric data during heating of the model solution (Figure 7) show, that the Cu(II)EDTA

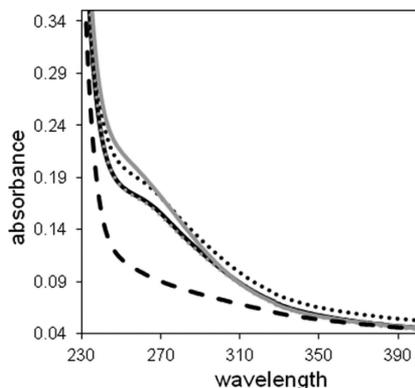


Figure 7. Absorption spectra of Cu complexes after combination of 18 μM Cu(II)EDTA with 250 μM Cys in model solution during heating. 20 °C (gray dotted); 40 °C (black); 60 °C (black, dotted); 95 °C (gray); model solution without addition (95 °C) (black, dashed).

spectrum was transferred into the signal of the Cys–Cu complex above 40 °C. This observation confirms the previously described findings (Figure 2). Also, H₂O₂ formation started after reaching of 40 °C, whereas highest levels of $\sim 71 \mu\text{M}$ were found, when reaching 95 °C, giving a stoichiometric ratio of Cys:H₂O₂ of 3.22:1. This ratio is in good agreement with the result of Pecci et al.²⁵ They found a ratio of Cys:oxygen consumption of 3.125:1. Also Kreitman et al.³³ reported the oxygen consumption found for the degradation of the thiol 6-sulfanyhexan-1-ol (6-SH) in the presence of 50 μM Cu(II) in oxygenated wine model to be 3.3:1. The molar ratios in each study, including the present, are similar, although the thiol:Cu ratio was ~ 5 in the former studies and ~ 13 in the present. This again strengthens the insignificance of the total amount of Cu on DMS oxidation as shown by RSM. Based on these results, we believe that the effect of Cys on DMS oxidation is based on H₂O₂ formation via autoxidation of Cys–Cu complexes with molecular oxygen. Complex formation between Cys and Cu is well documented in the literature.^{25,33,37,38}

A simplified reaction mechanism of the proposed effect of Cys and Cu(II) on DMS oxidation is illustrated in Figure 8.

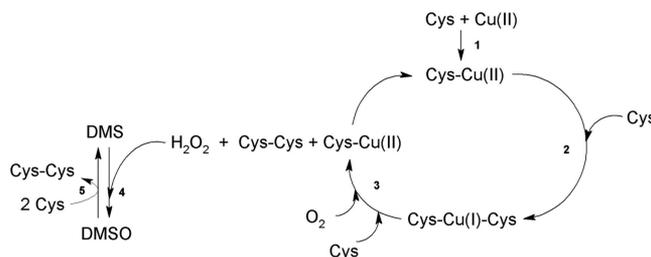


Figure 8. Proposed reaction mechanism of the effect of Cys and Cu(II) on DMS oxidation.

Initially, Cu(II) binds covalently to the sulfur atom of Cys leading to the Cys–Cu(II) complex (1). In molar excess of Cys, a bis-Cys complex (Cys–Cu(I)–Cys) is formed, in which Cu(II) is subsequently reduced (2).^{25,37,38} In the absence of oxygen, this complex possesses a relative high stability.^{25,38} In its presence, autoxidation proceeds through reaction with molecular oxygen and a further Cys to form cystine, Cys–Cu(II) and H₂O₂ (3).²⁵ We propose that H₂O₂ was then responsible for DMS oxidation (4). The autoxidation of Cys–Cu(I)–Cys involves a 2-electron reduction of molecular oxygen, initially originating from 2 Cys. Earlier studies by electron paramagnetic resonance (EPR) spectroscopy^{25,33} demonstrated that Cu remains as Cu(I) throughout Cys complexation if Cys is available. These observations are comparable with the finding of this study in which H₂O₂ reached its maximum after all Cys was consumed. In molar excess of Cys, which is also representative for the abundance of Cu and Cys in malting and brewing, it is therefore likely that Cu(II) is recycled via Cys complexation and H₂O₂ formation as long as available Cys is oxidized to cystine. Oxidation of Cys in the presence of Cu(II) was shown to be relatively fast.²⁵ Cys (100 μM) was degraded within ~ 10 min at room temperature. The high velocity of Cys oxidation was also observed in this study (Figure 6). Cys was almost completely degraded in 5 min during heating from 40 to 60 °C. The faster reaction rate is most likely related to the temperature-determined higher rate constants of Cys oxidation and H₂O₂ formation.

Involvement of Radical Species during Autoxidation of Cys–Copper Complexes. In the presence of transition

metal ions, like Cu(I) or Fe(II), H₂O₂ undergoes the Fenton reaction producing hydroxyl radicals. In our study, H₂O₂ exponentially declined after reaching its maximum at 95 °C. Therefore, the question arose if radicals were involved in the oxidation of DMS. It was reported that thiyl radical intermediates (R-S·) may be formed during Cu complexation by thiols.²⁵ However, Davis et al.³⁷ did not find evidence of free thiyl radicals. Also Kreitman et al.³³ showed that autoxidation of the thiol 6-sulfanyhexan-1-ol (6-SH) was unaffected by the addition of 5,5-dimethyl-1,1-pyrroline *N*-oxide (DMPO), a specific spin trapping agent for hydroxyl radicals and thiyl radicals. They also stated that 6-SH oxidation was unaffected by the addition of 4-methyl catechol, a specific thiyl radical scavenger.

Kachur et al.⁴⁰ reported formation of hydroxyl radicals in the copper-catalyzed oxidation of Cys. Hydroxyl radicals, react with Cys to form Cys sulfinic acid, cysteic acid, and Cys sulfonic acid. Though, data on such hydroxyl radical mediated reaction products of Cys oxidation are not available from the latter study.⁴⁰ In contrast, Pecci et al.²⁵ found that >95% of oxidized Cys was recovered as its disulfide cystine and only ~2% were found as Cys sulfinic acid and cysteic acid. Analogous observations were made for the Cu catalyzed oxidation of 6-SH, which was completely recovered as 6-SH disulfide.³³ Gilbert et al.⁴¹ showed that reoxidation of Cys–Cu(I)–Cys complexes by H₂O₂ can take place via radical and nonradical mechanisms. They stated that, if Cu(I) is highly aggregated by Cys, its oxidation by H₂O₂ would proceed without the release of hydroxyl radicals. At high ratios of Cys:Cu, “Fenton-like” reactions and hydroxyl radical formation may occur. Yet, only very weak EPR signals in the range of trace quantities of hydroxyl radicals were detected.⁴¹

When H₂O₂ degradation started in our experiments (Figure 6), according to the postulated mechanism (Figure 8), most Cu should be present as Cu (II). Therefore, reactions of H₂O₂ with Cys–Cu(II) are more likely to occur and should be considered (Figure 9). Under such conditions, thiolate Cu(II) peroxide

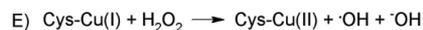
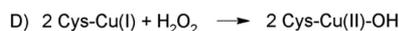
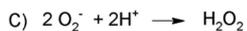


Figure 9. Proposed mechanisms of the re-oxidation of Cys–Cu(II) complexes by H₂O₂ (description in the text).

formation may occur (A). Cu(II) peroxides decompose slowly in aqueous solution leading to superoxide radical formation (B).⁴² In the investigation of the reoxidation of Cys–Cu(I)–Cys with H₂O₂, Gilbert et al.⁴¹ used DMPO as spin trapping agent for the detection of potentially formed hydroxyl radicals by EPR spectroscopy. Superoxide radicals, generated as proposed (B), react very slowly with DMPO under formation of instable spin adducts.⁴³ Consequently, DMPO is not used for the assessment of superoxide radicals by EPR spectroscopy. However, the weak EPR signals as found by Gilbert et al.⁴¹ may be related to DMPO-superoxide radical interactions, even though no hydroxyl radicals were present. The pK_a of the superoxide radical is 4.75, disproportionation to H₂O₂ (C) is

therefore unlikely to have occurred in our study (pH 5.5). Though, disproportionation can be expected to take place in wine and beer (pH < 4.5). The formed Cys–Cu(I) may then undergo nonradical reoxidation by H₂O₂ (D) as described by Davis et al.,³⁷ although a “Fenton-like” mechanism (E) and formation of hydroxyl radicals cannot be ruled out. We therefore propose that H₂O₂ was the primary source of DMS oxidation in this work.

Investigations from the field of environmental chemistry showed earlier that different species of peroxides represent sufficient oxidation ability of DMS in aqueous solution.^{44,45} In proximity of the stoichiometric required amount of H₂O₂ for DMS oxidation, the reaction follows a second order mechanism with DMSO as main product. Rate constants vary between 1.4 and 8.1 × 10⁻² M⁻¹ s⁻¹ (22–25 °C).⁴⁴ Under similar conditions, DMSO reacts much slower with H₂O₂ than DMS (0.5–4.5 × 10⁻⁵ M⁻¹ s⁻¹),⁴⁵ and DMSO was therefore suggested as main oxidation product.

However, these reactions have not been investigated at temperatures above 30 °C and at stoichiometric higher concentrations of H₂O₂ over DMS. At high Cu and Cys concentrations, we found H₂O₂ levels of ~71 μM, which represents a molar H₂O₂:DMS ratio of ~4. At these concentrations and the applied temperature of 95 °C, oxidation of DMSO to DMSO₂ may have been more apparent. However, DMSO₂ data are not available from our studies, therefore precise conclusions cannot be drawn at this point. Even though we believe H₂O₂ was the main oxidant in this work, it is noteworthy to mention that DMS would probably be oxidized to DMSO much faster when hydroxyl radicals were present ($K = 1.9 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ (22–25 °C)).⁴⁶ Further oxidation of DMSO with hydroxyl radicals would have produced methane sulfinic acid at slightly lower rate constants ($6 \pm 1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$).^{47–49}

The findings of this work can be transferred to the malting and brewing process as the abundance of peroxides, such as H₂O₂ or fatty acid hydroperoxides, in malt and beer production is well-known.²²

As mentioned before, no crossover to antioxidative properties of Cys, as for DMSO reduction and DMS increase was observed. Therefore, DMSO reduction by Cys was investigated to elucidate the scope of this reaction.

DMSO Reduction by Cys. In order to assess the antioxidant function by means of sulfoxide reduction (Figure 8, reaction 5), DMSO was incubated with Cys for 24 h at 95 °C in deoxygenated model solutions, before DMSO and DMS were analyzed. After 24 h, DMSO was reduced from 13 ± 0.02 μM to 11.8 ± 0.54 μM and 1.47 ± 0.11 μM DMS were formed, which shows the capability of Cys to reduce DMSO to DMS. However, within 24 h, the overall DMSO reduction was below 10% indicating that the reaction rate is low as compared to H₂O₂ production by Cys–Cu(I)–Cys autoxidation, in which for instance ~70% of DMS was oxidized within 3 h. Yet, there are no rate constants available, either for DMSO reduction by Cys as well as for the Cys–Cu(I)–Cys formation and autoxidation. Though, it is obvious that the prooxidative activity of Cys was predominant in this study and that sulfoxide reduction^{14,31,32} seems to play a subordinated role, probably due to much lower reaction rates. Even if DMS oxidation dominated in this work, H₂O₂ or hydroxyl radical scavenging by Cys may have also occurred. As for H₂O₂ scavenging, this mechanisms’ antioxidative contribution can be regarded rather marginal because of its low rate constants at room temperature

($10^{-2} \text{ M}^{-1} \text{ s}^{-1}$, pH 5). Also, Cys was not present anymore, when H_2O_2 reached its maximum. It was shown by Yang et al.¹⁰ that DMSO formation correlated with proteolytic modification (e.g., at high germination temperatures) of the malt which is substantiated commonly by the accompanied higher formation and release of SMM. Isolated correlations of proteolysis on both DMS precursors SMM and DMSO have not been drawn so far. However, the results of this study imply that elevated release of Cys during proteolytic modification may drive H_2O_2 formation in malting and brewing.

In this work, the reactions were followed in model solutions to minimize reactions with other reactants for the characterization of the reaction mechanism. As for every other complex food matrix, such reactions always require biochemical availability of reactants and high affinity of the respective reactants to react at remarkable reaction rates. In the malting process, the reactions described in this work would undoubtedly be affected by the multitude of competing reactions involving Cys, such as hydroxyl radical scavenging, Maillard reaction or complex formation with carbonyls, as reported by Baert et al.⁵⁰ In addition, especially during the withering process ($\sim 50\text{--}60 \text{ }^\circ\text{C}$), high enzymatic activities and synthetic pathways require thiols for their maintenance. Also, copper is required as a cofactor for certain enzymes. To a certain extent, the effect of the thiol oxidation-mediated H_2O_2 formation may be diminished by oxygen scavenging enzymes, such as peroxidase or catalase. At higher temperatures, where enzyme activity is limited, Cys–Cu(I)–Cys autoxidation may have a higher effect.

This work was able to demonstrate the high affinity of Cys for Cu(II) and the ability of substantial H_2O_2 formation at temperatures between 40 and 95 $^\circ\text{C}$. The concentration ranges of the reactants which were applied in this study are representative for the malting and brewing process. Also, the availability of oxygen throughout most parts of the malting and brewing process points to the potential relevance of the aforementioned reactions. With respect to the brewing process, Bamforth et al.²² highlighted that significant oxygen consumption by malt ingredients takes place during the brewing process. Muller²⁹ showed that oxidation of thiol-rich proteins can lead to H_2O_2 formation during mashing which is in good accordance with our results. Observations and deductions from this study may have transferred to other foodstuffs, e.g., in the production and processing of tomato paste, where DMS is one of the most important flavor compounds.¹ The oxidation of DMS in such products would be accompanied by aroma-deterioration.

The outcome of this work should serve as fundamental knowledge for kinetic investigations of the Cys–Cu(I)–Cys formation and autoxidation as well as interactions with other transition metal ions in malting and brewing. Also, the reactivity of further thiols (e.g., glutathione) or thiol-rich peptides (e.g., lipid transfer protein 1) and the formation and autoxidation of mixed thiol-metal complexes is very likely and needs to be studied. On the other hand the assessment of reaction kinetics of DMS oxidation by H_2O_2 as well as hydroxyl radicals combined with pH and temperature dependencies are necessary to evaluate their role in DMSO formation in foodstuffs.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b05472.

The experimental data of the RSM of the effect of Cys and Cu(II)EDTA on DMS oxidation and DMSO formation (PDF)

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Notes

The authors declare no competing financial interest.

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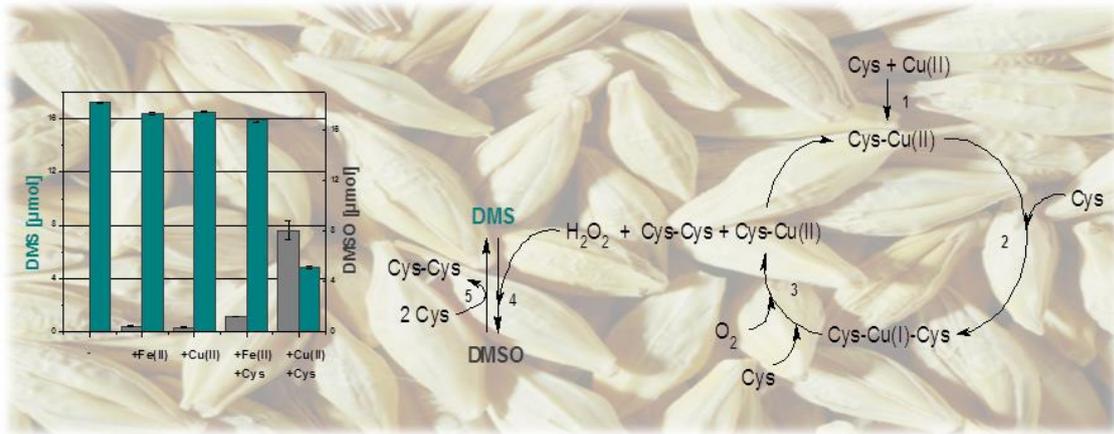
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Supporting information – Publication D

Table S1: 2-factorial experimental design for RSM of the effect of Cys and Cu(II)EDTA on DMS oxidation and DMSO formation. Results are presented as mean values (n=3), center point trials (0-0 combinations) are shown as single determinations (n=5).

trial _{rand}	trial	Cu(II)EDTA [μ M]	Cys [μ M]	DMSO [μ M]	DMS [μ M]
4	1	15.28 (+1)	213.39 (+1)	5.85	6.03
1	2	2.62 (-1)	36.61 (-1)	2.18	13.47
9	3	8.95 (0)	125.00 (0)	3.62	8.65
6	4	17.91 (+ α)	125.00 (0)	3.88	9.80
11	5	8.95 (0)	125.00 (0)	3.62	8.65
7	6	8.95 (0)	0.00 (- α)	0.00	16.08
10	7	8.95 (0)	125.00 (0)	3.73	8.81
3	8	2.62 (-1)	213.39 (+1)	8.67	4.87
12	9	8.95 (0)	125.00 (0)	4.23	10.13
8	10	8.95 (0)	250.00 (+ α)	7.38	5.23
5	11	0.00 (- α)	125.00 (0)	4.95	8.92
2	12	15.28 (+1)	36.61 (-1)	1.98	13.03
13	13	8.95 (0)	125.00 (0)	4.59	10.14

Table of contents graphic – Publication D



Publication E:

In the next publication a bigger variety of potential antioxidants (Cys, GSH, Asco, GA and sulfite) that can also be found in malt were tested on their effect on DMS oxidation in the presence of Cu^{2+} . In comparison to the previous study, also higher concentrations were applied to potentially overcome prooxidative effects and to assess the applicability of antioxidants as adjuvants for minimization of DMSO in malt. Moreover the antioxidants were added to an *in vivo* malting experiment to test the relevance of their effects on DMSO formation in malt.

The complete procedure, results and the discussion of the study were submitted to the *Journal of Agricultural and Food Chemistry*, in a paper entitled “*Effect of Malt-Derived Potential Antioxidants on Dimethyl Sulfide Oxidation*” (Publication E²⁰⁶), which is currently under revision.

In the study it was demonstrated that all substances tested had a prooxidative effect on DMS oxidation and DMSO formation. H_2O_2 was found as primary DMS oxidant for Cys, GSH, Asco and GA whereas it was evidently shown, that the much bigger effect of sulfite was evoked the formation of bisulfite radicals. The latter were proposed of forming peroxymonosulfate radicals that are capable of exhaustive DMS oxidation (~100%) over a wide concentration range. The prooxidative effects were confirmed in the malting experiments, whereas those were lower as compared to experiments in buffered model solutions. Potential reaction mechanisms and their contribution to the final levels on DMSO in malt were discussed.

Publication E

Effect of Malt-Derived Potential Antioxidants on Dimethyl Sulfide Oxidation

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Supporting Information

ABSTRACT: During malt kilning, dimethyl sulfide (DMS) is partly oxidized to dimethyl sulfoxide (DMSO), which can be reduced by yeast to generate DMS during fermentation. The aim of this study was to test the effect of malt-derived potential antioxidants on DMS oxidation and to assess their applicability for DMSO minimization. In the presence of 18 μM copper, all tested antioxidants (250 μM) catalyzed DMS oxidation to deviating extents (sulfite > ascorbic acid (Asco) > gallic acid (GA) > L-cysteine (Cys) > L-glutathione (GSH)). Hydrogen peroxide was found as primary DMS oxidant for each substance except for sulfite. Electron spin resonance spectroscopy provided evidence for the formation of bisulfite radicals and peroxymonosulfate radicals, which are proposed as being capable of exhaustive DMS oxidation ($\sim 100\%$) over a wide concentration. The data demonstrate that use of antioxidants per se cannot be suggested for the minimization of DMSO formation in malt and other foodstuffs. Potential shifts from pro- to antioxidative behavior of antioxidants and their implications on malt quality are discussed.

KEYWORDS: dimethyl sulfide oxidation, sulfite radical, hydrogen peroxide, thiols, gallic acid

INTRODUCTION

Dimethyl sulfide (DMS) is the smallest volatile thioether present in food. In beer DMS concentrations above its flavor threshold of $\sim 30\text{--}100\ \mu\text{g/L}$ ^{1,2} can evoke characteristic off-flavor impressions, often ascribed as “boiled-vegetable”-like. The primary precursor of DMS, S-methyl methionine (SMM) is synthesized during germination in diverse flowering plants, including brewing cereals, like barley.³

SMM is thermally unstable and thus is hydrolyzed to DMS and L-homoserine upon heating. In dependence of the malting- and kilning technology, substantial amounts of the generated DMS are oxidized to form DMSO.^{4,5} In comparison with other foodstuffs, DMSO is of certain importance to beer flavor as yeast is capable of DMSO reduction and accompanied DMS formation via methionine sulfoxide reductase systems during fermentation.² At this process step, surplus formation of DMS cannot be compensated by commonly applied evaporation techniques. Consequently, the levels of DMS formed during fermentation can increase the levels of DMS in beer. Therefore, the levels of DMSO in malt can crucially alter beer flavor.

The role and behavior of SMM as thermal precursor has been extensively investigated. Undoubtedly, DMSO originates from the oxidation of DMS. However, there is a substantial lack of knowledge regarding mechanisms of DMS oxidation and DMSO formation in malt.

Recently, it was reported that significant amounts of DMSO were formed via DMS oxidation by the reactive oxygen species (ROS) hydrogen peroxide and hydroxyl radicals.⁶

Antioxidants can generally be considered for the inactivation of ROS. Germinating brewing cereals, such as barley or wheat, possess a complex enzymatic antioxidative defense system,

consisting of superoxide dismutase, catalase, or ascorbate peroxidase.^{7,8} The combined action of such enzymes inactivate ROS and thereby protecting plant constituents, in particular DNA, lipids, and proteins from oxidative deterioration and accompanied loss of functionality.

Additionally, naturally abundant- or exogenously supplemented antioxidants, like thiols, polyphenols, reductones, or sulfite may potentially inactivate ROS via electron donation and/or acceptance. On the contrary, antioxidants are also capable of donating electrons to transition-metal ions, which can be considered as their prooxidative character. Thereby, the lower valence state of the transition-metal ions, such as Fe^{2+} or Cu^+ , is maintained, and consequently, oxygen activation and ROS formation is accelerated.^{9,10}

Recently, it was reported that the thiol Cys catalyzed DMS oxidation in the presence of transition-metal ions.¹¹ Besides thiols, malt consists of a plethora of further potential antioxidants that can potentially affect DMS oxidation. The aim of this study was therefore to investigate the behavior of selected malt-derived antioxidants on DMS oxidation and to further evaluate their applicability as additives for the minimization of DMSO in malt.

Besides Cys as investigated in our previous study, we tested GSH as a further representative thiol present in malt. The increase of phenolic compounds was reported to increase antioxidant malting was reported to increase antioxidant activity of malt.¹² Therefore, gallic acid was chosen as

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representative for water-soluble, galloyl-, or catechol-bearing phenolic compounds. Ascorbic acid was chosen on the basis of its abundance in malt⁷ as well as its well-characterized pro- and antioxidative properties in food. In particular, sulfite was aimed to be investigated on the basis of its capability of DMSO reduction¹³ and its high reactivity with hydrogen peroxide,¹⁴ which might oxidize DMS during malt kilning.⁶ Both mentioned properties suggest antioxidative functionality of sulfite regarding DMS oxidation. During malt kilning, sulfite is added in order to prevent the formation of nitrosamines,¹⁵ whereas prooxidative behavior was not reported before.

In this study, it was aimed to initially test the effect of the chosen antioxidants Asco, Cys, GSH, GA, and sulfite on DMS oxidation in the presence of Cu²⁺, in buffered model solutions to exclude side reactions and to elucidate potential mechanisms of anti- or prooxidative reactions. To also include potential side reactions including metabolic-biochemical as well as physical-chemical factors, an *in vivo* malting experiment should elucidate the effect and relevance of the addition of antioxidants and Cu²⁺ on the final levels of DMSO in malt. Outcomes of this work should serve for approaches of minimizing the levels of DMSO to decrease the risk of DMS formation during fermentation. Moreover, this work should further provide information about pro- and antioxidative effects of the applied substances in relation to thermal food processing in general.

MATERIAL AND METHODS

Chemicals. Dimethyl sulfide (DMS; $\geq 99\%$), iron(II) sulfate heptahydrate (Fe²⁺; $> 99\%$), iron(III) chloride hexahydrate (Fe³⁺, 98–102%), acetic acid ($\geq 99\%$), gallic acid monohydrate (GA; $> 99\%$), hydrochloric acid (HCl; 37%), and 5,5-dimethyl-1-pyrrolone N-oxide (DMPO; $> 97\%$) were purchased from Sigma-Aldrich (St. Louis, USA). Sodium acetate anhydrous ($\geq 99\%$) and horseradish peroxidase (HRP; grade I) were obtained from AppliChem (Darmstadt, Germany). Hydrogen peroxide (H₂O₂; 30%), L-cysteine (Cys; $> 99\%$), L-glutathione (GSH; $\geq 98\%$), L(+)-ascorbic acid (Asco; $\geq 99\%$), Leuco crystal violet (LCV, $\geq 98\%$), sodium chloride (NaCl; $> 99.8\%$) and copper(II) sulfate pentahydrate (Cu²⁺; $\geq 99\%$) were purchased from Carl Roth GmbH & Co KG (Karlsruhe, Germany). Nitrogen (99.999%) and synthetic air (20.5 \pm 0.5% O₂ in N₂) were obtained from Air Liquide GmbH (Berlin, Germany). Ethanol ($> 99.5\%$), ethylenedinitrotetraacetic acid disodium salt dihydrate (EDTA; 99–101%), sodium sulfite (sulfite; 97–10.5%), Disodium phosphate ($\geq 99\%$) and sodium dihydrogen phosphate (99.0–102%) were acquired from Merck Millipore (Darmstadt, Germany).

Incubation of DMS with Different Compositions of Antioxidant and Cu²⁺ in Model Solutions. Incubation of DMS combined with Cu²⁺ and antioxidants (Asco, Cys, GSH, GA, and sulfite) was conducted in 0.1 M sodium acetate/acetic acid buffer solution (pH 5.5) (model solution). All aqueous solutions were prepared with double-distilled, ultrapure water (Milli-Q). The final model solutions were purged with synthetic air for 20 min at 20 °C to reach oxygen concentrations of 8 mg/L as measured optically by a Visiferm DO 120 oxygen sensor (Hamilton GmbH, Planegg, Germany). 62 g of the model solution were filled in 50 mL Duran bottles (Schott AG, Mainz, Germany), whereby the gaseous headspace of the bottle was minimized in order to avoid extensive DMS diffusion out of the solution. The bottles were tightly closed with screw caps containing PTFE coated silicone seals and covered with aluminum foil to avoid UV-light exposure. After cooling to 3 °C specific combinations of aliquots of Cu²⁺ (9- or 18 μ M) and aqueous stock solutions of antioxidants were spiked into the bottles to reach the desired concentration as described in the separate trials. All spiking solutions were prepared volumetrically shortly before spiking at 20 °C.

In the last step, an ice-cold DMS–ethanol mixture was spiked to achieve a DMS concentration of 14 μ M. The DMS stock solution was kept in a tightly closed vial on ice to avoid volatilization. Immediately after spiking, the bottles were tightly closed and placed in a water bath, which was heated to 80 °C. The temperature was monitored using a HI935002 Dual Channel K-Type Thermocouple Thermometer (Hanna Instruments, Voehringen, Germany). After the solutions reached 80 °C, the samples were incubated for 0–180 min. At the end of incubation, the bottles were cooled stepwise with tap water and ice-cold water prior to analyzing DMS and DMSO.

Kinetics of DMS Oxidation in the Presence of 250 μ M of Sulfite and 18 μ M Cu²⁺ and Fe³⁺. DMS oxidation in model solution was monitored over time in the presence of 250 μ M sulfite and whether 9- or 18 μ M Cu²⁺ and 18 μ M Fe³⁺. Incubation was carried out as described before, whereas sulfite, Cu²⁺, Fe³⁺, and DMS were spiked at 20 °C, where the first sample was taken (0 min). The bottles were placed into the water bath (80 °C). Further samples were taken after 0.5 (40 °C), 1.5 (60 °C), 10 (80 °C), 25, 40, and 70 min. All samples were cooled to 1 °C before the analyses of DMS and DMSO.

Impact of Sulfite Concentration on DMS Oxidation in the Presence of 9 μ M of Cu²⁺. DMS oxidation and DMSO formation was assessed in model solution after the incubation of different sulfite concentrations (0–100 mM) in the presence of 9 μ M Cu²⁺ and ~14 μ M DMS. Sample preparation was conducted as described above.

Electron Spin (ESR) Resonance Spectroscopy. To test the presence of radical species potentially involved in DMS oxidation in the presence of sulfite and transition-metal ions ESR spectroscopy was applied. Therefore, the model solution with or without EDTA (0.18 mM) was spiked with different compositions of sulfite (500 μ M), Cu²⁺ (18 μ M) or Fe³⁺ (18 μ M), after DMPO addition (5-, 50- or 100 mM). To test the effect of sulfite ionization on potential radical formation the experiments were also conducted in a 0.1 M sodium phosphate buffer solution at pH 7. Initially, all model solutions were purged with N₂ for 15 min to reduce the dissolved oxygen concentration to $< 14 \mu$ M. Two minutes after spiking the ESR spectra were recorded using a Bruker X-band ESP 300E ESR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany). The parameters were as follows: Sweep with, 80000 G; Static Field, 3472200; microwave frequency, 9.786; attenuator, 0; microwave power, 8.92 mW; Receiver gain, 3.99×10^3 ; modulation frequency, 86 kHz; modulation Amplitude, 1.04 G; time constant, 40.96 ms; conversion time, 10.24 ms; sweep time, 5.24 ms, number of scans, 10 or 50. Spectra were simulated using the WinSim (NIEHS/NIH) software package based on algorithms developed by Duling.¹⁶

Impact of Antioxidants and Cu²⁺ on DMS Oxidation during Malting. For germination 18 g of barley variety Quench were weighed into 50 mL Falcon tubes perforated with 5 wholes (diameter 2 mm) at the cone for CO₂ removal. The tubes were placed in a stainless steel box (15 \times 60 \times 12 cm) which was used for steeping and germination. The box contained 6 air inlets at the top and a conical water/air/CO₂ outlet. The first wet steep was carried out in aerated 0.9% (w/v) saline solution at 16 °C for 4 h followed by an air rest of 20 h and a second wet steep of 2 h. On the first day of germination, the antioxidants and/or Cu²⁺ were supplemented to the steeping media required to reach a final steeping degree of 45%. The aimed concentrations were 500 μ M of antioxidant and 18 μ M Cu²⁺ (both in μ mol/kg barley dry matter). The upfollowing germination process was carried out for 5 days at 18 °C and a steeping degree of 45%, which was adjusted daily until germination day 3 by spraying the desired amount of saline solution onto the kernels. The progress of germination was assessed by analyzing the relative acrospire lengths according to MEBAK guidelines. For withering the green malt was transferred into aluminum bowls, which were incubated for 11 h at 60 °C in an incubator. Kilning was then carried out 85 °C for 3.5 h. After kilning, the rootlets were removed. The final malt was milled using a disc mill (DLFU, Bühler GmbH, Braunschweig, Germany) at a disc gap of 0.2 mm. Four grams of the malt grist were weighed into 50 mL Falcon tubes. Subsequently, 40 g of double-distilled ultrapure water were added to the tubes. The tubes were tightly closed and shaken at

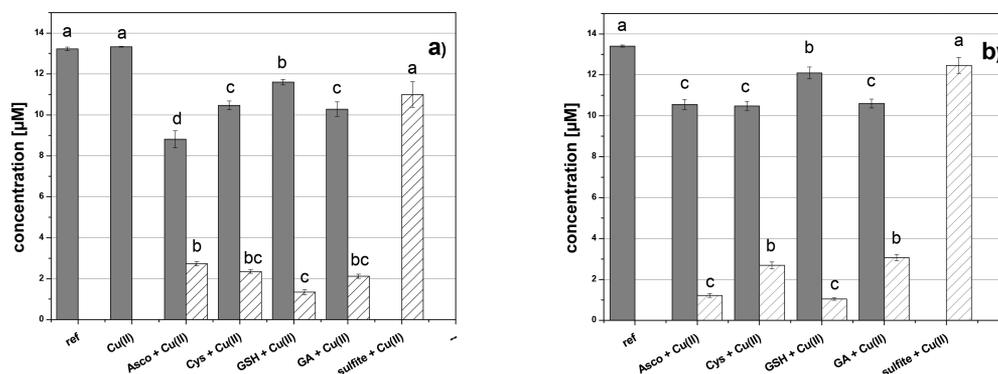


Figure 1. Effect of antioxidants on DMS oxidation in the presence of 18 μM Cu(II) in air-saturated model solutions at 80 $^{\circ}\text{C}$ for 180 min. DMS concentration (gray bars); DMSO concentration (white, dashed bars) in μM ; reference (model solution only containing DMS); (a) 250 μM antioxidant; (b) 500 μM antioxidant. Results are presented as mean values of triplicate trials ($n = 3$) \pm standard deviation (SD). Different letters indicate significant differences according to the Fisher (LSD) test.

175 rpm at room temperature for 20 min. The samples were cooled down and centrifuged at 1 $^{\circ}\text{C}$ at 7340g for 10 min. The supernatant was analyzed for DMS, SMM, and DMSO.

Determination of SMM, DMS, and DMSO. DMS and DMSO were analyzed according to a previously described method,¹⁴ which involves the reduction of DMSO by sodium metabisulfite. The generated DMS is subsequently analyzed by headspace gas chromatography and pulsed flame photometric detection (HS-GC-PFPD). SMM was determined according to the method of White and Wainwright,¹⁷ which includes a heat-alkaline treatment for SMM hydrolysis. The generated DMS was determined by HS-GC-PFPD as described for DMS and DMSO. The concentration of SMM, DMS and DMSO in malt is presented in μM ($\mu\text{mol}/\text{kg}$ malt).

Determination of H_2O_2 . Model solution containing 18 μM Cu^{2+} and whether 250- or 500 μM of Asco, Cys, GSH, GA, or sulfite was heated to 80 $^{\circ}\text{C}$ within 10 min. Afterward the samples were cooled to 20 $^{\circ}\text{C}$ and H_2O_2 was determined as described previously via the spectrophotometric measurement of the H_2O_2 -mediated formation of crystal violet cation (CV^+) from leuco crystal violet (LCV), which is catalyzed by HRP.¹¹

Statistics and Data Analyses. All experiments in this work were conducted in triplicate. Analysis of variance (ANOVA) was carried out at a confidence-level of 95% using Microsoft XLstat (version 2014.5.03, Addinsoft, USA), after verification of normal distribution of the data. Classification of significant different groups was then performed by Fisher's least significant difference (LSD) test at a confidence level of 95%.

RESULTS AND DISCUSSION

The germination process of brewing cereals follows the goal of fast cytolysis and proteolysis to ensure high malt quality and processability in the brewing process. Parameters accelerating the germination rate also enhance the formation of the primary DMS precursor, SMM. During kilning, the majority of SMM is thermally degraded to L-homoserine and DMS, whereas the latter is partly oxidized to DMSO. High levels of DMSO can be crucial for the resulting beer flavor as yeast is capable of DMSO reduction and concomitant DMS formation that cannot be compensated technologically anymore at this point. Thus, it is of major importance to gain information about the mechanisms of DMSO formation and to find possibilities of its minimization in malt. In general, antioxidants are potential antagonists toward reactive oxygen species and may be considered as additives to the malting process to reduce DMSO formation. However, the thiol Cys was recently reported to catalyze DMS oxidation in the presence of transition-metal ions in model solutions.¹¹

Therefore, the effect of further malt antioxidants on DMS oxidation was investigated (Figure 1). As transition-metal ions are also present during malting, all antioxidants were also tested in the presence of Cu^{2+} to furthermore cover potential prooxidative effects.

In accordance to the investigation of Cys in our previous study,¹¹ we tested the effect of representative malt-derived antioxidants at a concentration of 250 μM in the presence of 18 μM Cu^{2+} (Figure 1 a). As it is known that potential prooxidative effects of antioxidants can be overcome by increasing their concentration, we also tested the effects in the presence of 500 μM of antioxidant (Figure 1b).

While the heating of DMS in model solution as well as its combination with Cu^{2+} did not affect its oxidation, all tested potential antioxidants revealed a prooxidative behavior on DMS oxidation in the presence of Cu^{2+} , whereas GSH showed the smallest effectiveness.

Thiols (Cys and GSH). It was reported previously¹¹ that the increase of the Cys concentration from 0 to 250 μM yielded a steady increase of DMS oxidation. However, in this study, no significant effect was observed when increasing the Cys concentration from 250 to 500 μM (DMS oxidation; 250 μM : $\sim 21\%$; 500 μM : $\sim 22\%$). In the GSH- Cu^{2+} system, significantly less DMS was oxidized, but also the increase of the GSH concentration to 500 μM did not affect DMS oxidative degradation (250 μM : $\sim 12.3\%$; 500 μM : $\sim 9.7\%$) as well as DMSO formation (250 μM : $\sim 10\%$; 500 μM : $\sim 8\%$) significantly.

In our earlier study the prooxidative effect of Cys was explained by the reductive chelation and autoxidation of a bis-thiol-copper complex ($\text{RS-Cu}^+-\text{SR}$) yielding H_2O_2 via autoxidation as primary source for DMS oxidation.¹¹

Therefore, we also measured the levels of H_2O_2 during the heating period (Table 1). In the Cys- Cu^{2+} systems the highest levels of H_2O_2 ($>55 \mu\text{M}$) were observed, whereas GSH produced significant less amounts of H_2O_2 ($<20 \mu\text{M}$). These data are in good agreement with the particular extent of DMS oxidation. Therefore, we propose that the prooxidative effect of GSH is based on a similar mechanism as previously reported for Cys. The stability constants of Cys_2Cu^+ and GSH_2Cu^+ complexes are similar¹⁸ and can therefore not explain their significant different effects on H_2O_2 formation and DMS oxidation. However, it was reported by Kachur et al.¹⁹ that the oxygen consumption is lower in the presence of GSH_2Cu^+ compared to Cys_2Cu^+ , which is in line with the levels of H_2O_2

Table 1. H₂O₂ Formation (μM) in Model Solution Containing 18 μM Cu(II) and either 250 or 500 μM Antioxidant after Heating to 80 °C^a

	antioxidant concentration	
	250 μM	500 μM
Cu(II)	n.d.	n.d.
Asco + Cu(II)	2.4 \pm 0.4	n.d.
Cys + Cu(II)	56.9 \pm 1	77.9 \pm 0.7
GSH + Cu(II)	18.4 \pm 2.1	2.4 \pm 1.5
GA + Cu(II)	n.d.	n.d.
sulfite + Cu(II)	n.d.	n.d.

^aResults are presented as mean values ($n = 3$) \pm standard deviation. n.d. – not detectable.

detected in this study (Table 1). An explanation of this effect may be found in the interaction of the oxidation products (disulfides) of Cys (cystine) and GSH (GSSG) with Cu²⁺. Both disulfides are also capable of chelating Cu²⁺ via the amino- and carboxyl groups.^{20,21} While cystine can bind one Cu²⁺ molecule,²⁰ GSSG was reported to bind up to three Cu²⁺ molecules, mostly via the glutamic acid moieties.²¹

The higher capacity of GSSG to chelate Cu²⁺ may explain the lower H₂O₂ formation and DMS oxidation evoked by GSH as compared with Cys. Also, the low water solubility of cystine as compared with GSSG devalizes its contribution to Cu²⁺ chelation and may further substantiate the higher prooxidative effect of Cys on DMS oxidation. Furthermore, the lower pK_a value of the thiol group in Cys (8.45) compared with GSH (9.06) and the accompanied potential higher abundance of the more reactive thiolate anions (S⁻) for the Cys treatment may have further contributed to its higher prooxidative effect.

However, when the concentration of Cys was increased from 250 μM to 500 μM , a noncumulative increase of H₂O₂ from ~57- to ~78 μM was detected (Table 1) while DMS oxidation was not significantly affected (cp. Figure 1).

Therefore, we propose that in the range the applied concentration of 500 μM , a “reversal point” for both thiols, Cys and GSH, was reached where prooxidative- changed to antioxidative behavior. Although this reaction is expected to be relatively slow at ambient temperature, the reaction of Cys with hydrogen peroxide²² may be a reasonable explanation for this observation.

Cys is also capable of DMSO reduction,^{13,23} and a proportion of DMSO and may therefore have been reduced to DMS again at the applied concentration range.

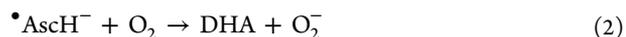
In contrast to Cys, the formation H₂O₂ was abated to ~87% when the GSH concentration was increased to 500 μM . This behavior may be explained by complexation of H₂O₂ by GSH via the carboxyl moieties, not involving the thiol group.²⁴ It is also likely that GSSG chelates H₂O₂. However, it is to mention, that such behavior was not found for the oxidation of Cys by H₂O₂.²² To conclude the effect of all thiols tested, we propose that H₂O₂ was the main DMS-oxidant formed by the interaction of thiols, molecular oxygen, and Cu²⁺.

Ascorbic Acid. In the presence of 250 μM ascorbic acid and 18 μM Cu²⁺, ~34% of DMS (CH₃SCH₃) were oxidized and ~2.7 μM were recovered as DMSO (CH₃SOCH₃). Asco was the only substance tested that significantly abated its effect on DMS oxidation (250 μM : ~33%; 500 μM : ~21%) when increasing its concentration. At 250 μM , ~2.4 μM H₂O₂ were detected while H₂O₂ was not detectable at 500 μM . This indicates an antioxidative effect even though it was insufficient

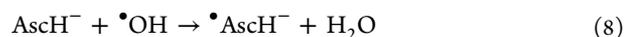
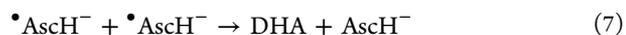
to prevent DMS oxidation. Prooxidative behavior of Asco in the presence of molecular oxygen and transition-metal ions was reported before in multiple applications, such as linoleic acid oxidation²⁵ or 1-butanol oxidation.²⁶

The reaction mechanism was described by Khan and Martell²⁷ to proceed via the initial reduction of Cu²⁺ by Asco, which should be present mostly as ascorbate anion (AscH⁻) at the applied pH of 5.5 (reaction 1). Thereby the ascorbyl radical ([•]Asc⁻) and the lower valence state of copper, Cu⁺ is formed. A further univalent oxidation of [•]Asc⁻ by molecular oxygen produces dehydro ascorbic acid (DHA) and the superoxide radical (reaction 2), whereas the latter is further reduced by AscH⁻ to H₂O₂ (reaction 3).

In comparison with the reductive chelation by the afore discussed thiols, Cu⁺ is released after its formation and can react with H₂O₂ in a “Fenton-like” mechanism to form [•]OH (reaction 4).⁹ Formation of [•]OH and 1-hydroxyethyl radicals by Asco addition $\leq 500 \mu\text{M}$ was also reported in beer.^{28,29} [•]OH formation may explain the relative high DMS oxidation at 250 μM , even though the H₂O₂ levels were much lower than for the Cys-Cu²⁺ treatment, for example. It is therefore proposed that besides H₂O₂, also [•]OH may have yielded the enhanced DMS oxidation in the presence of Asco, Cu²⁺ and molecular oxygen in these trials (reactions 5 and 6).



Compared with thiols, Asco was found to be incapable of DMSO reduction;¹³ therefore, we propose that the decline of DMS oxidation when increasing the Asco concentration was related to the following well-established antioxidative properties of Asco: the ascorbyl radical rapidly disproportionates to form a dimer (reaction 7)³⁰ which reduces the capability of oxygen reduction according to reactions 2–4. Also, Asco is capable of scavenging [•]OH (reaction 8) at diffusion-controlled reaction rates.³¹ Those reactions may have competed with [•]OH and H₂O₂ and thereby abated the extent of DMS oxidation at higher Asco concentration.



Galic Acid. Regardless of the concentration, the effect of GA on DMS oxidation (250 μM : ~22%; 500 μM : ~21%) and DMSO formation (250 μM : ~16%; 500 μM : ~23%) was in analogy to the observations for the Cys-Cu²⁺ treatments. In this study, H₂O₂ was not detected in the GA-Cu²⁺ treatments during the heating period. Akagawa et al.³² reported that oxidation of GA and other phenols by molecular oxygen and Cu²⁺ generated H₂O₂ up to ~180 μM in phosphate buffer at pH 7.4 after 24 h at 37 °C, whereas at pH 5, the H₂O₂ concentration was below 10 μM , probably resulting from the lower solubility of phenols at lower pH. It therefore seems likely that the H₂O₂ formation by GA-Cu²⁺ at pH 5.5 proceeds much slower than in the case of thiols and may accordingly

have affected DMS oxidation in the further progress of incubation at 80 °C. However, more investigations need to be conducted to characterize the prooxidative effect of GA on DMS oxidation.

Sulfite. Sulfite showed the highest prooxidative effect on DMS oxidation and DMSO formation of all substances tested. DMS was completely oxidized in the presence of 250 or 500 μM sulfite and 18 μM Cu^{2+} . This was also the case for 250 μM sulfite and 9 μM of Cu^{2+} (data not shown). H_2O_2 was not detected in the presence of 250 or 500 μM of sulfite. Because of the strong effect of sulfite, further investigations were focused to characterize the reactions of sulfite, transition-metal ions, and DMS. In the next step, the course of DMS oxidation was monitored in the presence of transition-metal ions and 250 μM sulfite in oxygenated model solution during heating up and incubation at 80 °C (Figure 2).

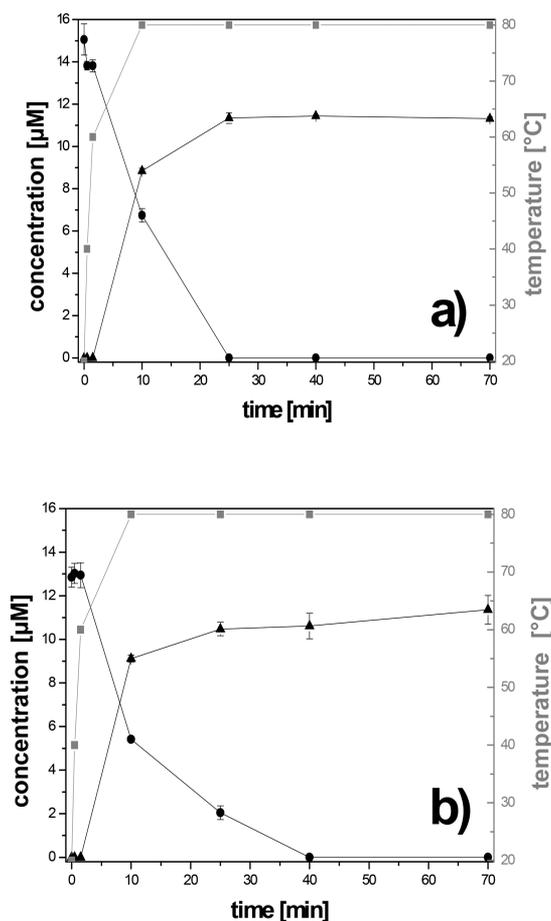


Figure 2. Kinetics of DMS oxidation in the presence 250 μM sulfite, 18 μM $\text{Cu}(\text{II})$ (a) and 18 μM $\text{Fe}(\text{III})$ (b) at 80 °C. DMS concentration (circles); DMSO concentration (triangles); temperature (squares), both in μM . Results are presented as mean values ($n = 3$) \pm standard deviation.

In the presence of 18 μM Cu^{2+} (Figure 2a), DMS concentration remained constant within the first 1.5 min until the solution reached a temperature of 60 °C. After 10 min, when the target temperature of 80 °C was reached, already $\sim 50\%$ of the DMS were oxidized. After 25 min, no DMS was detected anymore and a maximum DMSO concentration was found. Noticeably, only $\sim 82\%$ of the total amount of oxidized DMS was recovered as DMSO.

In the presence of 18 μM Fe^{3+} (Figure 2b), the behavior of DMS and DMSO was similar, but marginally slower. After 10 min $\sim 58\%$ of the DMS were oxidized and after 25 min, $\sim 2 \mu\text{M}$ of DMS were still detectable. Yet, after 40 min, the DMS concentration was below the detection limit. The total amount of DMSO recovered was $\sim 87\%$ which was slightly higher than for application of Cu^{2+} . Taken together, these data indicate that DMS oxidation in the presence of transition-metal ions and sulfite is significantly faster as compared with the oxidation by H_2O_2 or $\cdot\text{OH}$,⁶ suggesting the involvement of another reactive radical species. These aspects will be discussed in context to the ensuing experiments.

Besides its reactivity with H_2O_2 ,¹⁴ the capability of sulfite to reduce DMSO represents a secondary antioxidative property regarding DMS oxidation. Therefore, the scope of the prooxidative effect was investigated in the presence 9 μM of Cu^{2+} and varying concentrations of sulfite (Figure 3) to explore potential shifts to antioxidative behavior.

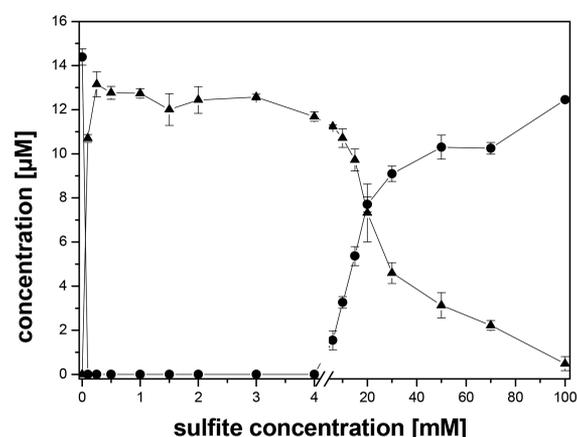


Figure 3. Effect of sulfite concentration on DMS oxidation in the presence of 9 μM $\text{Cu}(\text{II})$. Samples were incubated for 180 min at 80 °C. DMS concentration (circles); DMSO concentration (triangles), both in μM . Results are presented as mean values of triplicate trials ($n = 3$) \pm standard deviation.

In the absence of sulfite, DMS was not oxidized to DMSO. When 100 μM of sulfite was added to the system, no DMS was detectable anymore, and the DMSO concentration increased to $\sim 11 \mu\text{M}$. Further sulfite additions from 0.25 to 4 mM were followed by a complete oxidation of DMS which was recovered as DMSO at $\sim 83\%$. These results are in accordance to the previous trial (Figure 1), even though the Cu^{2+} concentration was only half as high, suggesting that Cu^{2+} concentration plays a subordinated role. This phenomenon was already reported for the prooxidative effect of Cys.¹¹ Around 6 mM of sulfite a reversal formation of $\sim 1.54 \mu\text{M}$ of DMS could be observed. By further increasing, the sulfite concentration the DMS concentration increased steadily while the DMSO concentration decreased again. This increase is proposed to be a result of a concentration-dependent shift of the equilibrium toward sulfite-mediated reduction of DMSO¹³ that prevents DMS from oxidizing. Another explanation may be that potential radical termination reactions predominated the yet unknown prooxidative effects of sulfite on DMS oxidation.

The experiment elucidated the broad field of the prooxidative effect of sulfite on DMS oxidation in the presence of Cu^{2+} . 100 mM of sulfite - which is far beyond supplementary additions of sulfite to foodstuffs - had to be added to reach the

maximum DMS ($\sim 12.5 \mu\text{M}$). At this point, $\sim 0.48 \mu\text{M}$ of DMSO were still detected. DMS oxidation could only be excluded when the model solution was purged with N_2 and when no transition-metal ions were added (data not shown).

To further clarify the source of sulfite's effect on DMS oxidation and to obtain insights into reaction mechanisms, we applied ESR spectroscopy to test the potential involvement of radical species. Initially, different combinations of Fe^{3+} , Cu^{2+} , and sulfite were combined with 5 mM of the spin trap DMPO. ESR spectra were recorded 2 min after spiking (Figure 4). No ESR signal was detected when 18 μM Fe^{3+} were combined with DMPO, exclusively (spectrum a). Though, the combination of 500 μM sulfite with whether 18 μM Fe^{3+} (spectrum b) or 18 μM Cu^{2+} (spectrum c) produced distinctive ESR signals, similar to the sulfite radical-DMPO ($\bullet\text{SO}_3/\text{DMPO}$) adduct

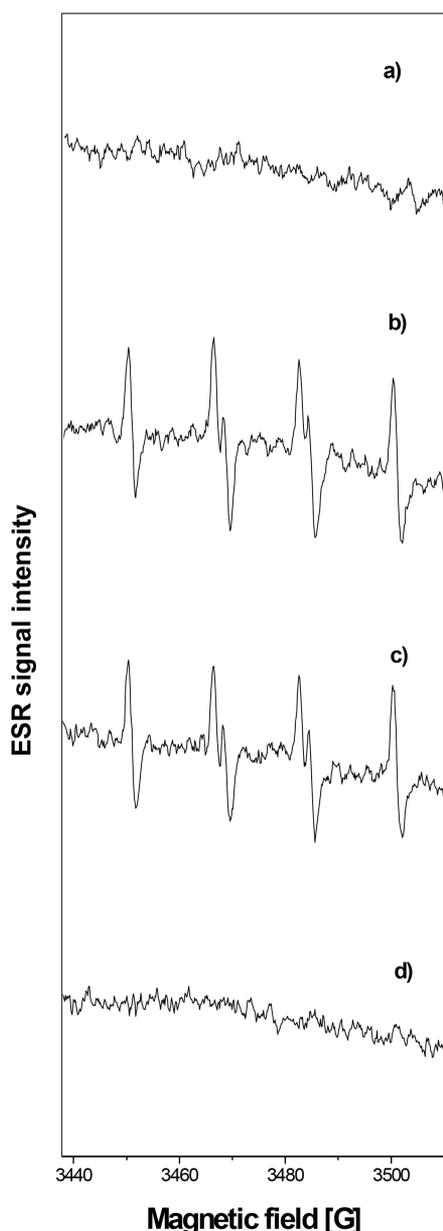
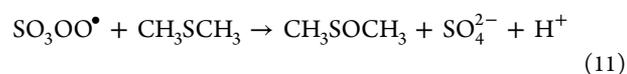
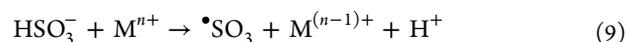


Figure 4. ESR spectra of DMPO (5 mM) spin adducts at room temperature: 18 μM $\text{Fe}(\text{III})$, (a); 18 μM $\text{Fe}(\text{III})$ + 500 μM sulfite, (b); 18 μM $\text{Cu}(\text{II})$ + 500 μM (c); 500 μM sulfite + 0.18 mM EDTA, (d). Spectra show the sum of 10 scans after 2 min of spiking.

reported before in relation to enzymatic oxidation of sulfite.^{33–35} The signal was not observed when the model solution as well as the sulfite stock solution contained EDTA (0.18 mM), which provides evidence for the formation of $\bullet\text{SO}_3$ by the interaction of sulfite with Cu^{2+} and Fe^{3+} .

The signal intensity of the $\bullet\text{SO}_3/\text{DMPO}$ spin adduct did not allow for accurate simulation and characterization of the radical species generated. Rangelova and Mason³⁶ reported that molecular oxygen competes with DMPO to react with $\bullet\text{SO}_3$. Even though we purged all solutions with N_2 , oxygen diffusion into the reaction system was inevitable in the progress of the investigation. Molecular oxygen may then have reacted with $\bullet\text{SO}_3$, thereby diminishing its detection via DMPO spin trapping and ESR spectroscopy. Therefore, we conducted a further ESR experiment with 50 mM DMPO (Figure 5). The combination of 18 μM Cu^{2+} and DMPO produced a distinctive ESR signal (spectrum a). The signal reveals similarities to a carbon-centered radical,³⁷ as probably generated from Cu^{2+} , acetate and DMPO interactions. However, the radical was not identified within this study and will be further designated as \bullet -radical. In the presence of sulfite, Cu^{2+} and DMPO the distinctive ESR signal of the $\bullet\text{SO}_3/\text{DMPO}$ spin adduct (+) evolved besides the \bullet -radical at a much higher intensity (spectrum b). The identity of $\bullet\text{SO}_3$ becomes even more obvious, when subtracting the signal of the \bullet -radical (spectrum a) from spectrum b (spectrum c). The $\bullet\text{SO}_3/\text{DMPO}$ spin adduct was also generated by the combination of sulfite with Fe^{3+} and DMPO, whereas the signal intensity was lower.

We simulated the experimental data of spectra c and d (Supporting Information, Figure S2), and we determined the hyperfine coupling constants for the $\bullet\text{SO}_3/\text{DMPO}$ spin adduct as generated by the interaction of sulfite with Cu^{2+} ($\alpha^{\text{N}} = 14.11$, $\alpha_{\beta}^{\text{H}} = 15.60$) and sulfite with Fe^{3+} ($\alpha^{\text{N}} = 14.12$ and $\alpha_{\beta}^{\text{H}} = 15.54$). The similarities of the hyperfine coupling constants indicate that the same radical was formed, irrespective of the transition metal used. Though, the constants are slightly smaller than reported for the $\bullet\text{SO}_3/\text{DMPO}$ adduct in oxidoreductase/ $\text{H}_2\text{O}_2/\text{Na}_2\text{SO}_3^-$ ($\alpha^{\text{N}} = 14.7$, $\alpha_{\beta}^{\text{H}} = 16.0$)^{36,38} at physiological pH. In those studies sulfite was present as a mixture of bisulfite anion and sulfite ($\text{HSO}_3^-/\text{SO}_3^{2-}$), whereas in our study (pH 5.5), sulfite should be present mostly as HSO_3 . Still, the same hyperfine splitting constants were also determined at pH 7. Moreover the result was not influenced by increasing the DMPO concentration to 100 mM. It is noteworthy to mention that in the previous studies,^{36,38} the transition-metal ions were present in an enzyme-bound state. In our study, the transition-metal ions can be presumed to be available for interactions, for example with DMPO³⁹ or $\bullet\text{SO}_3/\text{DMPO}$ spin adducts, which may explain the lower values of the hyperfine splitting constants. However, the prooxidative effect of sulfite on DMS oxidation when combined with the transition-metal ions (M^{n+}) Fe^{3+} or Cu^{2+} can be proposed as follows:



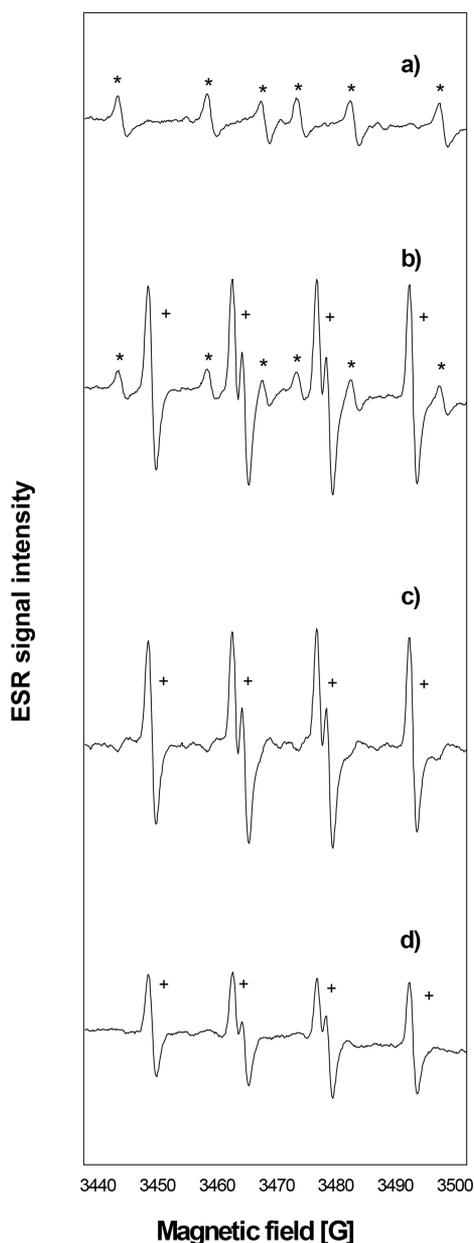
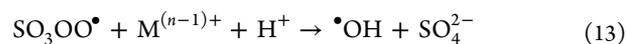
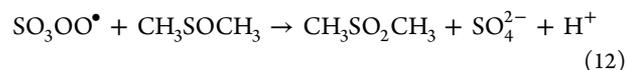


Figure 5. ESR spectra of DMPO (50 mM) spin adducts at room temperature: 18 μM Cu(II), (a); 18 μM Cu(II) + 500 μM sulfite, (b); spectrum (a) subtracted from (b) (c); 18 μM Fe(III) 500 μM sulfite (d). Spectra show the sum of 50 scans after 2 min of spiking.



Initially, the transition-metal ion (e.g., Fe^{3+}) donates an electron to HSO_3^- , whereupon its lower valence state and $\bullet\text{SO}_3$ are formed^{40,41} (reaction 9). As mentioned before, $\bullet\text{SO}_3$ rapidly reacts with molecular oxygen ($k_{[10]} = 1.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$)⁴² leading to the formation of a peroxymonosulfate radical ($\text{SO}_3\text{OO}^\bullet$)^{34,41,43} (reaction 10). We propose that $\text{SO}_3\text{OO}^\bullet$ was then primarily responsible for DMS oxidation and DMSO formation (reaction 11). As the sum of DMS and DMSO was mostly lower at the end of the experiment, it is also likely that DMSO was further oxidized to DMSO_2 (reaction 12). It was also reported that $\text{SO}_3\text{OO}^\bullet$ may be reduced, for example, by Fe^{2+} to form $\bullet\text{OH}$ ⁴⁴ (reaction 13), which is a powerful DMS oxidant as well (reaction 14).⁶ The holistic of those reactions is proposed of being the source of the prooxidative effects of sulfite on DMS oxidation and DMSO formation.

To our knowledge, the oxidation of DMS by $\text{SO}_3\text{OO}^\bullet$ has not been reported before. Though, in chemical synthesis potassium peroxymonosulfate, commonly known as “oxone” is used for the oxidative synthesis of sulfoxides and sulfones from sulfides,⁴⁵ which is in line with the findings of the present study.

DMS oxidation was investigated in model solutions to exclude potential side reaction of the substances used and to obtain information on the reaction mechanisms. Though, these investigations do not consider metabolic factors and bioavailability. An in vivo malting experiment was thus conducted to investigate the significance of the described reactions on DMS oxidation in malt. The antioxidants were added solely- or in combination with Cu^{2+} - to the calculated amount of saline solution that had to be added to achieve the desired steeping degree of 45% (w/w) at the beginning of germination. After the addition the germination was carried out for 5 days.

The relative acrospires lengths (Supporting Information, Figure S2) after germination in each treatment were significantly higher than the reference, whereas the highest acrospires length was for the Cys treatment, which was $\sim 12\%$ higher than the reference. However, in combination with Cu^{2+} , the acrospire length was reduced significantly to ~ 0.62 . For all other antioxidants there was no significant effect for their respective combination with Cu^{2+} . After germination, the green

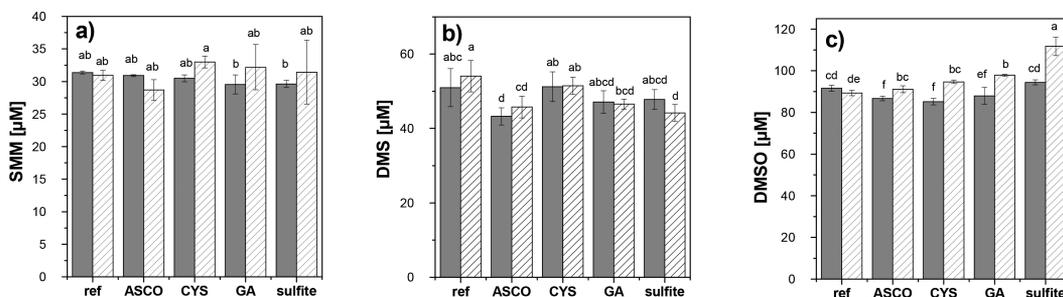


Figure 6. Impact of 500 μM antioxidant and 18 μM Cu(II) on the level of SMM (a); DMS (b) and DMSO (c) in malt presented in μM ($\mu\text{mol}/\text{kg}$ malt); without Cu(II) addition (gray bars), with addition of 18 μM Cu(II) (white, dashed bars). Results are presented as mean values of triplicate trials ($n = 3$) \pm SD. Different letters indicate significant differences according to the Fisher (LSD) test.

malts were dried stepwise in the withering and kilning process. The water content of the resulting malts were 4.3–4.5% (w/w) and were not significantly different from each other. The concentration of SMM, DMS and DMSO in the different malts are presented in Figure 6. Compared to the reference (no addition) the concentration of the primary DMS-precursor, SMM, was not significantly affected by the treatments, whereas the concentration in the Cys-Cu²⁺ treatment was significantly higher than in the GA- and sulfite treatment (< ~10.5%). However, the effect of the treatments toward SMM synthesis can be estimated to be relatively small. For the levels of DMS, only the treatments Asco, Asco-Cu²⁺ and sulfite-Cu²⁺ were significantly different from the reference (no addition), while the latter as well as GA showed significant lower DMS levels than the reference-Cu²⁺ treatment. The exclusive application of Asco and Cys generated significantly less DMSO which can be regarded as an antioxidative effect. However, the DMSO contents in those malts were solely ~7% lower than in the reference.

All antioxidant-Cu²⁺ treatments showed significant higher levels of DMSO than their respective exclusive application. Again, as compared with the model systems highest DMSO formation was found in the sulfite-Cu²⁺ followed by the GA-Cu²⁺ treatment. In the sulfite-Cu²⁺ treatment, the DMSO concentration was ~22% higher as compared with the reference. One possible explanation for the smaller prooxidative effects is probably the reaction of sulfite with respiration-derived hydrogen peroxide formation in the course of the germination process.⁶ Considering the plethora of potential side-reactions of sulfite and the other tested substances in malt, the observed prooxidative effects are still remarkable. Also, it is important to mention that the addition occurred ~5 days before SMM was subject to degradation and DMS formation during the kilning process. On the basis of these results, we propose that the aforementioned reaction mechanisms have relevance for the malting process. The aforementioned reactive species (e.g., H₂O₂ or the •SO₃/SO₃OO• system) may have accumulated before and during the withering and kilning process to evoke DMS oxidation and DMSO. Yet, further investigations of bioavailability and abundance of the reactive oxygen species derived from antioxidant-transition ion mediated reduction of oxygen during different steps of malting are necessary. Also, further research is needed to assess the effect of different contents and types of antioxidants, and transition-metal ions already present in barley as those can be regarded as crucial impact factors for pro- and/or antioxidant behavior. In particular, the yet-undiscovered abundance of sulfite-derived radical species in malt is important to be investigated as sulfite is commonly used in the malting process for the prevention of nitrosamines, for example.¹⁵ Sulfite and its radical intermediates were already assigned to induce damage, for example, to proteins³⁵ and polyunsaturated fatty acids.⁴⁶ Also, •SO₃ was already identified in wine,⁴⁷ where its autoxidation product SO₃OO• was found to play an important role in polyphenol oxidation.⁴⁸

In this study, we demonstrated the high reactivity of SO₃OO• with DMS. Though, reactions of sulfite-derived radicals with malt, wort, and beer ingredients (e.g., polyphenols, fatty acids, or amino acids) and their consequences for product quality are mostly unknown.

Sulfite is regarded as the primary, most effective antioxidant in beer.^{49,50} These findings are mainly based on evaluation of oxidative beer flavor stability using ESR spectroscopy after spin

trapping of hydroxyethyl radicals (generated from •OH and ethanol) with phenyl-*N-tert*-butylnitron (PBN)^{28,51} or α -(4-Pyridyl *N-oxide*)-*N-tert*-butylnitron (POBN).⁵² These spin traps do not form detectable adducts with sulfite-derived radicals, though. Also, •SO₃ is essentially unreactive toward ethanol and does therefore not generate hydroxyethyl radicals. Even though sulfite reacts with hydrogen peroxide ($k = 9.1 \times 10^7 \text{ M}^{-2} \text{ s}^{-1}$),¹⁴ the formation of •SO₃ via transition-metal-ion-mediated oxidation of sulfite and its autoxidation to SO₃OO• is very likely to occur ($k = 1.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$). Hence, it is necessary to further apply DMPO spin trapping and ESR spectroscopy to malt, wort, and beer for analyzing the abundance of sulfite-derived radicals and their relevance for malt and beer quality. In this study, we also demonstrated that thiols were capable of significant H₂O₂ formation in the presence of Cu²⁺ and molecular oxygen, which is in good agreement with previous studies.^{11,53} Based on their high naturally occurring levels in malt, wort and beer, their role in H₂O₂ formation and consequences for oxidative processes needs to be further investigated. The reactions described in this study are likely to also occur in miscellaneous other food systems that contain thiols, phenols, ascorbic acid, sulfite, and transition-metal ions. As such, prooxidative effects can lead to food quality deterioration those reactions need to be further addressed in the future to find approaches for increasing antioxidative functionalities, for example, by combining different ratios of antioxidants and to assess optima for synergisms.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b02906.

(PDF)

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Notes

The authors declare no competing financial interest.

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Supporting information – Publication E

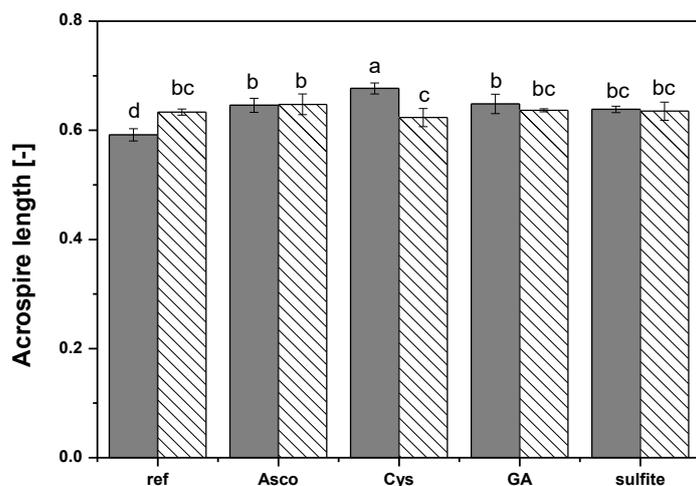


Figure S1: Relative acrospire lengths of the green malts treated with antioxidants, without Cu(II)(grey bars) and in combination with 18 µM Cu²⁺ (white, dashed bars). Results are presented as mean values of triplicate trials (n=3) ± standard deviation. Different letters indicate significant differences according to the Fisher (LSD) test.

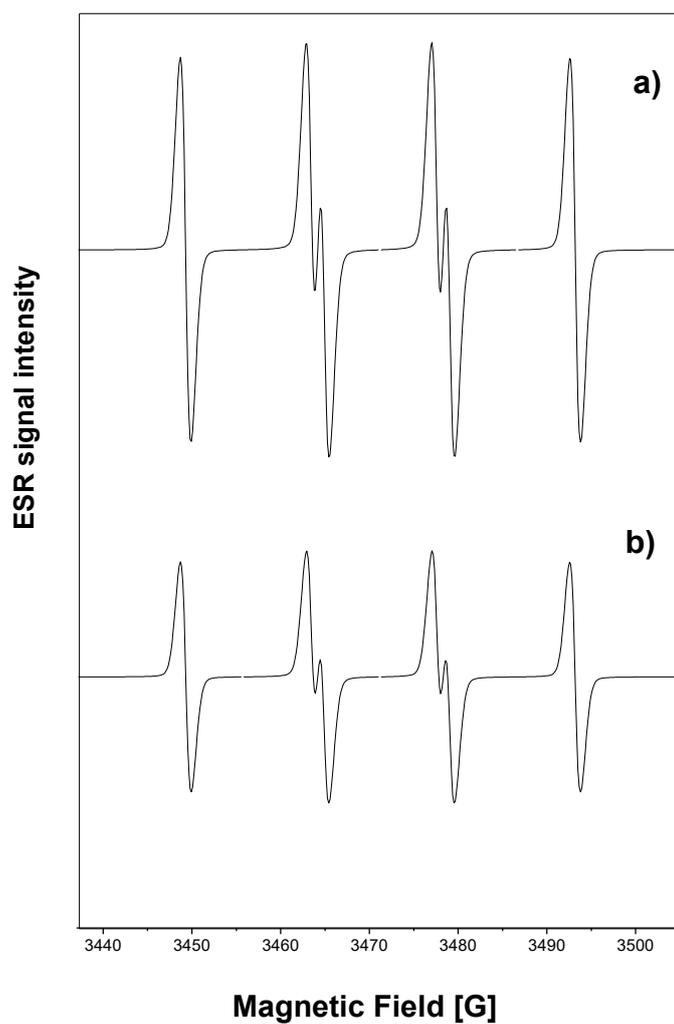


Figure S2: Simulated ESR spectra from the interaction of 500 μM sulfite, 50 mM DMPO and 18 μM Cu^{2+} (a), or 18 μM Fe^{3+} (b).

Additional data – Publication E

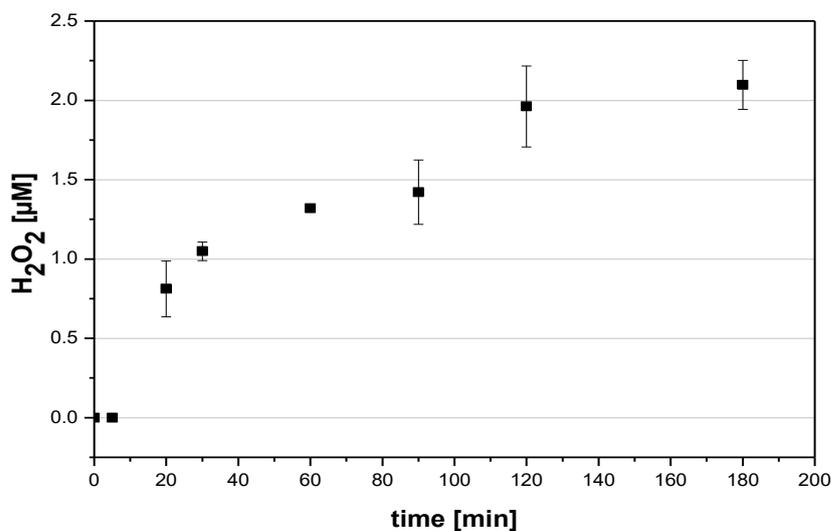


Figure 17: Formation of H₂O₂ in model solutions containing of 250 µM GA and 18 µM Cu²⁺ (T=80°C). Data are presented as mean value (n=3) ± standard deviation. Data were generated as described in publication E²⁰⁶ except that samples were also taken after 20-, 30-, 60-, 90-, 120- and 180 minutes.

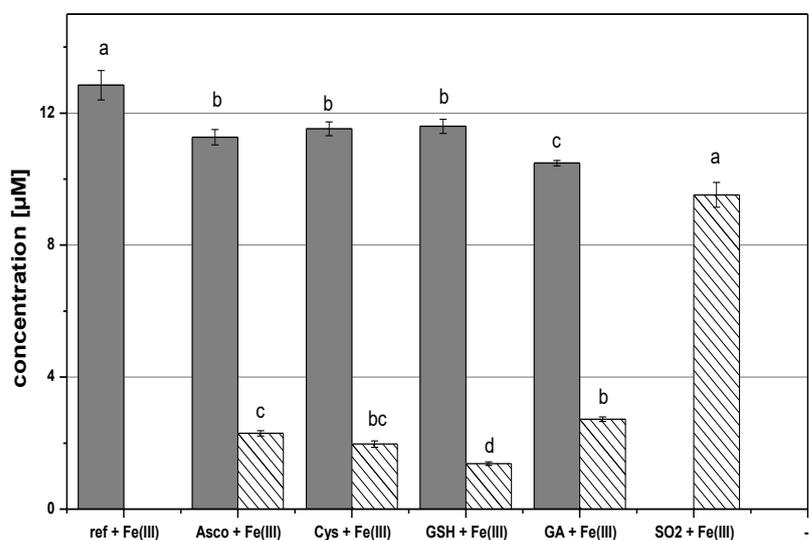
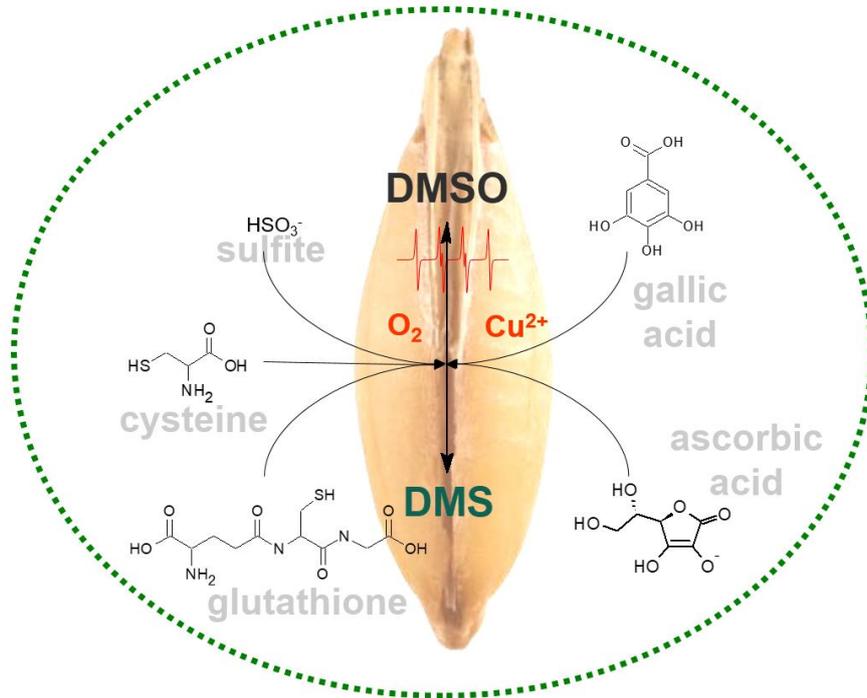


Figure 18: Effect of 250 µM antioxidants on DMS oxidation in the presence of 18 µM Fe³⁺ in air-saturated model solutions at 80°C for 180 minutes.

DMS concentration (gray bars); DMSO concentration (white, dashed bars); ref (model solution only containing DMS). Results are presented as mean values of triplicate trials (n=3) ± standard deviation. Different letters indicate significant differences according to the Fisher (LSD) test. Data were generated as described in publication E.²⁰⁶

Table of Contents Graphic – Publication E



Publication F:

In the previous investigations H_2O_2 was designated as a potential DMS oxidant. Therefore constitutive experiments were performed to characterize the reaction between DMS and H_2O_2 under consideration of physical-chemical parameters. Besides H_2O_2 also the extent of DMS oxidation in the presence of further (reactive) oxygen species, O_2 and $\cdot\text{OH}$, was explored.

The full background, methodologies and outcomes of the investigations were published in the *Journal of the American Society of Brewing Chemists*, in a paper entitled “*Response Surface and Kinetic Modeling of Dimethyl Sulfide Oxidation – On the Origin of DMSO in Malt*”(Publication F²⁰⁷).

Besides its origin from autoxidative reactions involving antioxidant- $\text{M}^{\text{n}+}$ -systems, H_2O_2 was detected in green malt as potentially derived from respiration as a side-product. These observations further justified the role of H_2O_2 as a source of DMSO in malt. CCD-RSM revealed that temperature as well as H_2O_2 concentration have a decisive impact on DMS oxidation, whereas the effect of pH was found to be insignificant. An ensuing kinetic experiment supported the relevance of DMS oxidation by H_2O_2 , whereas the reaction of $\cdot\text{OH}$ and DMS and the concomitant formation of DMSO was significantly faster. In conclusion potential reactions leading to the formation of DMSO in malt are presented.

Publication F

Response Surface and Kinetic Modeling of Dimethyl Sulfide Oxidation – On the Origin of Dimethyl Sulfoxide in Malt

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ABSTRACT

Dimethyl sulfide (DMS) contributes significantly to beer aroma. Malt contains considerable amounts of its oxidized form dimethyl sulfoxide (DMSO), the precursor of DMS during fermentation. Yet, detailed information regarding mechanisms of DMSO formation in malt is limited. Recently, hydrogen peroxide was suggested as a potential DMS oxidant in malt. The aim of this study was to characterize the reaction of hydrogen peroxide and DMS in buffered model solutions. Initially, hydrogen peroxide was identified in green malt using leuco crystal violet and horseradish peroxidase. Based on these findings the reaction of DMS with H₂O₂ was investigated in buffered model solutions at varying temperatures and pH using response surface modeling (RSM). Significant amounts of DMSO were formed by H₂O₂ mediated oxidation of DMS (~0–41%), which was determined by temperature and hydrogen peroxide concentration ($P \leq 0.003$). The effect of the pH was insignificant ($P \geq 0.4$). Kinetics of DMS oxidation by H₂O₂ (DMS:H₂O₂ = 1:3) at 95°C followed a pseudo first order mechanism ($k = 0.0041 \text{ min}^{-1}$). Molecular oxygen was not capable of oxidizing DMS, whereas hydroxyl radical mediated DMS oxidation was faster than DMS oxidation aroused by H₂O₂. Potential reaction mechanisms and their relevance in DMS oxidation are discussed as a first approach to minimize DMSO levels in malt.

KEYWORDS

DMS; oxidation; DMSO; hydrogen peroxide; hydroxyl radicals; Fenton; malting; stress

Introduction

The thioether dimethyl sulfide (DMS) has a considerable impact on the overall flavor impression of many foodstuffs. High levels of DMS can be found in heated truffle and asparagus.^[1,2] Moreover, DMS is present in soy beans,^[3] citrus fruits,^[4] wine,^[5,6] melons,^[7] and tomato products.^[8,9]

In beer, DMS is mostly considered an undesirable flavor compound. Above its flavor threshold of ~30–100 µg/L, aroma impressions, such as “cooked-vegetable”-like may appear.^[10,11] However, below its flavor threshold, DMS is also reported to contribute positively to beer flavor.^[10] The origin of DMS in beer can originally be retraced to the thermal decomposition of S-methyl methionine (SMM), which is synthesized during the germination of brewing cereals.^[12,13] Its synthesis mainly takes place in the embryo, eminently in rootlets, acrospire, and scutellum.^[13,14]

In the malt kilning process, at temperatures above ~70°C, SMM decomposes in a pH-dependent manner to DMS and L-homoserine.^[15,16] In addition to evaporation by the kiln’s ventilation air, considerable amounts of DMS oxidize to DMSO and minorities of dimethyl sulfone (DMSO₂) are formed.^[16–17]

Residual SMM remains a thermal precursor, which is generally eliminated in the upfollowing brewing process via consecutive degradation and DMS evaporation in the wort boiling process. The high polarity of the sulfoxide group as well as the pyramidal structure of DMSO in aqueous solution is responsible for its high water solubility and the high boiling point (189°C). Owing to these properties, DMSO cannot be removed

by evaporation and its entirety probably can be recovered in the pitching wort.

In contrast to other foodstuffs, DMSO reveals a peculiar role in brewing, since it acts as secondary DMS-precursor during fermentation.^[15,19,20–22] Yeast possesses thioredoxin dependent methionine sulfoxide reductases (MSRA), which respond to oxidative stress through recycling of oxidized methionine back into its reduced state. As a side effect, MSRA also deoxygenate the sulfoxide group of DMSO, which results in DMS formation.^[23–26]

Most lager yeast strains are able to reduce DMSO, in particular even up to 21%.^[19] Taking into account that pitching wort contains DMSO levels of more than ~500 µg/L, even a small reduction may increase the levels of DMS in beer significantly. Labelling studies^[18,27] clearly elucidated this effect. Leemans et al.^[18] even declared DMSO as the most potential source of DMS in beer as they found that 80% of the total DMS in beer originated from the addition of deuterated DMSO to the pitching wort.

Since DMSO is a potential source of DMS in beer, extensive DMS oxidation and DMSO formation should be minimized in malting and brewing. Oxidative reactions have been widely investigated and elucidated in malting and brewing, predominantly in relation to beer flavor stability.^[28–33] The role of transition metal ions was considered as key factor for the initial reduction and activation of oxygen, which leads to the formation of reactive oxygen species (ROS), for example superoxide radicals (O₂⁻), hydrogen peroxide (H₂O₂), or hydroxyl radicals (•OH).^[34]

However, there is a substantial lack of knowledge regarding reaction mechanisms of DMS oxidation in the malting process. It is important to gain information on the origin of DMSO in malt as a first approach for its minimization.

Considering other research sectors, the oxidation of DMS is of high interest, especially for the chemical industry, marine- and environmental sectors. It is believed that DMS and its oxidation products present $\sim 50\%$ of the total sulfur emitted to the atmosphere,^[35] where DMS is mainly oxidized by nitrate (NO_3) and hydroxyl radicals ($\cdot\text{OH}$) forming sulfate (SO_4) as the final product.^[36–38] In the late 1980s, the so called “CLAW-hypothesis” aroused huge attention owing to the assumption that DMS emission by phytoplankton directly correlates with the global climate and that DMS oxidation to SO_4 aerosols influences the earth’s radiation as well as climate regulation.^[39] Regarding this background the oxidative behavior of DMS was investigated by product analysis in a multitude of experimental set-ups in gas- and liquid phases. In terms of synthetic production of DMSO, which is widely used as organic solvent, investigations in aqueous solution were conducted by the chemical industry to optimize such processes. General oxidation products were reported to be DMSO, SO_2 , SO_4 , methane sulfinic acid (MSI), methane sulfonic acid (MSA), dimethyl sulfone (DMSO_2), and others.^[36–37,40–41] Adewuyi and Carmichael^[40] investigated the oxidation of DMS by H_2O_2 in acidic and alkaline aqueous media using spectrophotometry. They found the reaction to follow a first order mechanism with respect to DMS and H_2O_2 in an overall bimolecular mechanism. Amels et al.^[41] were able to confirm these findings and investigated the rates of further peroxides on the oxidation of DMS and DMSO. Regarding H_2O_2 , their work shows that the rate of oxidation of DMSO to DMSO_2 is very low with a half-life of several days at 293 K. The latter reactions were investigated at temperatures below 35°C at DMS- and H_2O_2 concentrations in the millimolar area.

DMS oxidation by H_2O_2 , however, has not been explored at temperatures and concentrations (micromolar), which are representative for thermal processing of foodstuffs, like malt. Recently, we reported that the thiol cysteine had a prooxidative effect on DMS oxidation, when it was combined with transition metal ions in malting and brewing related concentration ranges at 95°C. We found that H_2O_2 , generated via autoxidation of cysteine-copper complexes, was responsible for DMS oxidation and DMSO formation.^[42]

In addition to autoxidative reactions, H_2O_2 can also be formed during germination of plants as a by-product of respiration.^[43–46] In this case, H_2O_2 or superoxide radicals are formed through incomplete reduction of oxygen in the mitochondria as a result of stress conditions, such as elevated temperatures, fungi infestation, low or high water exposition.^[43,45–46]

The objective of this work was to initially test if H_2O_2 is also present in germinated barley (green malt). The main focus was then to characterize the reaction between DMS and H_2O_2 at different concentrations, temperatures, and pH values. Constitutively, a kinetic investigation was conducted to provide information regarding the reaction rate of DMS oxidation by H_2O_2 , which allows deductions on the potential contribution of this reaction to DMSO formation under kilning-like conditions, for example. In addition to H_2O_2 , molecular oxygen as well as

hydroxyl radicals were tested on DMS oxidation. Outcomes of this study will be discussed in relation to physical-biochemical as well as technological malting factors. The data should serve as a first approach to minimize DMS oxidation in malt.

Experimental

Chemicals

Dimethyl sulfide (DMS; $\geq 99\%$), dimethyl sulfoxide (DMSO; $> 99\%$), iron(II) sulfate heptahydrate (Fe(II), $\geq 99\%$), and acetic acid ($\geq 99\%$) were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). Sodium acetate anhydrous ($\geq 99\%$) and horseradish peroxidase (HRP, Grade I) was obtained from Applichem (Darmstadt, Germany). Leuco crystal violet (LCV, $\geq 98\%$), hydrogen peroxide (H_2O_2 , 30%), and chloroform (≥ 99.8) were purchased from Carl Roth GmbH & Co KG (Karlsruhe, Germany). Nitrogen (99.999%) and synthetic air (20.5 \pm 0.5% O_2 in N_2) were from Air Liquide GmbH (Berlin, Germany). Ethylenedinitrilotetraacetic acid disodium salt dehydrate (EDTA), disodium phosphate ($\geq 99\%$) and sodium dihydrogen phosphate (99.0–102%) were acquired from Merck (Darmstadt, Germany).

Identification of H_2O_2 in green malt

Germination

A 16 g aliquot of the barley variety Quench was weighed into 50-mL Falcon tubes, which were perforated with 5 holes (diameter 2 mm) at the cone. The tubes were placed in a stainless steel box (15 \times 60 \times 12 cm), which was used for steeping and germination. The box contained 6 air inlets at the top and a conical water and air/ CO_2 outlet. The barley was steeped for 4 h in aerated 0.9% (wt.) saline solution at 16°C. The first wet steep was followed by a 12 h air rest and a second wet steep of 2 h at 16°C. Afterward, the germination was carried out for 5 days at 18°C and a steeping degree of 45%, which was adjusted daily until germination day 3 by spraying the desired amount of saline solution onto the kernels.

H_2O_2 detection

The water used throughout the investigations was ultrapure water (Milli-Q®). A total of 2 g of the green malt was combined with 4 mL of sodium phosphate buffer (0.2 M, pH 6.5) and ground by mortar and pestle for 30 s. Afterward, 2 mL of LCV solution (60 mg/100 mL 0.5% (v/v) HCl) and 1 mL of HRP solution (30 mg/20 mL) were added. The reference sample contained 3 mL of bidistilled ultrapure water instead of HRP-, and LCV solution. Then, the mixture was diluted with another 15 mL of the phosphate buffer. Samples were transferred into 50 mL Falcon tubes (VWR GmbH, Darmstadt, Germany). The tubes were shaken at 175 rpm for 30 min at room temperature. Afterward, 10 mL of chloroform was added and the sample was mixed for another 10 min. The mixture was centrifuged (10 min, 7340 g). The chloroform phase was accumulated at the bottom and 15 mL of the aqueous phase was removed and substituted by a further addition of 14 mL of chloroform. A further extraction step of 10 min was carried out before the

mixture was centrifuged again. The chloroform phase was then withdrawn for the spectrophotometric analysis. A Lambda 25 spectrophotometer from Perkin Elmer (Waltham, MA, U.S.A.) was used for recording the absorption spectra between 200 and 700 nm using a quartz cuvette (path length 10 mm, VWR GmbH, Darmstadt, Germany).

Incubation of DMS under various conditions

The incubation of DMS in a selected range of pH, temperature, and H₂O₂ concentration was conducted in 0.1 M sodium acetate/acetic acid buffer solutions. All aqueous solutions were prepared with ultrapure water (Milli-Q®), which was boiled before use. The final buffer solutions were purged with nitrogen for 20 min at 20°C to reach final oxygen concentrations below 2.5 μM, measured by a Visiform DO 120 oxygen sensor from Hamilton GmbH, Planegg, Germany. An amount of 62.00 g of the respective buffer solution was then filled into 50-mL Duran® bottles (Schott AG, Mainz, Germany). Thereby, the headspace volume was minimized to avoid DMS diffusion out of the reaction system. The bottles were tightly closed with screw caps containing PTFE seals and covered with aluminum foil to avoid UV-light exposure. After the incubation period the bottles were cooled to 1°C. In the next step, a respective aliquot of an aqueous H₂O₂ stock solution was spiked into the bottle before an ice-cold DMS-Ethanol stock solution was added to reach initial DMS concentrations of 15.00 μM. The DMS stock solution was kept in a tightly closed vial with reduced headspace volume, located in an ice block in order to avoid volatilization. Immediately after spiking the bottles were tightly closed and placed in a water bath, which was adjusted to the desired temperature. The temperature increase was tracked using a HI935002 Dual Channel K-Type Thermocouple Thermometer (Hanna Instruments, Voehringen, Germany). After incubation the bottles were cooled down stepwise with tap water and ice cold water before analysis of DMS and DMSO was conducted using headspace gas chromatography combined with pulsed flame photometric detection (HS-GC-PFPD) according to a previously described method.^[20]

Experimental design of DMS oxidation by H₂O₂

Rotatable central composite design of the experimental set-up and response surface modeling (RSM) of the results was applied to engross the knowledge regarding the scope of DMS oxidation by H₂O₂. Factors investigated were H₂O₂ concentration, temperature, and pH. Each factor was varied over 5 levels including 2 axial points ($\pm\alpha$), 6 factorial points (± 1), and 5 center points (0) leading to 20 test runs (Table 1). Center point

Table 1. Experimental set-up for the Response Surface Design (RSM) for testing the effect of H₂O₂ concentration, temperature and pH on DMS oxidation and DMSO formation.

Factor	$-\alpha$	-1	0	$+1$	$+\alpha$
H ₂ O ₂ [μM]	0	7.5	18.4	29.4	37.0
temperature [°C]	49.8	60	75	90	100
pH [-]	3.32	4	5	6	6.7

trials were investigated 6 times, axial and factorial points in triplicate.

Kinetic of DMS oxidation

Sample preparation for the time-course of DMS oxidation by H₂O₂ was carried out as described before; whereas, H₂O₂ concentration was adjusted to 48.3 μM. Samples were taken after reaching 95°C (0 min), 60-, 120-, 180-, 240- and 300 min. The samples were analyzed for DMS and DMSO as described previously.

DMS oxidation by various oxygen species

The incubation of DMS in combination with different compositions of oxygen, H₂O₂ and Fe(II) was carried out as previously described with following deviations: The pH of the model solutions was adjusted to 5.5. Oxygen concentration of 1.25 mM was adjusted by purging the media with pure oxygen at 20°C for 40 min. In the experiments where H₂O₂ was added, the model solutions were purged with nitrogen to reach oxygen concentrations below 2.5 μM. Moreover, EDTA (45 μM) was added. Stock solutions of Fe(II) and H₂O₂ for the mediation of hydroxyl radicals were prepared with ultrapure water (Milli-Q®), which was boiled and purged with nitrogen before 48.3 μM of Fe(II) and H₂O₂ were applied (Fe(II):H₂O₂:DMS = 3:3:1). Table 2 gives an overview of the experimental set-up.

Statistics and data analyses

All experiments in this work have been carried out in triplicate. The design of experiments as well as the multivariate data analysis was constructed with Designexpert 6® (Ver. 7.0, Stat-Ease, Inc., Minneapolis, MN, U.S.A.). A backward elimination of model terms was carried out at $\alpha > 0.1$ at a confidence level of 95%. The kinetic data are presented as means \pm standard deviation (s.d.). Analysis of variance (ANOVA) was carried out at a confidence level of 95% using Microsoft XLstat (version 2014.5.03, Addinsoft, U.S.A.). Classification of significant different groups was then performed by the Tukey Kramer honest significant difference test (HSD) at a confidence level of 95%.

Results and discussion

In the malting process, the grain germinates at high rates to achieve a fast proteolysis and cytolysis. At this stage, the primary precursor of DMS, SMM is synthesized. In the upfollow-

Table 2. Experimental set-up for testing the effect of O₂, Fe(II) and hydrogen peroxide on DMS oxidation and DMSO formation.

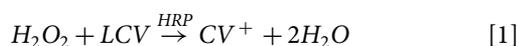
Experiment	Composition	Incubation time [min]
1	EDTA	0
2	O ₂ , EDTA	180
3	O ₂ + Fe(II)	180
4	H ₂ O ₂ , EDTA	60
5	H ₂ O ₂ , EDTA	180
6	H ₂ O ₂ + Fe(II)	60
7	H ₂ O ₂ + Fe(II)	180

ing kilning process, SMM is thermally degraded to DMS and homoserine. In addition to evaporation, substantial amounts of DMS are oxidized to DMSO, the precursor of DMS during fermentation.

Recently, we reported that the reaction of cysteine-copper complexes with molecular oxygen produced H_2O_2 , which was subsequently responsible for DMS oxidation.^[42] As mentioned in the introduction, the formation of H_2O_2 during natural germination of cereal seeds was reported before to correlate with certain stress conditions, which presents another pathway of H_2O_2 formation. However, to our knowledge, H_2O_2 formation was not investigated in barley germination conditions, which are representative for the malting process. This process can be regarded as a “forced germination.” To ascertain the abundance of H_2O_2 in germinated barley (green malt), samples were taken after the fifth day of germination. At this stage, the acrospire lengths were >0.8 related to overall grain length. The samples were processed as mentioned in the Material and Methods section.

Abundance of H_2O_2 in green malt

Figure 1 shows the absorption spectra of the chloroform extracts with different compositions. The green malt extract revealed only a slightly higher absorption between 250–350 nm than pure chloroform. The chloroform extract containing green malt, HRP, and LCV leads to a higher absorption between 250–350 nm, which was probably evoked by the dissolved enzyme itself. Moreover, a distinctive peak at ~ 590 nm was observable, which is in agreement with the findings of Mottola et al.^[47] They stated that the occurrence of this spectrum is related to the specific oxidation leuco crystal violet by H_2O_2 , which is catalyzed by HRP (equation 1):



In this reaction a H^+/e^- is abstracted from LCV thereby forming crystal violet (CV^+), which is able to absorb light in

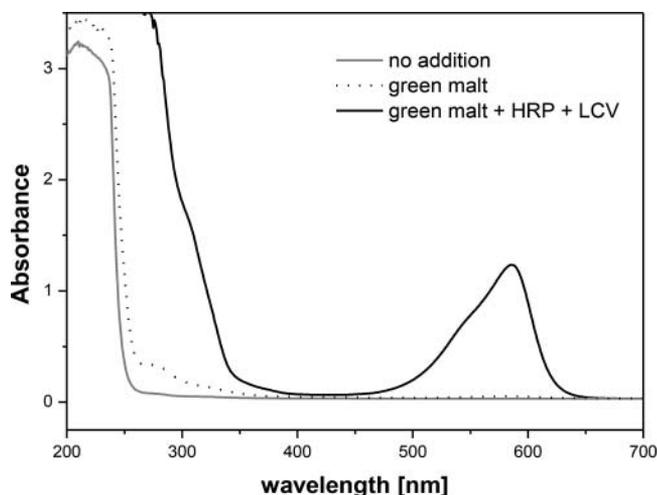


Figure 1. Identification of H_2O_2 in green malt. Absorption spectra of chloroform extracts with different compositions: no additions (gray line), green malt (black line, dashed), green malt with HRP and LCV (black line, solid).

Table 3. A 3-factorial experimental design for RSM of the effect of hydrogen peroxide concentration, temperature and pH on DMS oxidation and DMSO formation. Results are presented as mean values ($n = 3$), center point trials (0-0 combinations)* are shown as single determinations ($n = 5$).

Trial _{rand}	H_2O_2 [μM]	T [$^{\circ}C$]	pH [-]	DMS [μM]	DMSO [μM]
9	0.0	75.0	5.0	12.65	0.00
1	7.5	60.0	4.0	12.62	0.55
15*	18.5	75.0	5.0	10.32	2.15
20*	18.5	75.0	5.0	10.83	1.74
18*	18.5	75.0	5.0	10.92	1.78
12	18.5	100.2	5.0	10.19	2.51
13	18.5	75.0	3.3	10.89	2.25
14	18.5	75.0	6.7	10.92	1.64
4	29.5	90.0	4.0	7.55	4.41
7	7.5	90.0	6.0	11.48	0.61
10	37.0	75.0	5.0	9.70	2.57
2	29.5	60.0	4.0	12.58	0.56
19*	18.5	75.0	5.0	10.43	1.78
6	29.5	60.0	6.0	11.96	1.63
5	7.5	60.0	6.0	12.32	0.81
11	18.5	49.8	5.0	12.55	0.72
3	7.5	90.0	4.0	11.08	1.14
8	29.5	90.0	6.0	8.83	3.35
16*	18.5	75.0	5.0	10.91	1.70
17*	18.5	75.0	5.0	10.67	1.73

Table 4. ANOVA results of 2-factor interaction model (2-FI) terms of DMS oxidation and DMSO formation.

Factor /interactions	DMS		DMSO	
	F-value	p-value ^a	F-value	p-value ^a
2FI- model	30.1	< 0.0001	52.1	< 0.0001
A- H_2O_2 [μM]	48.7	< 0.0001	104.6	< 0.0001
B-temperature [$^{\circ}C$]	76.6	< 0.0001	67.6	< 0.0001
C-pH [-]	0.2	0.6289	1.4	0.2604
AB	20.8	0.0004	38.6	< 0.0001

^a probability that f-value of this size is determined if model terms are insignificant. Insignificant model terms exceeding the confidence level of $\alpha = 0.1$ were removed via backwards elimination

the indicated range. In the fate of the reaction, HRP donates a further H^+ that results in the accompanied formation of 2 water molecules. This specific reaction clearly provides evidence for the presence of H_2O_2 in the green malt and substantiates its potential role as a DMS oxidant in the upfollowing kilning process. The reaction between H_2O_2 and DMS was investigated in the upfollowing experiments.

Response surface modeling (RSM) of DMS oxidation by H_2O_2

In this step, RSM was used to ascertain the effect of physical-chemical parameters on the scope of DMS oxidation by H_2O_2 to evaluate its potential contribution to the levels of DMSO in malt. Even though we identified H_2O_2 in green malt, we do not have information regarding its actual quantity at this point. However, for the purpose of investigating the effect of pH and temperature, concentrations of H_2O_2 in a stoichiometric range required for DMS oxidation (H_2O_2/DMS : ~ 0.5 – 2.5) were used. The experimental design was leading to 20 test runs. Within this range, significant DMS oxidation (0–41.6%) as well as DMSO formation (0–35.2%) was observed (Table 3). Analysis of variance (ANOVA, Table 4) demonstrates that temperature,

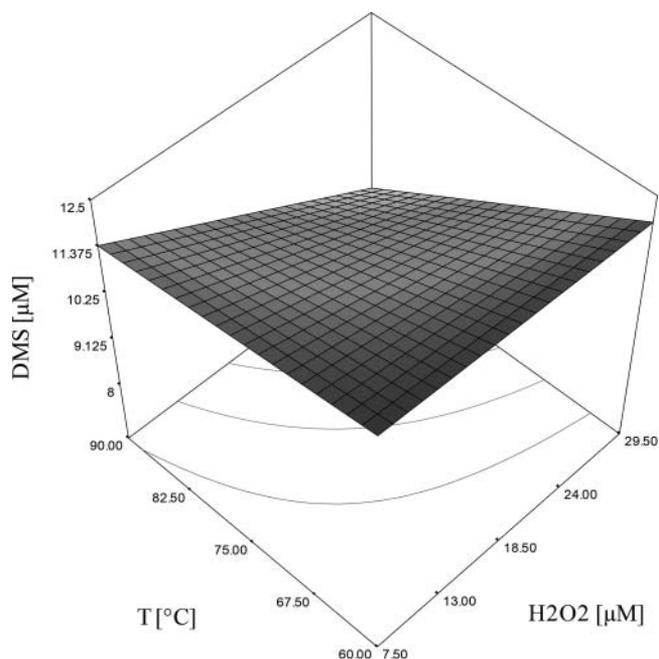


Figure 2. Response surface of the 2-factor interaction (2-FI) model of DMS oxidation by H_2O_2 at pH 5.5.

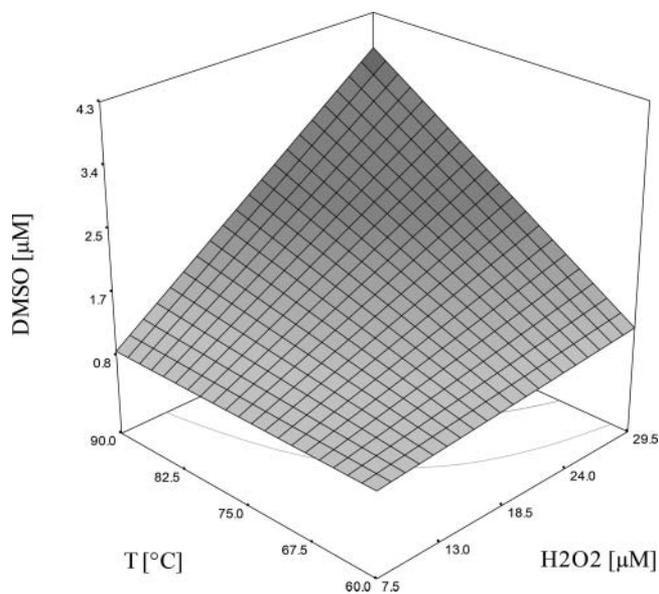


Figure 3. Response surface of the 2-FI model of DMSO formation through DMS oxidation by H_2O_2 at pH 5.5.

H_2O_2 concentration as well as their interaction had a significant impact on DMS oxidation as well as on DMSO formation. However, the effect of pH was found to be insignificant.

For both substances, 2-factor interaction models (2-FI) were chosen owing to their highly correlating coefficients of determination (R^2). For DMS, the coefficients of determination of the 2-FI model were determined to be: R^2 : 0.9149, adjusted R^2 (R^2_{adj}): 0.8845, predicted R^2 (R^2_{pred}): 0.8015. The correlation coefficients of the 2-FI model for the attributed formation of DMSO were R^2 : 0.9413, R^2_{adj} : 0.9203, R^2_{pred} : 0.8582. For both models the lack of fit (LOF) was insignificant (p -value ≥ 0.05), which further substantiates the applicability of the chosen models. Owing to the ANOVA data, the DMS oxidation in the

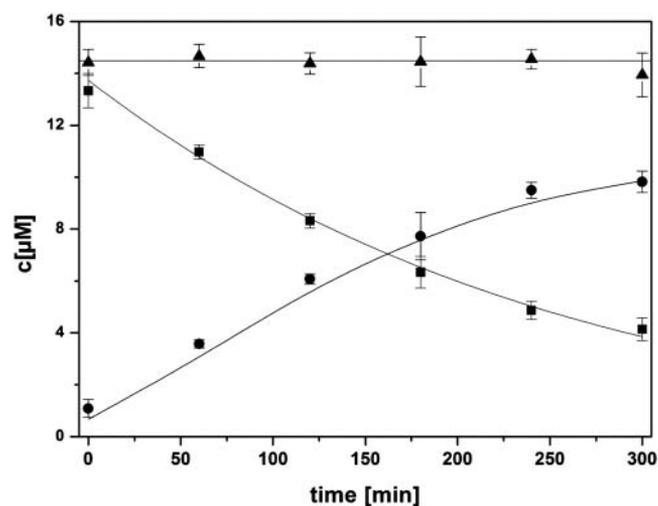


Figure 4. Reaction kinetics of DMS oxidation by H_2O_2 (1:3) at 95°C. DMS (squares), DMSO (circles), sum (DMS+DMSO) (triangles). Results are shown as mean values ($n = 3$) \pm standard deviation (s.d.).

applied experimental set-up was found to be best predicted by the following equations (coded factors):

$$DMS = 10.97 - 0.85 \times [H_2O_2] - 1.06 \times T - 0.72 \times [H_2O_2] \times T \quad [2]$$

$$DMSO = 1.68 + 0.82 \times [H_2O_2] + 0.66 \times T + 0.65 \times [H_2O_2] \times T \quad [3]$$

The three-dimensional response surfaces of the 2-FI models of the DMS oxidation and the DMSO formation are illustrated at pH 5.5 in Figure 2 and Figure 3. The figures depict a parallel oxidative consumption of DMS and a concomitant formation of DMSO. Within the variations of the H_2O_2 : DMS ratio, no significant difference was found between the respective sums of DMS and DMSO. This was reported before for DMS oxidation by H_2O_2 below 35°C^[40] and suggests that no further oxidation of DMSO took place, even though much higher temperatures and H_2O_2 :DMS ratios were applied in our study. The observed insignificant effect of pH is also in line with the results of Adewuyi and Carmichael.^[40] In their investigations DMS oxidation by H_2O_2 did not significantly deviate between pH ~ 1.5 –9 at 20–30°C. They stated, that H_2O_2 may be subject to protonation ($H_3O_2^+$), which can donate OH^+ much easier to the nucleophile sulfur atom. Thus, only at pH < 1, sufficient amounts of H_2O_2 can be protonated to increase the rate constant. Near stoichiometric equilibrium of DMS and H_2O_2 , they established DMSO as only oxidation product.

Kinetics of DMS oxidation by H_2O_2

A chemical reaction in food can only be relevant to the subject of investigation, if the reaction rate proceeds fast enough to compete with other reactions. To gain information regarding the reaction rate between DMS and H_2O_2 , we monitored the DMS consumption and DMSO formation over time at 95°C (Figure 4). The reaction between DMS and H_2O_2 can generally

be written as follows:



This reaction was reported to follow an overall bimolecular second order mechanism in which the consumption of the respective reaction partners is a first order process.^[40,41] As the effect of pH was shown to be insignificant, we investigated the oxidation of DMS by H₂O₂ only at a pH of 5.5.

In our case, an exponential decline of DMS (R^2 : 0.9937) was observed, which indicates that the oxidative consumption of DMS at the given experimental parameters follows a first order reaction. In general, such “pseudo” first order reactions are mostly known for reactions in which one reaction partner is present in high excess. In this study the H₂O₂ concentration was merely 3 times higher than the DMS concentration. Still, DMS oxidation can be calculated by the integrated first order rate law (equation 4), by which the rate constant was determined to be 0.00403 min⁻¹.

$$\text{DMS} = \text{DMS}_{t=0} \times e^{-kt} \quad [5]$$

In comparison to the thermal hydrolysis of SMM in wort at 95°C and a pH of 5.3 ($k = 0.01 \text{ min}^{-1}$),^[48] DMS oxidation by a 3 fold overshoot of H₂O₂ is merely ~2.5 times lower. This relationship supports the theory that H₂O₂ is a potent DMS oxidant in malt. Even though H₂O₂ is very likely to react with a multitude of further malt endogenous substances, like thiols, fatty acids or transition metal ions, this result may explain the relative high levels of DMSO in malt.

Oxidation of DMS by O₂, H₂O₂, and •OH

After the physical-chemical characterizations of the reaction between DMS and H₂O₂, the impact of further oxygen species on DMS oxidation was investigated. To assess the effect of potential oxidants, 7 trials were carried out under different conditions, such as abundance and absence of oxygen, H₂O₂ and Fe(II), and the metal ion chelator EDTA. The different combinations were incubated with DMS in model solutions at 95°C for either 60 or 180 min (Figure 5). The oxygen concentration was adjusted by purging pure oxygen into the model solution, resulting in 1.25 mM, which is five times higher than the maximum atmospheric concentration of oxygen in water at 20°C. To exclude the role of potentially abundant trace levels of transition metal ions, which can potentially reduce oxygen to states of higher reactivity, EDTA was applied in overshoot to chelate and inactivate those metal ions. The levels of DMS did not significantly alter within 180 min at 95°C (column 2), compared to the reference (column 1), which was analyzed after reaching of 95°C. Also, no DMSO formation was observed.

From these results, it can be concluded that molecular oxygen in its ground state is not capable to oxidize DMS under such conditions. When Fe(II) was introduced to the oxygenated system, significant DMS consumption and DMSO formation took place. However, the oxidative consumption of DMS was solely ~10%. In this case Fe(II) may have donated an electron

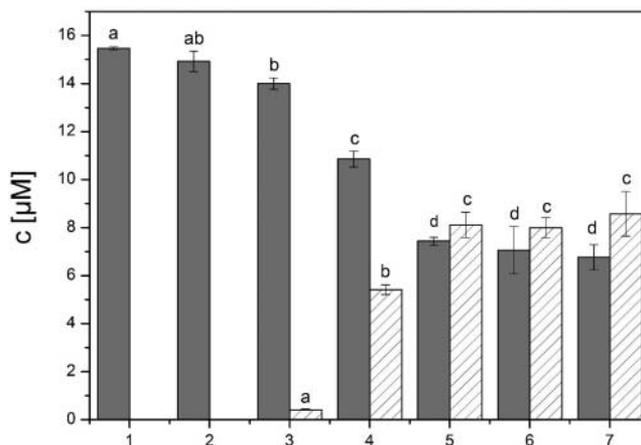
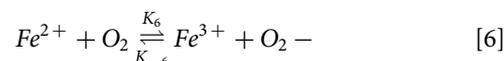


Figure 5. Effect of O₂, Fe(II) and H₂O₂ on DMS oxidation. DMS concentration (gray bars) and DMSO concentration (dashed white bars) after incubation of DMS at 95°C in buffered model systems. 0 min (1), 180 min, oxygenated, EDTA (2), 180 min, 48.3 μM Fe(II), oxygenated (3), 48.3 μM H₂O₂, EDTA, 60 min (4), 48.3 μM H₂O₂, EDTA, 180 min (5), 48.3 μM H₂O₂, 48.3 μM Fe(II), 60 min (6) 48.3 μM H₂O₂, 48.3 μM Fe(II), 180 min (7). Mean values (n = 3) are presented with error bars (±standard deviation), letters indicate significant differences according to Tukey (HSD) test at p < 0.05.

to oxygen, thereby forming superoxide radicals according to equation 6.



However, contribution of equation 6 to elevated formation of superoxide radicals is disputable. The redox potential difference of O₂/O₂⁻ and Fe³⁺/Fe²⁺ ($E^0_{(\text{O}_2/\text{O}_2^-)} = -0.16 \text{ V}$, $E^0_{(\text{Fe}^{3+}/\text{Fe}^{2+})} = 0.77 \text{ V}$) indicates that the reaction is less thermodynamically favorable and requires energy for its maintenance (endergonic reaction). We applied the Gibbs free energy differences (ΔG) to demonstrate the scope of this reaction (n : 1 M, R : ideal gas constant, F : Faraday constant).

$$\Delta G = -n \times F \times E^0_{\text{O}_2} = R \times T \times \ln(K_{\text{O}_2}) \quad [7]$$

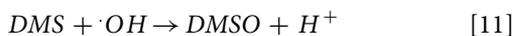
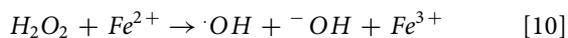
$$\Delta G = -n \times F \times E^0_{\text{Fe}} = R \times T \times \ln(K_{\text{Fe}}) \quad [8]$$

By subtraction of equation 8 from equation 7 and exponentiation, the ratio of the equilibrium constants can be calculated according to Wood^[49] (equation 9).

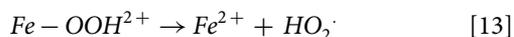
$$\frac{K_6}{K_{-6}} = \exp \left[\left(\frac{F}{R \times T} \right) \times (E^0_{\text{O}_2} - E^0_{\text{Fe}}) \right] = 10^{-16} \quad [9]$$

The small value of the K-ratio demonstrates that the equilibrium is rather on the left side of the reaction since K_6 is 10¹⁶ times smaller than K_{-6} , meaning that the backward reaction diminishes excessive superoxide anion accumulation. This circumstance may explain the low amount of DMS oxidation probably aroused by the small quantities of O₂⁻ or lower valence states of oxygen (H₂O₂ or •OH). A much higher DMS oxidation was aroused, when the 2-electron reduced state of oxygen, namely H₂O₂ was present (~30% after 60 min, ~52% after 180 min). The potential reaction mechanism (equation 4) was previously discussed.

EDTA was used to prevent Fenton-like reactions of H_2O_2 with potentially abundant transition metal ions. In the absence of EDTA and presence of $48.3 \mu M$ Fe(II) and $48.3 \mu M$ of H_2O_2 , DMS was already consumed to $\sim 55\%$ after 60 min (column 6). The DMS consumption at this stage was significantly higher compared to the exclusive application of H_2O_2 . However, after 180 min, no significant difference between the overall oxidative DMS consumption could be detected. The formation of $\cdot OH$ via the Fenton reaction (equation 10) is well known. $\cdot OH$ may then have oxidized DMS according to equation 11.



We believe that DMS was oxidized by $\cdot OH$ much faster than by H_2O_2 as it was also reported for reactions at ambient temperatures.^[40,50] However, the fact that levels of DMS and DMSO did not significantly differ for $\cdot OH$ and H_2O_2 after 180 min may have been aroused by iron peroxide ($Fe-OOH^{2+}$) formation according to Perez-Benito.^[51]



After $\cdot OH$ formation through the Fenton reaction, Fe^{3+} may have reacted with residual H_2O_2 in a side reaction to form $Fe-OOH^{2+}$ (equation 12), which decomposes slowly to the hydroperoxyl radical (equation 13). The latter is generally less reactive than $\cdot OH$. These reactions may have abated the $\cdot OH$ mediated DMS oxidation.

More investigations have to be carried out to determine the rate constants of DMS with H_2O_2 and $\cdot OH$ in malt and malt-like matrices at different concentration ratios to further evaluate the impact of these reactions on the levels of DMSO in malt.

Conclusions

Based on the results of this study, a proposal on the origin of DMSO in malt is presented in Figure 6. During malting the grain is forced to germinate at high rates by factors, like com-

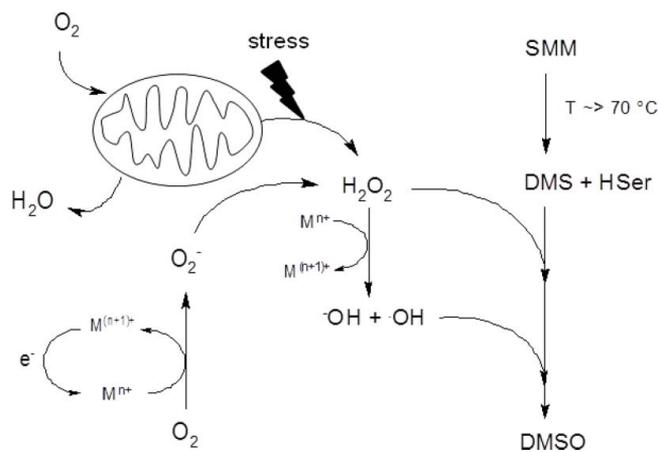


Figure 6. Proposed reaction mechanisms of DMSO formation during malting.

bined wet- and dry steeping procedures, increased temperatures as well as aeration. From the plant physiological perspective, those unnatural treatments are representative abiotic stress factors, which lead to H_2O_2 formation in the embryo during respiration in the mitochondria. Especially the transition from germination to the withering procedure, where the majority of water is removed at temperatures between $45-65^\circ C$, can probably be regarded as crucial point for H_2O_2 formation. As the majority of SMM is also synthesized and hydrolyzed to DMS in the embryo,^[13] it is likely that accumulated H_2O_2 is primarily responsible for the formation of DMSO in malt. Yang et al.^[16] reported that barley genotype has a decisive impact on the levels on SMM, DMSO, and DMS. The extent to which barley variety also influences the formation H_2O_2 remains to be investigated. In addition to the enzymatic formation of H_2O_2 , autoxidative reactions during malting may also lead to oxygen reduction and formation of ROS, which are able of DMS oxidation. Although this work demonstrated that the transition metal ion Fe^{2+} was not capable to form elevated amounts of ROS in oxygenated model solutions, such reactions may be catalyzed by certain reductones or antioxidants,^[42,52,53] which act as electron donor for transition metal ions (M^{n+}).

Certainly, the amount of SMM formation and -degradation determines the levels of DMS being released during kilning, which can be oxidized to DMSO. The scope of DMS oxidation depends on the abundance of reactive oxygen species which are able to oxidize DMS to DMSO. In this work, H_2O_2 as well as hydroxyl radicals were shown to be powerful DMS oxidants. It is also possible, that superoxide - or nitric oxide radicals are capable of DMS oxidation; however, this was not investigated in this work.

If the current germination technology leads to elevated H_2O_2 formation, the generation of hydroxyl radicals is also very likely to occur via the Fenton reaction as the steeping water and the brewing cereals contain significant amounts of transition metal ions, like iron or copper. Therefore, the aim for prospective research needs to be identification of the main factors leading to H_2O_2 during germination, withering, and curing in order to prevent extensive DMSO formation. Through such approaches, it may be possible to minimize the levels of DMSO in the pitching wort, which will reduce the risk of DMS formation during fermentation.

In addition to its role as a DMS precursor, DMSO may find applicability as metabolic indicator of oxidative reactions in connection to a bigger scope of malt endogenous substances susceptible to oxidation. For example, fatty acid autoxidation leads to fatty acid hydroperoxides,^[54] which may possess a certain DMS oxidation potential as well. Moreover, they are precursors for beer staling aldehydes like trans-2-nonenal or hexanal.^[55] Also, amino acid oxidation was shown recently to be a source of Strecker aldehydes in beer.^[33] The formed aldehydes can be bound to cysteine complexes^[56] or sulfite complexes^[56-58] in the early stages of malt and beer production and may be released during beer ageing.

Oxidation of proteins, in particular thiol groups may diminish enzyme activity. Also, H_2O_2 was reported to increase wort turbidity.^[32] All these reactions are potentially affected by H_2O_2 formation in the malting process.

Considering steps for minimization of oxidative reaction, antioxidants can be regarded as powerful antagonists. Plants consist of a complex enzymatic antioxidative defense system (e.g., superoxide dismutase, catalase, glutathione reductase, or MSRA) that protect plant constituents, in particular DNA, lipids, and proteins from deterioration by ROS. Additionally, naturally abundant or exogenously supplemented antioxidants, such as thiols, ascorbic acid, (poly-)phenols, or sulfite, reveal the ability to scavenge ROS and support the antioxidative defense system. However, some of the mentioned antioxidants were also reported to elevate oxidative reactions via transition metal ion reduction and the concomitant maintenance of ROS formation via oxygen reduction.^[42,52,59,60] Today, the actual conditions to which antioxidants reveal antioxidative and/or pro-oxidative behavior are still not well understood and need further research to increase the beneficial effect of these substances.

Based on the methodology of H₂O₂ assessment in this work, we are currently developing a method for the quantitation of H₂O₂ in diverse samples from the raw grain up to malt, mash, and wort. Combined with other powerful tools, such as EPR spectroscopy,^[53] the quantitation of H₂O₂ should provide maltsters and brewers with an additional tool to evaluate and optimize their processes with respect to oxidative reactions and their consequences for processability, as well as wort- and beer quality.

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4 Summarizing discussion

Beer flavor is the key factor in creating consumer demand and consequently for the economic success of a brewery. The dialkyl sulfide DMS has a critical impact on beer flavor as it occurs in most beers at concentrations above the lowest reported flavor threshold of 25 $\mu\text{g/L}$.^{26,208,209} With an odor activity value of 59, DMS was also proposed as being an important contributor to the overall aroma of a Pilsner-type beer.²⁷ DMS can also lead to undesirable, “cooked cabbage”- or “canned maize”-like aroma impressions that may cause beer to become undrinkable and be rejected by customers. In order to control the levels of DMS in beer it is vital to completely understand its formation pathways and origins in beer. The role of SMM in releasing DMS upon heating and its technological implementations are well understood. However, there are significant knowledge gaps and discrepancies regarding the contribution of DMSO to beer DMS levels.

4.1 Method development for DMSO quantification

There was no established method for quantifying DMSO in common international malting- and brewing-related compilations of analytical methods.^{54,55,201} The starting point of this dissertation work was therefore to develop a method for DMSO quantification that can be used for diverse malting- and brewing-related samples to investigate the redox behavior of DMS and DMSO. As a result of the high water solubility and the high boiling point of DMSO, it is not possible to directly apply headspace gas chromatographic techniques for its quantification. Its distribution coefficient (ratio of DMSO concentration in the aqueous sample and in the headspace) can be expected to be <1 . Consequently, the levels of DMSO in the headspace at equilibrium state cannot be determined by flame photometric detection, for example. Therefore the first approach was to reduce DMSO to the equivalent amount of volatile DMS, which can be quantified by established headspace gas chromatography (Publication A²⁰²).

The first experiment aimed to verify the applicability of sodium metabisulfite as a DMSO-reducing agent in a buffered model solution as proposed by Dickenson²¹⁰ (Publication A,²⁰² Figure 1 a). The chromatogram generated after incubating a buffered model solution containing 288 $\mu\text{g/L}$ DMSO and 2 g sodium metabisulfite using the isothermal oven program⁵⁴ illustrated that the DMS signal overlapped with another signal derived from sodium metabisulfite. This was already observed by Yang and Schwarz,⁶⁵ which prompted the authors to reject sodium metabisulfite as a DMSO-reducing agent. In this study, the issue was

overcome by using a different GC oven program that started at 35 °C, which is below the boiling point of DMS. The program included a heating gradient of 7 °C/min to 55 °C and a faster temperature increase (60 °C/min) up to 150 °C. The chromatogram of the optimized oven program (Publication A,²⁰² Figure 1 b) shows an appropriate, clear separation of the DMS signal and the signal derived from sodium metabisulfite. The latter consists of at least two substances, one of which can be expected to be SO₂, as it is released into the headspace from heating aqueous bisulfite anion (HSO₃⁻) solutions. Further substances that are potentially included could not be identified in this work. However, the optimization of the GC oven program was sufficient to further test this methodology for its application in malting- and brewing-related media. Sulfite is a naturally occurring substance in beer and compared with other methods^{198,199} its application for DMSO reduction represents rather mild reducing conditions. It was therefore of interest to test more potential reducing substances derived from malt and beer in terms of their capability and hence applicability for DMSO reduction. Besides sodium metabisulfite, the thiols L-cysteine (Cys) and L-glutathione (GSH), ascorbic acid (Asco) and the phenols gallic acid (GA) and ferulic acid (FA) were combined with increasing concentrations of DMSO in buffered model solutions. While Asco and the phenolic compounds did not generate DMS from DMSO, the DMS concentration increased exponentially (PFPD response) in the case of the thiols and sodium metabisulfite (Publication A,²⁰² Figure 2). However, sodium metabisulfite revealed the highest DMSO-reducing capability by far under the applied experimental conditions. The sample preparation and concentration was therefore optimized further for sodium metabisulfite as DMSO-reducing agent. As a premise for the application of this substance for DMSO reduction and subsequent analysis of DMS, it was assured that other potential precursors of DMS present in malt, wort or beer (SMM, DMSO₂, Cys, L-cystine, GSH, MetSO, H₂S) were unaffected by this procedure and did not contribute to the quantification of DMSO (Publication A,²⁰² Table 1). The method (as described in detail in the Materials and Method section of Publication A²⁰²) was now used to assess the recovery rates of DMSO in a 100% Pilsner malt pitching wort (12°P) as well as in a Pilsner-type beer (Publication A,²⁰² Figure 3). The addition of DMSO in different concentrations led to a linear increase and adequate recovery rates for the pitching wort (R²:0.9998; recovery rate: ~96.5 %) and for the Pilsner-type beer (R²:0.9984; recovery rate: ~99 %), indicating low matrix effects of the method. These data therefore demonstrated that the method could be used to quantify DMSO in malt extracts, wort and beer (Publication A,²⁰² Table 2). It was striking that most of the commercially available malt types tested contain significantly different amounts of DMSO (roasted malt < wheat malt < Pilsner malt <

Munich malt I < Munich malt II), which are also relatively high compared with the commonly accepted threshold for the primary DMS precursor, SMM, which is 5-7 $\mu\text{g/g}$.²¹¹ However, there is no threshold for DMSO in malt. The levels of DMSO found in the malts are also higher than reported in the early 1980s.^{46,49} In the roasted malt no DMSO could be detected (<0.06 $\mu\text{g/g}$). Those malts are generally treated with a short germination process, also resulting in low levels of SMM^{38,39} that can potentially be converted into DMS and DMSO during the kilning process. Even though it is unlikely to occur at temperatures below 100 °C, DMS may have been completely oxidized to DMSO₂⁴⁶ at the high temperatures generally used for roasted malts (~180-220 °C).²¹² Compared with Pilsner barley malt, the DMSO concentration was significantly higher in the dark barley malts (Munich malt I & II), whereas the highest levels of ~10.3 $\mu\text{g/g}$ was found in the darker Munich malt II. Munich-type malts are commercially produced at higher steeping degrees and higher germination temperatures to achieve greater modification and higher formation of low molecular peptides, amino acids and carbohydrates. The latter undergo an accelerated Maillard reaction leading to a darker malt color and more intense aroma compound formation during the withering and kilning processes. Higher temperatures (80-105 °C) are also applied here than for Pilsner malt (80-85 °C).²¹² These technological parameters lead to a significant higher formation of SMM during germination and withering as well as greater hydrolysis of SMM during kilning.^{38,39,41,213} The higher availability of DMS combined with the higher temperatures is what is thought to be responsible for the higher levels of DMSO in the Munich-type malts. Wheat malt contained significantly less DMSO than the Pilsner malt. A possible explanation for this may be in the acrospires of wheat malt that contain a large proportion of SMM, which is removed during cleaning. It is expected that a major release of DMS and its oxidation to DMSO takes place in the acrospires, which is also removed by the cleaning process. Also, wheat malt does not contain husks, which may act as a boundary layer of DMS that retains its evaporation from the grain and thus increases the probability of DMS oxidation. However, more precise deductions on the different levels of DMSO in malt cannot be drawn because no specific production parameters were available from the commercially obtained malts. The data did give an insight into the significant quantities of DMSO in different malt types and the method was proven to be applicable to investigate the redox behavior between DMS and DMSO in the brewing process.

4.2 Relevance of DMSO as a DMS precursor in beer

The next milestone of this work was to assess the behavior of DMSO in the brewing process. Specifically, the objective was to investigate the extraction of DMSO as well as the redox behavior in terms of DMS oxidation in wort and DMSO reduction by diverse yeast strains during fermentation (Publication B²⁰³).

The first experiment of this publication was carried out to test if DMS undergoes oxidation during mashing. Mashing was therefore conducted in a closed system to avoid extensive DMS evaporation and to increase its availability of being oxidized. The same mashing regime was applied in an open system and the data on DMS, SMM and DMSO were compared before- and after mashing. In the open mashing system the DMS concentration was significantly reduced to ~63 %. As a result of preventing evaporation, the DMS concentration in the closed system was similar to the levels of DMS found after mashing-in (5 minutes). Even though the residence time of DMS was higher in the closed system, the concentration of DMSO was similar to that found in the open system. The levels of SMM in both systems remained approximately constant during mashing and did therefore not contribute to DMS formation. Consequently, it was proposed that the increase of DMSO was related to an extraction process from the malt particles into the aqueous mash rather than to DMS oxidation. It was assumed that potentially abundant reactive oxygen species (ROS) from mash⁷⁶ have preferably reacted with other constituents that are known to be susceptible to oxidation, for example fatty acids,^{68,214} thiols¹⁷⁷ or polyphenols.^{83,196}

Another explanation may be found in the presence of oxygen-scavenging enzymes,^{76,196} such as superoxide dismutase or (per)oxidases that may have diminished the availability of ROS for DMS oxidation. This suggestion would be in line with the findings of Muller¹⁷⁷ as he was not able to detect H₂O₂ in mash before acidic treatment, whereas after treatment peroxidases were inactivated in the mash and H₂O₂ was detectable. Still, the possibility that a DMS redox reaction did occur in the mash should not be excluded even if the data did not indicate this. The results also indicated that the contribution of such reactions to the DMSO levels in the mash is negligible.

However, such aspects could probably be explained by adding deuterated DMS (DMS-d₆) to the mash and assessing DMSO-d₆ after mashing via mass spectrometry. Based on the unavailability of such a method and the aforementioned low probability of DMS oxidation, no such effort was made within the scope of Publication B.²⁰³ In a consecutive trial, the levels of DMS and its precursors SMM and DMSO were monitored over time in an isothermal (62 °C),

open mashing regime (Publication B,²⁰³ Figure 2) Within the first 5 minutes of mashing the DMS concentration was already reduced to ~30 %. The relative volatility of DMS at 60 °C was determined by Scheuren et al.²¹⁵ to 657. This index demonstrates that, at equilibrium state of an aqueous DMS solution, the DMS concentration in the gas phase is 657 times higher than its concentration in the liquid phase. It was therefore proposed that rapid diffusion of DMS in the initial stage of mashing was related to its high relative volatility and to the concentration gradient between aqueous and gas phase (DMS concentration in the gas phase at $t=0$ min: ~0 $\mu\text{g/L}$). In the further course of mashing the decline of the DMS concentration decelerated, which was explained by a smaller DMS concentration gradient and a consequently slower diffusion of DMS through the small opening of the mashing beaker cap. The concentration of DMSO was significantly increased in the first 10 minutes of mashing, whereas no significant change was observed from this point onwards until the end of mashing. The data demonstrate that DMSO is extracted from the grist particles into the mash within 10 minutes. Based on the DMS diffusion behavior and aspects of ROS availability, oxidation of DMS is unlikely to have caused the observed increase of DMSO. The SMM concentration remained constant during mashing and was explained by its relative stability against thermal decomposition below 70 °C, which was in accordance with previous investigations.^{39,42,42} Even though both compounds reveal high water solubility, the extraction of SMM was faster compared with DMSO and not measurable with the chosen experimental time intervals. The chemical-physical properties of DMSO seem to be a reasonable explanation for this phenomenon. Based on the amphiphilicity of the sulfoxide group (see 1.2.1), DMSO is capable of interacting with proteins²¹⁶ and amylose²¹⁷ by displacing water and thereby acting as a hydrogen binding competitor. It is notable that the interaction of DMSO with hemicelluloses, such as β -glucans or pentosans, was suggested to be likely to occur in mash as well. In this case, together with the aspects of protein interactions, DMSO extractability would correlate with malt modification. Those aspects remain to be investigated, though. In the following experiment the behavior of DMS and its precursors was investigated during an atmospheric wort boiling process (Publication B,²⁰³ Figure 3). SMM was exponentially degraded following a first order mechanism (cp. 1.2.1, Equation 1). The determined rate constant of $k=0.021 \text{ min}^{-1}$ was consistent with the data reported by Dickenson.⁴⁴ It was therefore proposed that SMM was thermally degraded to DMS and homoserine. After 30 minutes of wort boiling none of the DMS present at the beginning of boiling (~150 $\mu\text{g/L}$), nor any of the generated DMS could be detected. This observation was explained by the well-established evaporation behavior of DMS.^{42,44,51,53,215,218,219} The limiting separation factor at infinite

dilution (K^∞) of DMS at 98.55 °C was determined by Hertel et al.²¹⁸ to be 75.6. This value is much lower compared to the relative volatility at 60 °C as discussed in relation of DMS desorption during mashing. This was in line with the findings of Scheuren et al.,²¹⁸ which demonstrated that the relative volatility of DMS decreases with rising temperature. According to Dohnal et al.²²⁰ K^∞ should be equal to the relative volatility (α^∞) (Equation 2), which can be determined using a distillation method by recording the total mass of a binary mixture (e.g. DMS and water) (n) and the concentration (x) of the highly diluted substance in the liquid phase (in this case DMS) before and after distillation.

$$\alpha_{i,j}^\infty = \lim_{\substack{x_i \rightarrow 0 \\ x_j \rightarrow 1}} \left(\frac{y_i/x_i}{y_j/x_j} \right) \approx \lim_{\substack{x_i \rightarrow 0 \\ x_j \rightarrow 1}} \left(\frac{y_i}{x_i} \right) = K_i^\infty \quad \text{E2}$$

$$\alpha_{1,2}^\infty = \frac{\ln \left(\frac{x_{1,1}}{x_{1,0}} \right)}{\ln \left(\frac{n_1}{n_0} \right)} + 1 \quad \text{E3}$$

A possible explanation for the temperature dependency of α^∞ can be found in Equation 3. The fact that the operation temperature in the study of Hertel et al.²¹⁸ was close to the boiling point of the solvent (water) implies that more water also enters the gaseous headspace and consequently compensates for the high excess in DMS in the gas phase according to Equation 3. However, this condition does not imply that less DMS can be evaporated at higher temperatures as the diffusion rate of DMS and therefore the time required to reach such equilibria is not supported by these data. According to the Stokes-Einstein-Relation (Equation 4: D : diffusion coefficient; k : Boltzmann's constant; T : temperature; η : dynamic viscosity; r : radius of molecule (simplified)), the diffusion coefficient of dissolved molecules rises considerably with temperature.

$$D = \frac{kT}{6\pi\eta r} \quad \text{E4}$$

Therefore, future research should assess the temperature-dependent diffusion coefficients to provide an overall kinetic characterization of the evaporation behavior of DMS and other volatiles during wort production. However, it can be expected that DMS was readily lost via

diffusion and evaporation of water as carrier, and that its residence time in the hot wort was transitory. The linear increase in the DMSO concentration was therefore proposed as being a result of a concentrating process, in which the loss of water increased the relative concentration of DMSO in the wort. DMS oxidation was presumed to be unlikely in this context. This assumption was proven by using a rectification column in which the overall water evaporation was reduced from ~16 % to ~0.2 %. In this regard the DMSO concentration was not significantly affected. This finding also provided evidence for the non-volatile nature of DMSO in wort, which was only suspected previously.⁴² Retaining the heat of the wort in hermetically closed glass bottles excluded water evaporation and loss of DMS via diffusion. As expected, SMM was also degraded exponentially (Publication B,²⁰³ Figure 3). However, this time the rate constant was determined as $k=0.017 \text{ min}^{-1}$, which was lower than found for the atmospheric boiling process. This observation was explained by the lower temperature reached in the bottles (98 °C) as a result of insufficient temperature gradient from the samples to the water bath (99.9 °C).

By summarizing the difference between SMM at $t=0$ minutes and SMM at $t=30$ minutes according to Equation 1 (SMM degradation) together with the levels of DMS analyzed at $t=0$ minutes, ~439 µg/L DMS were generally available for oxidation. From this quantity ~70 µg/L were recovered as DMSO. The lower increase in DMS after 30 minutes (~380 µg/L) substantiated the fact that DMS was oxidized to DMSO in this period. Further wort heat retention did not significantly affect the levels of DMSO and the overall increase of DMSO was determined as ~15 %. It was proposed that significant oxygen activation and formation of ROS, such as hydrogen peroxide or hydroxyl radicals took place, especially during the heating period from 20 °C to ~98 °C as oxygen is depleted at higher temperatures with decreasing solubility. ROS may then have oxidized DMS especially at >70 °C, where significant SMM hydrolysis starts. Compared with the levels of DMSO introduced by the malt, it was concluded that the influence of wort production on the final levels of DMSO in wort is relatively small and that the majority of DMSO is formed in the malting process. Subsequent experiments in Publication B²⁰³ aimed to investigate the reduction of DMSO during fermentation. To test the impact of wort composition, the grist composition was varied by using different malt types. The behavior of DMS, DMSO and SMM in mash and wort containing dark-/specialty malt or a barley proportion was similar to the behavior described when using a 100 % Pilsner malt. However, the different concentrations of DMSO in the malt types, as determined in Publication A,²⁰² were proportionally recovered in the mash (unpublished data, page 46). Consequently, the resulting pitching worts contained

significantly different DMSO concentrations. Surprisingly, irrespective of the raw materials used, the DMSO concentration did not change substantially during fermentation (Publication B,²⁰³ Figure 4 a) but there was still significant DMS formation (8-14 µg/L) (Publication B,²⁰³ Figure 4 b). However, the respective increase in the DMS concentration did not correlate with the quantity of DMSO in the pitching worts. It was reported that the free amino nitrogen content and the methionine concentration in particular have an important influence on DMSO reduction during fermentation via nitrogen catabolite repression.^{221,222} The investigated worts contained FAN levels of 116-184 mg/L and methionine levels of 8-21 mg/L (Publication B,²⁰³ Table 1) but those factors did not correlate with DMS formation either. In the study by Gibson et al.²²¹, the reduction of DMSO by lager yeast decreased from ~70 % to ~20-30 % when the FAN concentration was increased from 70- to 3500 mg/L. Sugai et al.²²² observed a decline of ~20-30 % in the activity of MSRA (DMSO-reducing enzyme) in lager yeast by adding ~597 and ~2984 mg/L of methionine to a glucose salt medium. In both studies,^{221,222} the FAN and methionine concentrations were extremely high and not representative of the levels generally found in wort. Therefore, it was proposed that the variations in FAN and methionine in the pitching worts were insufficient to cause differences in DMSO reduction and DMS formation. In another study by Gibson et al.,²²³ methionine sulfoxide (MetSO) was reported to inhibit DMSO reduction during fermentation. This seems reasonable because it is the actual substrate of MSRA (cp. 1.2, Figure 7). Unfortunately, no data were available on the levels of MetSO in the pitching worts in Gibson's or our study. But it is likely that different levels of MetSO in the pitching worts produced from different malts suppressed DMSO reduction to deviating, yet unknown extents.

It is well established that SMM cannot be converted to DMS by yeast.^{42,44} Also, no contaminants capable of forming DMS from SMM were found in any of the pitching worts. Therefore the question of the DMS origin remained to be resolved. It is important to mention that the DMSO concentration before fermentation (Publication B,²⁰³ Figure 4a) was determined from the supernatant after yeast addition and centrifugation, implying that the yeast was already in contact with wort ingredients. In the next experiment, the behavior of DMSO was investigated starting from the pitching wort and across the entire fermentation process (Publication B,²⁰³ Figure 5). The first data point of DMSO represented the DMSO concentration in the pitching wort. After yeast addition and centrifugation, the DMSO concentration was determined in the supernatant (approximately 30 minutes after yeast addition). During this period the DMSO content was already reduced to ~13 %. No DMS could be detected at this time. 24 h after yeast addition the DMSO concentration was already

reduced to ~22 % and the DMS concentration was increased to ~8 $\mu\text{g/L}$. During fermentation, a slight increase of DMSO was noted. The overall DMSO reduction was ~11 % and 9-12 $\mu\text{g/L}$ DMS were formed. An ultrasonic treatment and accompanied extraction of the yeast after fermentation day 4 revealed that substantial amounts of DMSO could be recovered from the cells. It was thereby deduced that the yeast had quickly absorbed and retained a part of the DMSO from the wort. The results also indicate that an uptake of DMSO is not strictly related to its enzymatic reduction to DMS. Again, the amphiphilicity of DMSO (cp. 1.2.1) was proposed as a reasonable explanation. DMSO is widely used in pharmacology as a drug penetration enhancer.²²⁴ In biology it serves as a cryoprotectant for enzymes and cell cultures as it induces dehydration inbetween phospholipid bilayers whereby it avoids ice formation.²²⁵ It was reported by Leekumjorn and Sum^{225,226} that a proportion of DMSO diffuses rapidly into phospholipid bilayers where it accumulates. Chang and Dea²²⁷ suggested a mechanism in which the amphiphilic DMSO molecules interact with the positively charged choline groups and the negatively charged phosphate head groups. Based on their findings it was proposed that part of the DMSO from the wort accumulated at the plasma membrane of the yeast. For demonstration phosphatidylcholine was used as a representative phospholipid from yeast plasma membranes (Figure 19).

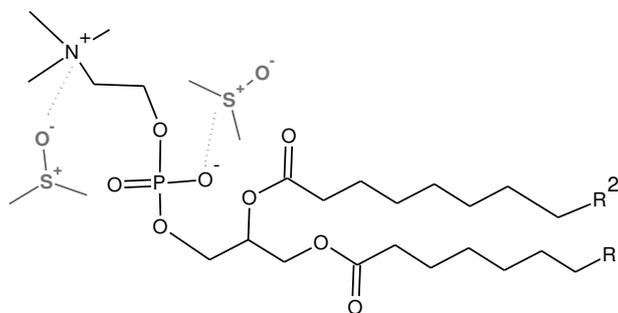


Figure 19: Proposed interaction of DMSO with phosphatidylcholine of yeast plasma membrane adapted from Chang and Dea²²⁷ (dotted, gray lines: non-polar interactions; +/- on DMSO illustrates the polarization, not the atom charge).

The fact that MSRA is located in the cytosol and mitochondria of the yeast cells,²²⁸ was proposed as explanation for the relative low DMSO reduction in yeast as compared to *Escherichia coli*, for example. In such Enterobacteriaceae, the DMSO-reductase is located in the plasma membrane where the availability of DMSO, according to Figure 18, can be presumed to be higher. However, the accumulation of DMSO in the plasma membrane further indicates that yeast may already contain DMSO from propagation steps, and that propagation techniques as well as yeast re-pitching could affect DMS formation from DMSO. Yet, these

aspects were not investigated any further within the scope of this work but they certainly deserve more attention in prospective investigations.

In the next experiment potential differences among different yeast strains regarding their capability of DMSO reduction and DMS formation were investigated (Publication B,²⁰³ Figure 6). The yeast strains were systematically chosen based on their proven genetic diversity as reported by Gonçalves et al.²²⁹ Based on population genomics and whole-genome sequencing they showed that top-fermenting *Saccharomyces cerevisiae* strains are polyphyletic, meaning that they may reveal similar characteristics even though not having the same origin (ancestor). Gonçalves et al.²²⁹ categorized the genetic diverse strains in different clusters. The latter were used for the selection of yeast strains in Publication B.²⁰³

It was noted that the top-fermenting yeast reduced substantial higher amounts of DMSO (13-26 %) and thus generating higher levels of DMS (16-32 µg/L). Highest DMSO reduction and DMS formation was found for TUM 149 and TUM 68, both used for wheat beer production. However, there was no correlation found between yeast domestication and DMSO reduction. This was explained by the potential ubiquitous abundance of MSRA in the tested yeast strains. According to Figure 6 (cp. 1.2.1) MSRA is an antioxidant enzyme as it recycles oxidized methionine back to its reduced form. Throughout their evolution all tested yeast strains were exposed to O₂ and potentially also to ROS. Hence, it is likely that also MSRA was developed to similar extents from adapting to ROS. Still, more investigations are necessary to further characterize the differences in MSRA activity throughout the tested yeast strains. The DMSO reduction by TUM 149 throughout the fermentation process followed a similar pattern than observed for TUM 34/70, whereas again, DMSO reduction (~19 %) and DMS formation (~38 µg/L) was higher (Publication B,²⁰³ Figure 7). However, DMSO reduction and DMS formation for both yeast strains mainly occurred in the first period of fermentation suggesting a connection to aerobic growth.

It was concluded from Publication B,²⁰³ that DMSO significantly contributed to the levels of DMS in beer. Even though DMS formation by top-fermenting yeast was above the lowest reported DMS flavor threshold of 25 µg/L,²⁶ the occurrence of a DMS off-flavor (>80 µg/L²⁶) in these beers is unlikely to have occurred. Though, it is important to mention here that the pitching worts did not contain detectable levels of DMS before fermentation, which is generally not the case in industrial brewing. When applying malt that contains high levels of SMM while not adapting the wort boiling time for sufficient degradation and DMS evaporation, the pitching wort DMS levels can be crucially increased during hot break

separation, for example. In such cases, a surplus formation of DMS during fermentation may be critical for beer flavor.

Role of DMSO as a DMS precursor during beer storage – Impact of Antioxidants

Publication B²⁰³ revealed that DMSO was readily solubilized within 10 minutes of mashing and that its quantity was not significantly affected in a conventional wort production process. During fermentation, the maximum DMSO reduction by yeast was ~26 %, indicating that the majority of DMSO from malt can be recovered in the final beer. Considering the DMS flavor threshold in this context, even minor DMSO reduction may produce significant amounts of DMS. Beer consists of a plethora of antioxidants (cp. 1.5) which can also be classified as reducing substances based on their property of donating electrons. It has been demonstrated (Publication A²⁰²) that sodium metabisulfite can be used to reduce DMSO at high concentrations. In aqueous solution at wort- and beer pH (3.5-5.5) it mainly forms HSO_3^- , which is also released by yeast via the synthesis of the sulfur-containing amino acids Cys and methionine. It is therefore a natural constituent of beer and was considered to be its most effective antioxidant.^{86,106,193,194} In addition, the thiols Cys and GSH, which are also potential antioxidants from beer^{86,110,179} were shown to be capable of reducing DMSO and forming DMS (Publication A²⁰²). It was therefore of interest to test and compare the aforementioned antioxidants (sulfite and thiols) with other selected antioxidants (GA and Asco) in terms of their capability to reduce DMSO and to evaluate their potential contribution to the levels of DMS in beer (Publication C²⁰⁴). The first approach was to test the relevance of the sulfite-mediated DMSO reduction on DMS formation in buffered model solutions containing only sulfite (50 mg/L) and DMSO (1.5 mg/L) as a precondition for further investigations in beer (Publication C,²⁰⁴ Figure 1). Within 25 days of storage (40 °C) a linear increase of DMS up to ~125 µg/L was observed. These data provided evidence that DMSO reduction by sulfite also takes place at much lower concentrations of sulfite compared to its application (>0.4 g/mL) in Publication A.²⁰² It was shown that the sulfite concentration remained approximately constant during the storage period. This was explained by the relative excess of sulfite over DMSO (sulfite:DMSO ~33). Even though DMS concentrations of ~125 µg/L would probably cause an off-flavor in pale lager beer,^{26,42} only ~12 % of the DMSO was reduced. Assuming an equimolar stoichiometry of the reaction between sulfite and DMSO, the DMS formation observed corresponds to only about 0.24 % sulfite. These changes could not be detected by the method used for sulfite quantification. This observation further demonstrated that nitrogen preparation and the use of oxygen-scavenging crown corks was sufficient to prevent sulfite from oxidative deterioration.

The screening of the antioxidants (Publication C,²⁰⁴ Table 2) showed that, besides sulfite, Cys and GSH were capable of significant DMSO reduction. While no significant difference was found regarding DMSO reduction among the latter, sulfite generated more DMS ($\sim 174 \mu\text{g/L}$) than Cys ($\sim 155 \mu\text{g/L}$) and GSH ($\sim 132 \mu\text{g/L}$). The combination of sulfite and GSH produced the highest DMS formation ($\sim 258 \mu\text{g/L}$), although the increase was not cumulative compared with using these substances on their own. This observation may suggest antagonistic behavior between the substances that may have diminished their availability for DMSO reduction. A possible interaction was proposed in which sulfite may have been bound to the keto group of the C-5 atom of the glutamic acid moiety of GSH, similar to the reaction of sulfite with glucose (Figure 20).²³⁰

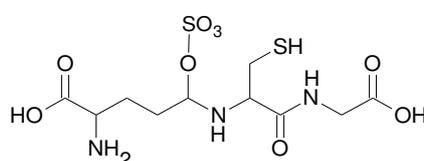


Figure 20: Proposed adduct between sulfite and GSH.

Assuming that GSH will be oxidized to GSSG in the case of DMSO reduction (reaction mechanism will be discussed later) it is also likely that HSO_3^- reduces part of the disulfide (GSSG) whereby one molecule of S-sulfo-GSH and GSH were formed (Figure 21).²³¹

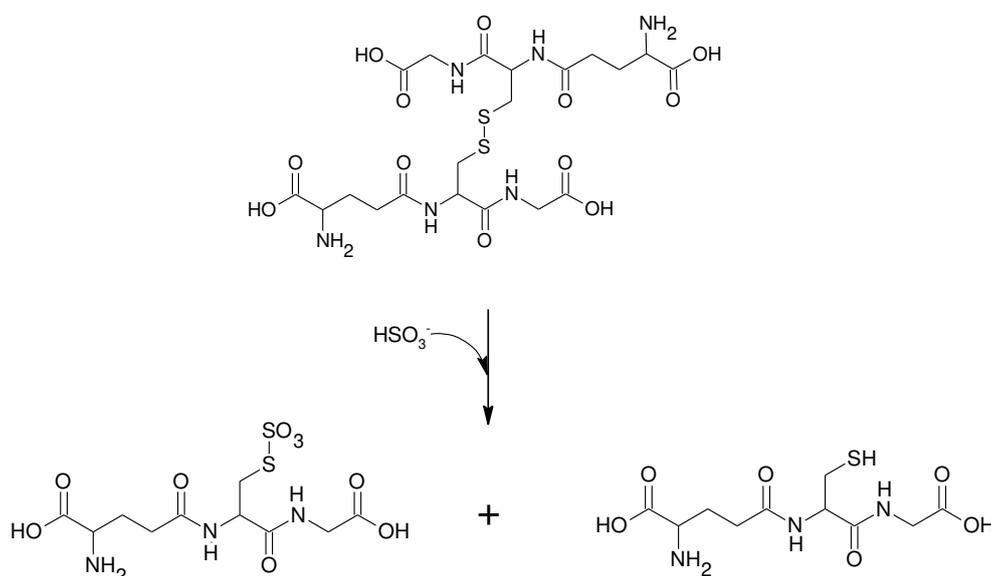


Figure 21: Reduction of glutathione disulfide (GSSG) by sulfite.²³¹

In S-sulfo-GSH both functional groups (sulfite and thiol) are sterically hindered to react with DMSO which may further explain the antagonistic effect. The Asco treatment induced a minor, but significant DMSO reduction. As no DMS was detected in the samples it was

suggested that the effect was a result of interactions other than DMSO reduction. The addition of Asco together with sulfite and GSH was without effect compared to the exclusive addition of sulfite and GSH. As Asco was not able to form DMS from DMSO, the only reasonable effect would have been protection of sulfite and GSH from oxidation which would increase their availability for DMSO. This was not observed, probably because there was no significant oxidative consumption of sulfite (cp. Publication C,²⁰⁴ Figure 2) and presumably also of GSH in the model solution. No effect, whether for DMSO nor DMS could be observed for GA.

As the previous trials did not consider interactions of DMSO, sulfite and thiols with other beer ingredients, further effort was made to determine the significance of their reactions on the final levels of DMS in beer (Publication C,²⁰⁴ Figure 2). Therefore different concentrations of DMSO, sulfite and GSH (Publication C,²⁰⁴ Table 1) were added to Pilsner beer, which was then stored over 120 days at a more moderate temperature of 28 °C. In contrast to the investigations in buffered model solution (Publication C,²⁰⁴ Figure 1), the sulfite concentration was consumed to ~50 % in all treatments, whereas it was slower in the first 14 days of storage. This observation was explained by the initial low abundance of oxygen as a result of preparation under nitrogen. As the closed bottles were stored under atmospheric conditions, diffusion of oxygen into the bottles was certainly inevitable.²³² The faster decline of sulfite during further storage was presumed to be related to the limited capacity of the sulfite-based oxygen scavengers in the crown corks. Consequently, more oxygen diffused in the beer where it was probably successively reduced by transition metal ion/reductone systems leading to hydrogen peroxide formation,^{79,87-89,233} which is known to react rapidly with sulfite to form sulfate.^{195,234} Interestingly, the sulfite consumption decelerated considerably when GSH was added to the beer and after 120 days the sulfite concentration was ~30 % higher than in the reference (no additions). This observation clearly indicates that GSH had protected part of the sulfite from oxidative degradation and was inconsistent with previous reports,^{86,90} which suggested that thiols act as secondary antioxidants not significantly before sulfite depletion. However, the observation in Publication C²⁰⁴ was explained by the initial relative excess of GSH over sulfite (GSH:sulfite ~1.7), which is mostly not the case in fresh pale lager beers but rather representative of later stages of beer ageing.⁸⁶ It was proposed that GSH reacted with H₂O₂,¹⁶⁴ for example, even though it is known that this reaction proceeds much slower than with sulfite.^{195,234} However, the emphasis was to assess the impact of sulfite and thiols on DMS formation via DMSO reduction (Publication C,²⁰⁴ Figure 3). The data confirmed the previous observations and

provided evidence that the sulfite-mediated reduction of DMSO also occurs in beer. This was substantiated by a significant DMSO reduction as well as a significant DMS formation at high sulfite (~50 mg/L) and DMSO (~1.0 mg/L) addition. In this context the DMS concentration of the beer was increased linearly from ~35 µg/L to ~100 µg/L within 120 days. For the lower sulfite concentration (25 mg/L) the overall DMSO reduction was not significant, whereas the DMS concentration was significantly increased to ~51 µg/L. However, together with GSH (50 mg/L) the DMSO content was significantly reduced and the DMS increase (~68 µg/L) was significantly higher than detected in the beers where sulfite and DMSO were added exclusively. GSH (50 mg/L) and DMSO (0.5 mg/L) alone did not evoke a significant DMSO reduction in the beer. But again, significant DMS formation was observed (~51 µg). The lowest DMS increase from ~35 µg/L to ~43 µg/L was found for the reference (no addition). An important aspect was that the SMM concentration in the fresh Pilsner beer (~13 µg/L) was degraded below the LOD in all samples. As reported by Hysert et al.,⁵⁰ SMM can also be subject to degradation to DMS in the final beer. Nevertheless, an equivalent increase in DMS from potential SMM decomposition (13 µg/L) could not explain the observed rates of DMS formation in Publication C,²⁰⁴ except for the reference. It was therefore concluded that sulfite and the thiol GSH were capable of DMSO reduction and DMS formation in the tested beer. Although DMSO was relatively stable, an already non-detectable reduction of DMSO (<5 %) (cp. trial 2 and 5) can evoke significant formation of DMS in beer.

The formation of DMS from reacting DMSO with sulfite and/or thiols in this study was proven. In summary, reaction mechanisms of DMSO reduction during beer storage were proposed (Publication C,²⁰⁴ Figure 4 & 5). The reaction mechanism of DMSO reduction by sulfite has not been reported before. However, based on literature findings regarding related sulfoxides (e.g. methionine sulfoxide),^{235–237} a reaction mechanism was proposed that may explain the sulfite-mediated formation of DMS in beer (Publication C,²⁰⁴ Figure 4). The reaction is initiated by DMSO protonation whereupon a nucleophilic attack of the bisulfite anion (HSO_3^-) forms a DMSO-bisulfite adduct. The latter was proposed as being subject to decomposition to DMS and sulfate as final products. The reaction mechanism of the reaction between DMSO and thiols was reported before in other systems.^{238–240} According to Wallace,²³⁸ the reaction also requires protonation of DMSO which is then susceptible to nucleophilic addition by the thiol. The thiol-sulfoxide adduct is further reduced by another thiol leading to the corresponding disulfide, water and DMS as final products (Publication C,²⁰⁴ Figure 5). Depending on the initial levels of DMS in beer, an increase in DMS of >50 µg/L as observed in this study can certainly produce off-flavors or at least significantly

contribute to beer flavor. However, this requires DMSO levels of > 1 mg/L and sulfite levels of > 50 mg/L, which is generally not the case for pale lager beers. From the results of Publication C²⁰⁴ it can be concluded that the formation of elevated levels of DMS via DMSO reduction is rather unlikely to occur in these beers as the levels of sulfite are mostly < 10 mg/L. Still, brewers should be aware of those reactions, especially when the levels of DMS at racking are already in the upper threshold range (e.g. 70-100 μ g/L). This occurs when the boiling process was not adapted to higher SMM levels of malt, for example. In such a case, DMS can reform rapidly during hot break removal. Part may be desorbed by CO₂ during fermentation but considering the contribution of enzymatic DMSO reduction (cp. Publication B) a substantial amount of the DMSO enters the beer. In this case, even smaller rates of DMSO reduction by sulfite and thiols during beer storage might be crucial for beer flavor. The implications of the attributed reactions might be important to breweries that use sulfite additives in their beer. For example, Brazil allows the addition of up to 50 mg/L of sulfite to beer.²⁴¹ The European Union (Food Information Regulation) allows the addition of more than 10 mg/L sulfite, when declared on the label. Germany is an exception to this as its “Rheinheitsgebot” (Beer Purity Law) prohibits using any additives in the beer.

In Publication C²⁰⁴ it was observed that sulfite was depleted to ~ 50 % in the course of beer storage. It can therefore be assumed that every approach which decelerates this oxidative consumption and helps to retain the levels of sulfite and thiols in beer promotes the risk of DMSO reduction. In other words, higher oxidative stability may lead to higher levels of DMS in beer when the concentration of the reaction partners is sufficient.

The wine industry, for example, already uses sealing material that can significantly decrease oxygen ingress and the associated consumption of antioxidants.^{242–244} DMS is also an issue in wine,^{21–23,245} which contains much higher levels of sulfite than beer, even up to 400 mg/L.²⁴⁶ De Mora et al.²⁴⁷ reported DMSO levels in wine of up to 1230 μ g/L. As wine is also longer stored than beer these conditions seemed suitable for DMSO reduction by sulfite as described in Publication C.²⁰⁴ To test this assumption we investigated 12 different European white wines and 12 different European red wines on their levels of DMSO and sulfite (data not published) to assess their potential contribution to DMS levels in wine, which ranged from below 5 to 45 μ g/L (data not published). Even though the wines possessed varying sulfite concentrations (73-112 mg/L), none of them contained significant levels of DMSO (all below 19 μ g/L), which was in contrast to the findings of de Mora et al.²⁴⁷ However, the origin of DMSO in wine grapes and must is doubtful as no thermal treatment is applied that may hydrolyze the relative low amounts of SMM,²² that may then be oxidized to DMSO. Based on

these findings the relevance of DMSO reduction on DMS formation in these wines was estimated to be low.

4.3 Reaction mechanisms of DMS oxidation and DMSO formation - Impact of Antioxidants

After the explanation of the significance of DMSO as a precursor of DMS during fermentation (Publication B²⁰³) and also during beer storage (Publication C²⁰⁴), the next step was to investigate the potential origins of DMSO. A key outcome of Publication B²⁰³ was that DMS was relatively stable to oxidation to DMSO in the brewing process indicating that the relative high amounts of DMSO mostly originate from malt. These findings designate the germination- and kilning process as the most auspicious process steps for controlling DMS oxidation. In the following publications the oxidation of DMS was investigated in buffered model solutions to minimize side reactions of potential DMS oxidants with other malt constituents. Furthermore, such an approach helps to clarify reaction mechanisms and their dependence on physical-chemical parameters, which are of fundamental importance for technological considerations. In this context, special attention was again set on the effect of antioxidants as their enrichment in malt may diminish DMS oxidation in two potential ways. Firstly by directly inactivating the reactive oxygen species that may be able to oxidize DMS. Secondly, as explained in Publication C,²⁰⁴ sulfite and thiols can reduce DMSO thereby providing a recycling mechanism that may shift the redox equilibrium towards DMS. However, as described in the introduction, most antioxidants also reveal prooxidative effects in combination with transition metal ions and O₂.

In the next publication O₂ and transition metal ions were tested on their effect on DMS oxidation in aqueous model solution. Specifically the effect of thiols should be investigated. Their levels were rarely quantified in malt, however, based on the relatively high amounts released during mashing^{76,177,181} they are probably one of the most abundant naturally occurring antioxidants in malt. Yet, their role in the oxidative reactions in malt, specifically in DMS oxidation, is unclear as they possess ambiguous pro-and antioxidative properties. Therefore, the effect of Cys as a representative thiol of malt was investigated in combination with the aforementioned transition metal ions (Publication D²⁰⁵).

Initially, DMSO formation was explored during the incubation of air-saturated buffered model solutions containing DMS and different compositions of Fe²⁺, Cu²⁺ and Cys (95 °C, 180 min) (Publication D,²⁰⁵ Figure 1). In agreement with previous findings^{46,65} no DMSO was formed when DMS was used exclusively implying that DMS was not subject to oxidation in

the absence of a catalyst. In the presence of either Fe^{2+} or Cu^{2+} , significant DMSO formation took place, however the oxidative consumption of DMS was insignificant (<2 %). The results indicated that, for example, Fe^{2+} and O_2 alone did not generate sufficient amounts of reduced oxygen species that could have oxidized DMS. It was described that the standard redox potential difference (ΔE_0) between the couples $\text{Fe}^{2+}/\text{Fe}^{3+}$ and O_2/O_2^- is probably too small to account for the subsequent formation of ROS, which in turn may oxidize DMS (this phenomenon will be further discussed in Publication F). The effect of Cys was significantly prooxidative with respect to DMS oxidation and the associated DMSO formation when combined with the transition metal ions, whereas its combination with Cu^{2+} resulted in the highest DMS oxidation (~40 %). It was proposed that a redox cycling process was responsible for the prooxidative behavior in which Cys acted as reducing agent for the metal ions, thereby maintaining their catalytic active lower valence state capable for oxygen reduction and formation of ROS. It is known that both metal ions form a complex with Cys, whereas the greater effect of Cu^{2+} was explained by its higher stability constant which consequently makes it more susceptible to oxidation by O_2 . In later stages of the study the focus was on Cys-Cu interactions.

It was important to consider that the yet uncharacterized reactions involving Cys, Cu^{2+} and O_2 may have taken place especially during the heating period of the buffered model solutions from 1 °C up to 95 °C (20 min), which was not a representative temperature profile for the withering and kilning process. Unfortunately, it was not reproducibly possible to introduce DMS into the model solutions at temperatures where it is actually formed from SMM in the malting process (>70 °C). Also proteolysis and the release of peptides including thiols such as Cys essentially starts above 50 °C. Therefore the approach was to postpone the availability of Cu^{2+} for Cys during the heating period by chelating it with EDTA in a stoichiometric ratio of 1:1. Spectrophotometric measurements (Publication D,²⁰⁵ Figure 2) showed that this approach could be used to delay the reactions towards higher temperatures as the signal of the $\text{Cu}^{2+}\text{EDTA}$ complex was completely transferred into the signal that was observed when non-chelated Cu^{2+} was combined with Cys. Another benefit of this approach would be that after exchange of Cu^{2+} to Cys, EDTA is available to chelate other potentially abundant traces of reactive metal ions originating from model and stock solutions. The latter interactions were previously reported to be a result of the formation of a bis-cysteine-copper complex (Cys- Cu^+ -Cys).^{174,248,249}

Based on these findings, $\text{Cu}^{2+}\text{EDTA}$ was used in subsequent experiments, in which its combination with Cys revealed an even greater effect on DMS oxidation (Publication D²⁰⁵,

Figure 3), probably because of the shift of Cu^{2+} availability for Cys at higher temperatures, where the reaction rates can also be assumed to be accelerated. It is well known for antioxidants, like thiols, that potential prooxidative behavior may be compensated by increasing their concentration. This shifts their reactivity towards antioxidative modes of action, such as hydrogen peroxide scavenging,¹⁶⁴ or reaction with hydroxyl radicals.¹⁶⁵

As the concentration of Cu^{2+} may also influence this concentration dependency of pro- and antioxidative behavior, response surface modeling (RSM) was chosen to investigate the effects of Cu^{2+} EDTA and Cys on DMS oxidation over a wider, malt- and wort-related concentration range of the reaction partners. Specifically, we used a rotatable central composite design response surface methodology (CCD-RSM), as it can also be used to describe more complex interactions among the response variables, for example polynomial function modeling, without conducting a full three level factorial design. The CCD-RSM experimental set-up for showing the effect of the two chosen variables (Cu^{2+} EDTA, Cys) on the two responses of interest (DMS, DMSO) comprised high- (+1), low- (-1), medium- (0) and extreme levels ($\pm\alpha$) levels of the chosen variables. The experimental response data of each composition are presented as supporting information (Publication D,²⁰⁵ page 58) These data, for example DMSO concentration, formed by the respective combinations of Cu^{2+} and Cys were plotted in a three-dimensional “cube” space. Mathematical statistical fits, such as linear, polynomial or other interaction models were then applied and evaluated using analysis of variance (ANOVA). Based on the ANOVA data, a model is chosen that best fits the data. This model can then be used to minimize or maximize the reactions or processes of interest. The goal of this study was to use CCD-RSM to clarify the regions of the prooxidative behavior of Cys and to identify concentration-dependent reversal points to antioxidative behavior in order to understand its contribution to DMSO formation.

However, the behavior of Cys within the experimental data was strictly prooxidative. Based on ANOVA, a linear model was chosen for the oxidative consumption of DMS (Publication D,²⁰⁵ Equation 1), whereas a 2-factor interaction model was chosen for the behavior of DMSO (Publication D,²⁰⁵ Equation 2). According to the models and the respective *F*-values (Publication D,²⁰⁵ Table 2) the quantity of Cys had the biggest impact on DMS oxidation and DMSO formation. This becomes especially apparent in the three-dimensional plots of the models for DMS and DMSO (Publication D,²⁰⁵ Figures 4 & 5). However, the concentration of Cu^{2+} was evaluated as being insignificant for DMS while it was significant for the levels of DMSO in terms of lowering its quantity with increasing Cu^{2+} and Cys concentration. In all the experiments in this study, the molar sum of DMS and DMSO was lower than the initial

amount of DMS added. Therefore it was proposed that, especially at high Cys- and Cu^{2+} levels, further oxidation of DMSO to DMSO_2 was responsible for these effects. However, taking the data in Figure 5 into account, the potential overall formation of DMSO_2 was estimated as being relatively low. This was further substantiated by the low F -value for the effect of Cu^{2+} (8.19), compared with the effect of Cys (172.24). However, no data on DMSO_2 were available from the study.

The CCD-RSM provided useful information and clarified the prooxidative behavior of Cys for DMS oxidation. Nevertheless, it was curious that the concentration of Cu^{2+} was insignificant, which may also indicate that it was Cys alone that catalyzed DMS oxidation. Only by using an excess of EDTA compared with potentially abundant Cu^{2+} ions or any other transition metal ions was it possible to avoid DMS oxidation (Publication D, Table 3). This finding revealed that Cys catalyzes DMS oxidation even in the presence of traces transition metal ions. As assessed using ICP-OES, these traces originated from the commercially obtained Cys lyophilisate, even though the highest purity grade was used. It was concluded from these results that, even if there are just traces of Cu^{2+} , their overall quantity plays a subordinate role in the prooxidative nature of Cys. Similar findings were recently observed for the effect of iron on the oxidative formation of Strecker aldehydes.^{73,250} To obtain insights into the reaction mechanism responsible for the prooxidative behavior of Cys, the levels of Cys along with H_2O_2 , a potential reduced-oxygen species formed during metal-catalyzed autoxidative reactions, were monitored during heating and incubation of buffered model solution. Additionally, we monitored the competitive complex formation of Cu^{2+} from EDTA to Cys (Publication D, Figure 7). Only minor amounts of Cys were degraded below 40 °C, and Cu^{2+} was still bound to EDTA. Between 40 and 60 °C, Cu^{2+} was released from EDTA and promoted a rapid and almost complete consumption of Cys. At that very moment the H_2O_2 concentration reached its maximum of ~71 μM , which was approximately one third of the initial concentration of Cys. This observation was in accordance with the findings of Pecci et al.¹⁷⁴ and to oxygen consumption experiments in relation of Cu^{2+} and 6-sulfanylhexasan-1-ol, a thiol compound present in wine, reported by Kreitman et al.²⁵¹ The thiol: Cu^{2+} ratio in these studies (~5:1) was different to that of the present study (~13:1) which again points to the insignificance of the total amount of Cu^{2+} . Based on the findings on the stoichiometry of the reaction together with the complex formation between Cys and Cu^{2+} it was possible to propose a reaction mechanism that describes the likely prooxidative behavior of Cys (Publication D,²⁰⁵ Figure 8). As mentioned earlier the reaction involves the formation of a bis-Cys- Cu^+ complex in which the Cu^{2+} was reduced by one of the Cys molecules. In the

presence of oxygen and a further Cys molecule, Cys-Cu⁺-Cys is autoxidized thereby forming Cys-Cu²⁺, the disulfide cystine and H₂O₂ as final products. It was then proposed that H₂O₂ was the primary oxidant that has oxidized DMS to DMSO in this work. Based on the fact that H₂O₂ was reproducibly quantifiable together with literature findings that describe the absence of free thiyl radicals^{174,251} in the case of thiol oxidation by Cu²⁺ and O₂, it was proposed that the reaction of thiyl radicals with DMS was a rather unlikely source for DMSO formation. It is well known that H₂O₂ can undergo “Fenton-like” reactions to generate [•]OH in the presence of transition metal ions in their reduced state. The H₂O₂ maximum occurred when all Cys was consumed, implying that also Cu was no longer present as Cu⁺ that could have potentially reacted with H₂O₂ to form [•]OH. It can also be presumed that other transition metal ions from the used chemicals were chelated in preference to EDTA, which was available after Cu²⁺ release and exchange to Cys. Therefore, the contribution of [•]OH to the levels of DMSO was also evaluated as being unlikely. The relative slow decline of H₂O₂ also indicated the absence of a radical mechanism as such reactions proceed mostly at immeasurable, diffusion-controlled rates. According to the proposed mechanism, one of the most likely substances to react with H₂O₂ is Cys-Cu²⁺. Cystine is also formed in equal amounts, but its low water solubility diminishes its reactivity in the applied system. The formation of copper peroxides from the reaction of Cu²⁺ with H₂O₂ was reported by Perez-Benito.²⁵² Based on his findings, a proposal for the formation of Cys-Cu²⁺-OOH was presented (Publication D²⁰⁵, Figure 9). In accordance with our data, these complexes were shown to decompose slowly in aqueous solution to release Cys-Cu⁺ and O₂⁻.²⁵²

As a result of the predominating prooxidative behavior, no reduction of DMSO by Cys as observed in Publication C²⁰⁴ was detected within the design space. The essential differences compared with the previous study were a higher temperature, higher pH and higher availability of O₂ and, even though it was not a significant factor for DMS oxidation, supplementation of Cu²⁺. However, DMS was not completely oxidized in any of the experimental data. Although expected to be small, the potential contribution of DMSO reduction by Cys to the results was unknown. Therefore, another experiment was conducted to test the extent and relevance of DMSO reduction by Cys at 95 °C. To exclude side reactions with Cu²⁺ and O₂, the model solution was deoxygenated and an excess of EDTA was used. The results showed that within 24 h less than 10 % of the DMSO was reduced to DMS. Taking these numbers in relation to the Cu²⁺-catalyzed autoxidation of Cys responsible for the oxidation of DMS to about 70 % within 3 h (Publication D,²⁰⁵ Figure 3, column 5), the contribution of DMSO reduction was estimated as negligible.

In the previous publication a strict prooxidative behavior of Cys on DMS oxidation was discovered and characterized. On this basis it was of particular interest to also test the effect of other malt-derived potential antioxidants on their effect of DMS oxidation and to assess their potential application to minimize the levels of DMSO in malt (Publication E²⁰⁶).

The antioxidants chosen for the investigations were, in accordance to Publication C,²⁰⁴ Cys, GSH, Asco, GA and sulfite. All of those substances are naturally abundant in malt, while sulfite is also used as an additive to minimize nitrosamine formation.¹⁹² GA was chosen as it is a water-soluble phenol bearing vicinal hydroxy groups. The latter property can also be found in a plethora of other (poly)phenols, such as di- and trihydroxy benzoic acids as well as flavanols (e.g. catechine, epicatechine). Asco was chosen as reductone representative, its abundance in malt,¹¹¹ and also because it is one of the most used adjuvants for minimizing oxidative reactions in food. Hence, its pro- and antioxidative properties are well documented in the literature.^{117,121,253–255} A main focus was on the effect of sulfite as its DMSO reduction (Publication A²⁰² and B²⁰³), and inactivation of H₂O₂¹⁹⁵ properties indicate general antioxidative behavior towards DMS oxidation. To clarify the reaction mechanism of H₂O₂ formation via Cys-Cu interactions, EDTA was used in Publication D²⁰⁵ to postpone complex formation of Cys and Cu²⁺ towards temperatures above 60 °C. Also, via the exchange of Cu²⁺ from Cu²⁺EDTA to Cys, EDTA was released into the media thereby potentially chelating traces of transition metal ions and therefore prevented Fenton-like reactions. However, EDTA was not used in the following study for reasons of comparability among the diverse antioxidants tested. In the first experiment the effect of 250 µM of each antioxidant was tested on DMS oxidation. The tests were performed in the presence of 18 µM of Cu²⁺ to also cover potential prooxidative effects. In Publication D²⁰⁵ 250 µM Cys was prooxidative in the presence of Cu²⁺. To test if the prooxidative behavior of Cys, and potentially also of the other tested antioxidants, can be overcome at a higher concentration the same experiment was also conducted at an antioxidant concentration of 500 µM (Publication E,²⁰⁶ Figure 1). The results demonstrated that at 250 µM all tested antioxidants showed a prooxidative effect on DMS oxidation in the following order: GSH < Cys ≤ GA ≤ Asco < sulfite. The increase to 500 µM antioxidant concentration only diminished the extent of DMS oxidation catalyzed by the Asco-Cu²⁺ system while no significant difference was observed for the other antioxidants. For the thiols, the effect of Cys was higher compared with GSH. The extent of DMS oxidation was in accordance with the respective levels of H₂O₂ detected in the systems after heating to 80 °C (Publication E,²⁰⁶ Table 1). For Cys these data confirmed the findings of Publication D²⁰⁵ and it was proposed that the prooxidative behavior of GSH was based on a similar

mechanism, which involves the formation and autoxidation of a bis-thiol-copper complex. The significantly lower amounts of H_2O_2 formed in the GSH-Cu^{2+} system was explained by the fact that the GSH oxidation product, glutathione disulfide (GSSG), is capable of binding up to three Cu^{2+} molecules, essentially via the glutamic acid moieties.²⁵⁶ Thereby the accessibility of Cu^{2+} for the remaining free thiol groups is hindered which was proposed as being the reason for the lower levels of H_2O_2 and consequently, also for the lower extent of DMS oxidation. The oxidation product of Cys (cystine) is only capable of binding one Cu^{2+} molecule²⁵⁷ and also, it is not soluble in aqueous solution. Therefore a lower effect of cystine on Cu^{2+} chelation was anticipated. A further explanation may be found in the lower pK_a value of the Cys thiol group (8.45) compared with the pK_a of the GSH thiol group (9.06). These numbers indicate that a higher quantity of thiolate anions (RS^-) may have been present in the Cys treatments. These are generally known to reveal higher reactivity than RSH ,²⁵⁸ which may further substantiate the higher levels of H_2O_2 . A non-cumulative increase of H_2O_2 was observed when the Cys concentration was increased to $500\ \mu\text{M}$, suggesting that around the applied concentration range a “reversal-behavior” was reached from prooxidative to antioxidative behavior. Based on the higher abundance of Cys in these trials, it is likely that part of the residual thiols reacted with H_2O_2 to form water and cystine as final products.¹⁶⁴ The fact that DMS oxidation was unaffected by increasing the Cys concentration may be explained by the property of Cys to reduce DMSO ,^{202,204,259} even though higher H_2O_2 levels were present. In contrast to Cys, using $500\ \mu\text{M}$ GSH reduced the levels of H_2O_2 to about 87 %. This effect was explained by the property of GSH to chelate H_2O_2 as proposed by Abedinzadeh et al.²⁶⁰ (Figure 22 a). Taking into account the aforementioned water solubility of GSSG, its interactions with H_2O_2 may also have occurred in the investigated systems (Figure 22 b).

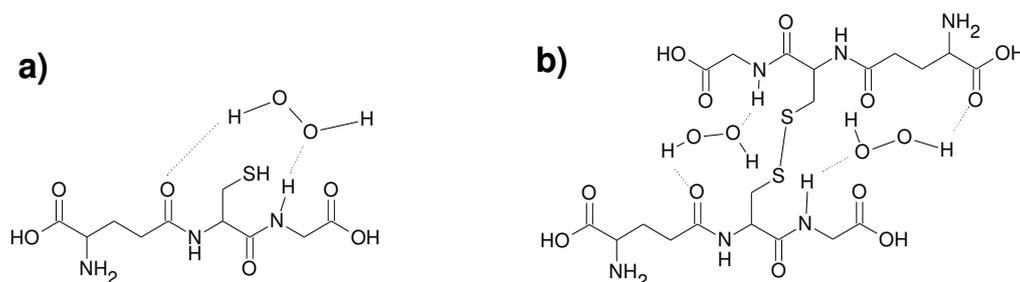


Figure 22: Proposed interaction of H_2O_2 with GSH ²⁶⁰ and GSSG.

However, more investigations into the interactions of those compounds are necessary to provide evidence for such behavior.

As previously mentioned, Asco was the only substance where DMS oxidation was significantly abated at 500 μM compared to a concentration of 250 μM . Such phenomena, where a prooxidative effect of Asco can be partly compensated by increasing its concentration has been previously reported.^{87,261,262} Based on findings of Khan and Martell,¹¹⁷ the prooxidative behavior of Asco towards DMS oxidation was also explained by a Cu^{2+} -catalyzed formation of H_2O_2 , as also detected in the systems treated with 250 μM Asco and Cu^{2+} (Publication E,²⁰⁶ Table 1). The reaction mechanisms are illustrated in Publication E²⁰⁶ (Reactions 1-3). Asco (AscH^-) also donates an electron to Cu^{2+} while, in contrast to the thiols, Cu^+ is released from the ascorbyl radical ($^{\bullet}\text{AscH}^-$). O_2 is then successively reduced to H_2O_2 by $^{\bullet}\text{AscH}^-$ and AscH^- . Cu^+ may then react with H_2O_2 in a “Fenton-like” mechanism to form hydroxyl radicals ($^{\bullet}\text{OH}$) (Publication E,²⁰⁶ Reaction 4), which also explained the relatively low amounts of H_2O_2 detected in this system (Publication E,²⁰⁶ Table 1). Based on these findings H_2O_2 and $^{\bullet}\text{OH}$ were proposed as potential oxidants of DMS, originating from the Cu^{2+} -catalyzed oxidation of Asco (Publication E,²⁰⁶ Reactions 5 & 6). The lower DMS oxidation detected at the higher Asco concentration was consistent with the absence of H_2O_2 (Publication E,²⁰⁶ Table 1). This shift towards antioxidative behavior was explained by the fast disproportionation of $^{\bullet}\text{AscH}^-$ ¹²⁰ (radical termination) as well as the more effective ability of Asco to scavenge $^{\bullet}\text{OH}$ at higher concentration.¹¹⁹

The prooxidative effect of GA was as high as that found for the Cys- Cu^{2+} interactions. It was reported by Akagawa²⁶³ that the oxidation of GA by O_2 and traces of Cu^{2+} generated significant amounts of H_2O_2 at pH 7 within 24 h at 37 $^{\circ}\text{C}$, while H_2O_2 formation was significantly lower at pH 5, probably because of the lower solubility of GA at pH 5 as compared to pH 7. In Publication E²⁰⁶ no H_2O_2 was detected in the oxygenated GA- Cu^{2+} systems after heating to 80 $^{\circ}\text{C}$. Although not directly comparable, the data of Akagawa²⁶³ suggest that H_2O_2 in such systems proceeds much slower in comparison with thiols, for example. It is therefore likely that H_2O_2 formation in the GA- Cu^{2+} treatments took place within the 180 minutes of incubation at 80 $^{\circ}\text{C}$. To test this hypothesis, an additional experiment was carried out where the fate of H_2O_2 was monitored over time (additional data, Figure 17, page 83). The experiment confirmed the hypothesis as the H_2O_2 concentration significantly increased after reaching 80 $^{\circ}\text{C}$ (10 minutes) in the bottles. Although the maximum H_2O_2 detected was merely $\sim 2 \mu\text{M}$, the data provide evidence that H_2O_2 was also involved in the prooxidate effect of GA and Cu^{2+} on DMS oxidation. $^{\bullet}\text{OH}$ formation could not be excluded either.²⁶²

Surprisingly, sulfite, which was shown to reduce DMSO in low oxygen environments in the previous publications,^{202,204} revealed the highest prooxidative effect on DMS oxidation in the presence of O₂ and Cu²⁺. Irrespective of the sulfite concentration, DMS was completely oxidized and recovered as DMSO to approximately 90 %. The same behaviour was observed in the presence of 4- and 9 μM of Cu²⁺ (data not shown). H₂O₂ was not detected in these treatments which suggests that another reactive species was involved in the sulfite-Cu²⁺-catalysed DMS oxidation. In general, similar effects were observed of all antioxidants tested in the presence of Fe³⁺ instead of Cu²⁺ (Additional data, Figure 18, page 83).

Based on this tremendous effect of sulfite, the next steps focused on further clarifying the potential reactions involved. An ensuing kinetic investigation (Publication E,²⁰⁶ Figure 2) demonstrated that more than 50 % of the DMS was already oxidized after reaching the target temperature of 80 °C. DMS oxidation and DMSO formation was completed after ~25 minutes in the presence of sulfite and Cu²⁺, whereas it was slightly slower when sulfite was combined with Fe³⁺ (completion after ~40 minutes). The relatively high velocities of those reactions highlight the potential relevance of the sulfite/transition metal ion-catalyzed DMS oxidation.

As sodium metabisulfite, which dissociates in aqueous solution to form 2 equivalents of sulfite, was used throughout this dissertation work for the quantitative reduction of DMSO, it was important to identify the scope of its prooxidative effect. Therefore another experiment with different sulfite concentrations and 9 μM Cu²⁺ tested DMS oxidation (Publication E,²⁰⁶ Figure 3). The results clarified that DMS was completely oxidized over a wide concentration range (100 μM – 4 mM). At 6 mM a reversal formation of DMS or, a decreased extent of DMS oxidation, was detected. Further increasing the sulfite concentration saw a higher recovery of DMS. At the highest concentration (100 mM) ~94 % of the added DMS was recovered but traces of DMSO were still detected. The effect could only be eliminated completely when the systems were supplemented with EDTA and also purged with nitrogen. The sulfite concentration used for the quantification of DMSO (Publication A²⁰²) is far beyond the maximum solubility of sulfite in water (~2.75 M) and also, all samples are purged with nitrogen. Under these conditions prooxidative effects of sulfite can be excluded, which again underlines the applicability of the method for analyzing DMSO in malting- and brewing related media. However, further information was necessary to characterize the prooxidative scope of oxygenated sulfite-Mⁿ⁺ systems on DMS oxidation. Therefore, ESR spectroscopy combined with DMPO spin trapping was used to identify the potential abundance of sulfite-derived radicals in the investigated systems and to obtain information on potential reaction mechanisms.

Although it is not a common technique in the field of malting and brewing, DMPO was used because it is a more versatile spin trap that reacts with O-, N-, S-, and C- radicals, whereas PBN or POBN mostly react with O- and C-centered radicals (e.g. hydroxyl- and hydroxyl ethyl radicals).^{79,233,264} All solutions were purged with nitrogen in the experiments to minimize the reaction of potentially formed radicals with O₂ to increase their detectivity. The combination of 5 mM DMPO with 500 μM sulfite and transition metal ions led to the formation of symmetric singlet-duplet-duplet-singlet signals (Publication E,²⁰⁶ Figure 4), which was distinctively different to DMPO-hydroxyl radical spin adducts, for example (four singlet signals).²⁶⁵ Similar signals were observed previously from the interaction of sulfite with oxidoreductases from which the radical was identified as sulfite radical (*SO₃).^{266,267} Even though similar signals were obtained in Publication E,²⁰⁶ (Figure 4), the signal intensity was too low to simulate and identify the radical species involved. Rangelova and Mason reported that O₂ competes with *SO₃ to react with DMPO. Even though we purged the model solutions with nitrogen and the preparation time until analysis was short it was certainly inevitable that oxygen diffused into the system. To further minimize the effect of oxygen, the DMPO concentration was increased to 50 mM (Publication E,²⁰⁶ Figure 5), which resulted in significantly higher signals. The simulated spectra (Publication E,²⁰⁶ supporting information) were used to determine the hyperfine splitting constants of the radical. In comparison with previous reports at physiological pH ($\alpha^N=14.7$ G, $\alpha_\beta^H=16.0$ G),^{266,267} the hyperfine splitting constants were slightly lower (Cu²⁺: $\alpha^N=14.11$ G, $\alpha_\beta^H=15.60$ G; Fe³⁺: $\alpha^N=14.12$ G, $\alpha_\beta^H=15.54$ G). To test if a protonated bisulfite radical was detected (H*SO₃) at the lower pH in Publication E,²⁰⁶ spectra were also recorded at pH 7, but still, the same hyperfine splitting constants were determined from the simulations and the hypothesis had to be rejected. It was therefore proposed that the small deviations were related to DMPO-Mⁿ⁺ interactions. These can be presumed to not have occurred in the previous studies as Mⁿ⁺ were enzyme-bound. Based on the evidence for the formation of bisulfite radical in the systems tested mechanisms of the sulfite-Mⁿ⁺ mediated DMS oxidation were proposed (Publication E,²⁰⁶ reactions 9-14). After reduction of the respective transition metal ion, its reaction with O₂ to the peroxymonosulfate radical (SO₃OO*) seems to be inevitable as the rate constant ($k=1.5 \times 10^9$ M⁻¹s⁻¹)²⁶⁸ is close to diffusion-control. This indicates that as soon as *SO₃ encounters O₂ it will react. In contrast to H*SO₃, which is a sulfur-centered radical, the free radical in SO₃OO* is oxygen centered. It was proposed for the first time that SO₃OO* was the main DMS oxidant resulting in DMSO and sulfate as final products. Interestingly, (non-radical) sodium peroxymonosulfate, that was already reported to sufficiently oxidize organic sulfides,²⁶⁹

underlined this assumption. The previous investigations in model systems were able to clarify the potential reaction mechanisms responsible for the prooxidative effects of the selected antioxidants for DMS oxidation. However, those investigations did not consider bioavailability and biochemical factors. To test the relevance of the described reactions in malt, an *in vivo* experiment was carried out to which the antioxidants and/or Cu^{2+} were added to the steeping water of germinating barley. After germination and kilning the resulting malts were analyzed on SMM-, DMS- and DMSO concentrations (Publication E,²⁰⁶ Figure 6). The addition of Asco and GA was accompanied by significant lower levels of DMSO in the malt, suggesting antioxidative behavior. However, the DMSO levels were merely ~7 % lower compared with the reference and the technological relevance was estimated as being marginal. However, in combination with Cu^{2+} , all antioxidant treatments significantly increased the levels of DMSO in malt. According to the effects observed in model solutions (Publication E,²⁰⁶ Figure 1 b), the highest levels of DMSO were found in the sulfite- Cu^{2+} treated malts where the DMSO concentration was ~22 % higher compared with the reference. Although the data confirmed the relevance of the prooxidative effects as found in the model solutions, the effect was clearly smaller. This was explained by potential side reactions of Cu^{2+} and antioxidants with malt ingredients. Especially the high reactivity of sulfite with carbonyls,¹⁹⁰ anthocyanins,²⁷⁰ or H_2O_2 ,^{195,234} all of which are present in malt, have probably competed with the reactions described before. Once formed from $\cdot\text{SO}_3$ and O_2 , the reaction of $\text{SO}_3\text{OO}\cdot$ with other malt constituents is also likely to occur and needs further scientific attention.

Also, the extent to which the antioxidants were absorbed into cell compartments, such as the embryo, where the majority of SMM is synthesized and degraded,³⁷⁻³⁹ remains unknown. The levels of M^{n+} in the barley were determined according to a previously described method²⁵⁰ as Cu: $50 \pm 5 \mu\text{M}$, Fe: $357 \pm 23 \mu\text{M}$ and Mn: $141 \pm 8 \mu\text{M}$ (unpublished data). The relative high levels already present in barley together with the lower levels of DMSO in the treatments with antioxidants without Cu^{2+} addition implies that they are present in rather unreactive, bound-state.²⁸³ The structures or the chelates of M^{n+} with barley ingredients are unknown. Therefore, also investigations on such chelates and their impact on the availability of M^{n+} are necessary.

Still, from the data obtained from Publication E,²⁰⁶ the application of the investigated antioxidants as additives to malt for minimizing the DMSO concentration cannot be suggested, *per se*. Further investigations, focused on topics such as the kinetics of DMS oxidation in the presence of antioxidants, M^{n+} as well as their interactions, pH- and concentration dependencies, are needed to ascertain the required approaches to increase their antioxidative properties to minimize oxidative reactions in food.

Investigation of DMS oxidation by reactive oxygen species and their potential role in DMSO formation in malt

The previous publications have shown that H₂O₂ is a potential primary oxidant of DMS. For the purpose of chemical synthesis of DMSO for its wide range of application in biology and organic chemistry (cp. 1.2.1) and also for other reasons, such as the deodorization of waste waters, the reaction between DMS and H₂O₂ has been investigated.^{271,272} Those investigations were carried out in the millimolar concentration range below 35 °C using spectrophotometric analyses. Yet, the reaction between DMS and H₂O₂ was not explored in relation to thermal food processing, such as the malting process, which uses temperatures above 60 °C. The aim of the following investigations was to characterize the oxidation of DMS by H₂O₂ at varying pH and temperatures representative of the withering and kilning process (50-100 °C). Another objective was to evaluate the role of further (reactive) oxygen species as potential DMS oxidants. The holistic consideration of these investigations together with the outcome of the previous publications (D&E) should then provide information for proposals on the origin of DMSO in malt (Publication F²⁰⁷).

During malt production the cereal is modified in a forced aerobic germination process to achieve high rates of cytolysis and proteolysis. Significant amounts of free amino acids including thiols, such as Cys, GSH or higher molecular thiols present in a protein- or peptide bound state. Also, Asco¹¹¹ and low molecular phenolic compounds¹⁵⁵ are synthesized and released during barley germination. Together with barley- and steeping water-derived transition metal ions, such as Cu²⁺ they may catalyze the formation of H₂O₂. From the plant-physiological point of view H₂O₂ may also derive as a side product of respiration,^{98,273-275} and can especially accumulate under different biotic and abiotic stress conditions.^{96,274,276,277} The forced germination conditions during malt production, such as high temperatures and high steeping degrees can be regarded as unnatural conditions to which the seedlings are not evolutionarily adapted because they are processed into beer. Based on this hypothesis there was a certain curiosity to test the presence of H₂O₂ in germinated barley (green malt) as a justification and precondition for the characterization of its reaction with DMS in the subsequent kilning process. We modified the method used for H₂O₂ assessment from Publication D²⁰⁵ for application in green malt. Besides adjusting the concentration of leuco crystal violet (LCV) and horseradish peroxidase (HRP), the biggest challenge was certainly to extract the bluish dye (CV⁺), as formed from the HRP-catalyzed oxidation of LCV by H₂O₂, from protein-starch or cellulose aggregates. A screening of diverse solvents revealed that double extraction using chloroform was sufficient to recover CV⁺ to more than 99 % (data not

published). The obtained chloroform spectra (Publication F,²⁰⁷ Figure 1) clearly provided evidence for the presence of H₂O₂ in the green malt as the typical signal of CV⁺ was observed.^{205,278}

As those results underlined the potential relevance of H₂O₂ as a potential oxidant in the subsequent kilning process its reactivity with DMS was investigated in the next step under consideration of H₂O₂ concentration, temperature (T) and pH in buffered model solution. For this approach rotatable central composite design response surface methodology (CCD-RSM) was used again as it was demonstrated before in Publication D²⁰⁵ to be a useful tool to clarify the factors that influence DMS oxidation. The data should provide basic information about crucial areas of DMS oxidation related to potential interactions among the investigated factors to ascertain technological approaches to reduce DMS oxidation, for example by pH-temperature adjustments.

Within the experimental data DMS oxidation ranged from 0-41.6 % and resulted in DMSO formation of 0-35.2 % (Publication F,²⁰⁷ Table 3). As the sum of DMS and DMSO in each experiment was not significantly different from the initial DMS concentration added to the samples it was proposed that no further oxidation of DMSO, e.g. to DMSO₂ occurred within the design space. The CCD-RSM followed by ANOVA (Publication F,²⁰⁷ Table 4) revealed that 2-factor-interaction models (2-FI) could best model the extent of DMS oxidation and DMSO formation (see coefficients of determination). H₂O₂ concentration (p<0.0001), temperature (p<0.0001) as well as their interactions (p≤0.0004) were significant impact factors for DMS oxidation and DMSO formation (p<0.0001) whereas the effect of pH on DMS oxidation to DMSO by H₂O₂ was found to be insignificant (p>0.26). The latter observation was in accordance with the findings of Adewuyi and Carmichael.²⁷¹ They investigated the oxidation of ~1.4 mM DMS by equimolar amounts of H₂O₂ at 20-30 °C. Within a pH range of ~1.5-9 the rates of DMS oxidation were similar. They stated that only below pH ~ 1.5 significant amounts of protonated H₂O₂ (H₂O⁺OH) are formed which can preferably add OH to the nucleophilic sulfur atom of DMS, and thus increasing the extent of DMS oxidation. However, the formation of H₂O⁺OH is unlikely to occur in malting and brewing and its relevance for the formation of DMSO can therefore be estimated as negligible.

Therefore pH was disregarded in the 2-FI models for DMS oxidation (Publication F,²⁰⁷ Equation 2) and DMSO formation (Publication F,²⁰⁷ Equation 3). The equations become more obvious in the three-dimensional plots illustrating the oxidative DMS consumption

(Publication F,²⁰⁷ Figure 2) and accompanied DMSO formation (Publication F²⁰⁷, Figure 3) at pH 5.5.

Another important factor for the relevance of a chemical reaction in malt is its reaction rate. Therefore the kinetics of DMS oxidation by H₂O₂ were investigated to test if this reaction is potentially capable of competing with other relevant reactions in malting, for example thiol oxidation¹⁶⁴ or lipid oxidation.²⁷⁹

DMS oxidation by 3-fold excess of H₂O₂ over time at 95 °C followed an exponential decline suggesting a pseudo-first order reaction (Publication F,²⁰⁷ Figure 4). The rate constant was determined to be $k=0.00403 \text{ min}^{-1}$. Considering that the thermal degradation of SMM is only three times higher ($k=0.01 \text{ min}^{-1}$)⁴⁸ it is likely that part of the generated DMS is subject to oxidation to DMSO during kilning before it diffuses out of the grain and evaporates into the ventilation air. However, DMS oxidation by H₂O₂ is a bimolecular reaction ($A+B \rightarrow \text{products}$), therefore the data of Publication F²⁰⁷ were further processed to determine the 2nd order rate constant for reasons of comparability to other reactions of H₂O₂. The following approach was therefore taken. Equation 4 (Publication F²⁰⁷) implies that the oxidative generation of one molecule DMSO is restricted to the consumption of one DMS- and/or H₂O₂ molecule. In general the second order rate constant ($k \text{ [M}^{-1}\text{s}^{-1}\text{]}$) can be determined from 2nd order rate law (Equation 5):

$$r = \frac{d[DMS]}{dt} = -k[DMS][H_2O_2] \quad \text{E5}$$

Considering the concentration of DMS at any given reaction time, its consumption is equivalent to the difference between the initial DMS concentration and the concentration of a product (DMSO or H₂O, further assigned as X). The same principle is applicable for H₂O₂.

$$[DMS] = [DMS]_0 - X \quad \text{E6}$$

$$[H_2O_2] = [H_2O_2]_0 - X \quad \text{E7}$$

The derived differential equation for the reaction rate can then be written as follows:

$$\frac{dx}{dt} = -k([DMS]_0 - x)([H_2O_2]_0 - x) \quad \text{E8}$$

From our study, no data for hydrogen peroxide were available, therefore a substitution method²⁷¹ was applied. Specifically, x was substituted by the introduction of the

dimensionless quantities of “reacted” DMS (z , Equation 9) and the dimensionless ratio of reactants (Q , Equation 10).

$$z = \frac{x}{[DMS]_0} \quad \mathbf{E9}$$

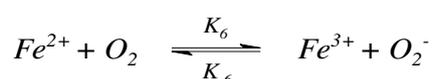
$$Q = \frac{[H_2O_2]_0}{[DMS]_0} \quad \mathbf{E10}$$

After substitution and integration the 2nd order rate constant can be determined from Equation 11:

$$\ln\left(\frac{Q-z}{Q(1-z)}\right) = [DMS]_0(Q-1)kt \quad \mathbf{E11}$$

In our case Q was 3 and z was determined for all sampling points. The plot of the left side of Equation 11 over time resulted in a linear correlation ($r^2:0.9853$), which confirmed the 2nd order nature of the reaction between DMS and H_2O_2 . Equation 11 was further applied to determine the rate constant of $k= 0.0000733 \text{ M}^{-1}\text{s}^{-1}$. This relatively low value compared with the reaction of H_2O_2 with Cys, for example ($k= \sim 0.009 \text{ M}^{-1}\text{s}^{-1}$, $\text{pH}=5.0$; $\text{T}=25^\circ\text{C}$) indicates that the reaction of H_2O_2 with Cys would probably be preferred over its reaction with DMS. Nevertheless, considerable amounts of DMSO are present in malt.^{41,46,202,203} A potential explanation for this may be found in the relatively high concentrations of SMM in green malt, which is in particular ~ 10 - 20 times higher than in the final malt. For example, $20 \mu\text{g/g}$ SMM would release $\sim 13.6 \mu\text{g/g}$ DMS in a kilning process comprising of 11h withering (65°C) and 3h curing (85°C) (calculated via Equation 1 and half-lives from Dickenson^{44,49}). Irrespective of the barley variety, Yang et al.⁴¹ recovered ~ 12 - 15% of the green malt SMM as DMSO in the final malt (5.2 - $9.3 \mu\text{g/g}$), which was in line with the data of Annes and Bamforth.⁴² They also recovered $\sim 15 \%$ DMSO from the initial green malt SMM, nonetheless a deviating kilning regime was applied. Even though merely 15% were oxidized, those numbers are significant considering the fact that it would result in pitching wort DMSO levels of ~ 650 - $900 \mu\text{g/L}$ (12 - 14°P). The H_2O_2 that was detected in the green malt was proposed to be a by-product of respiration meaning that most of the H_2O_2 formed during germination probably originates from the embryo, where also the majority of SMM is synthesized.³⁷⁻³⁹ This implies that the bioavailability of DMS for H_2O_2 is high. As mentioned before, the reaction of H_2O_2 with protein thiols, e.g. Cys residues would be preferred. However, lower molecular weight thiols are subject to synthesis of higher molecular proteins in the embryo.²⁸⁰ Such enzyme-

catalyzed reactions are much higher than autoxidative reactions thereby diminishing their availability for H₂O₂. The barley embryo is also rich in lipids as it contains about one third of the total barley lipids (~2%),²⁸¹ consequently they are also available for H₂O₂. The reaction rate of H₂O₂ with a mixture of linoleic-, oleic-, palmitic-, stearic and linolenic acid was reported to be $k=0.0000296 \text{ M}^{-1}\text{s}^{-1}$ ²⁸² which is lower but still in a similar range than the rate constant determined for the reaction of H₂O₂ with DMS in this dissertation work. This again strengthens the potential relevance of DMS oxidation by respiration-derived H₂O₂ during kilning. In plants, transition metal ions such as iron, are essentially present in a bound-state as they have developed mechanisms for iron uptake and homeostasis. This includes incorporation into enzymes or chelative transport whereby the amount of free iron in cellular compartments is minimized.²⁸³ However, as a consequence of higher iron levels in the soil or in the steeping water, free Fe²⁺ may be present in the grain, e.g. in the embryo, where H₂O₂ is also released. In this case •OH formation via the Fenton reaction (cp. 1.4) would be inevitable (diffusion-controlled reaction). Therefore, an additional experiment was performed to ascertain the reactivity of DMS with •OH in comparison with H₂O₂, O₂ and O₂ / Fe²⁺ as all of those systems are potentially abundant in malt. A particular focus was set on the relevance of the reaction between O₂ and Fe²⁺ to generate reduced oxygen species as this reaction is considered to be the key-initiating step of ROS formation in malting and brewing (Reaction 14).^{73,83–90}

**R14**

In accordance with the data of Publication D²⁰⁵ DMS was not subject to oxidation when heated in aqueous model solution (Publication F,²⁰⁷ Figure 5) even though this time the dissolved O₂ concentration was about five times higher. This observation confirmed that O₂ in ground state is not capable of reacting with DMS to form DMSO. The addition of Fe²⁺, in contrast to the data of Publication F²⁰⁷ (Figure 5), caused significant DMS oxidation as well as DMSO formation. This can probably be explained by the higher Fe²⁺- and O₂ concentrations used this time. However, considering the fact that the model systems were adjusted to extreme levels of Fe²⁺ and O₂, the oxidative DMS consumption was estimated to be as low as ~10 %. Even though electron donation from Fe²⁺ to O₂ and the attributed formation of O₂⁻ and H₂O₂ most likely resulted in the low amount of DMS oxidation, the contribution of Reaction 14 to elevated formation of ROS is probably overestimated. This became even more obvious by the standard redox potential differences of Fe³⁺/Fe²⁺ and

O_2/O_2^- , which were used to determine the Gibbs free energy differences (Publication F²⁰⁷, Equations 7-9). The approach demonstrated that the backwards reaction towards Fe^{2+} and O_2 is thermodynamically favored over O_2^- formation and that there is only a vanishingly small proportion O_2^- present at equilibrium state. These data were in agreement with the low amounts of DMSO formed in these systems. In agreement with the previous findings of this work, a much higher DMS oxidation was noted in the presence of H_2O_2 . When using H_2O_2/Fe^{2+} , a significantly higher DMS oxidation and DMSO formation was noted after 60 minutes suggesting that the reaction of DMS with $\cdot OH$ proceeds at a higher rate than with H_2O_2 . These observations were consistent with literature findings of DMS oxidation at ambient temperatures.^{271,284} However, after 180 minutes no significant differences was found for DMS oxidation between H_2O_2 and $\cdot OH$. A possible explanation was proposed in which Fe^{3+} (Fenton reaction product) reacted with residual H_2O_2 to form $Fe-OOH^+$ according to Perez-Benito.²⁸⁵ The latter decomposes slowly to form the hydroperoxyl radical ($HOO\cdot$) and thereby probably decelerated DMS oxidation in the investigated systems.

In conclusion of the findings of Publication F²⁰⁷ the H_2O_2 detection in green malt together with the characterization of its reaction with DMS via response surface- and kinetic modeling highlighted the potential relevance of H_2O_2 , and concomitantly also of $\cdot OH$, on the origin of DMSO in malt. Respiration combined with autoxidative reactions involving antioxidants and M^{n+} (cp. Publications D²⁰⁵ & E²⁰⁶) were proposed as main sources for H_2O_2 in malt. Furthermore, it was proposed that the reactions described in this work are also likely to be involved in other oxidative reactions (cp. 1.4) that are vital for processability and beer quality.

5 Conclusions

In accordance with the objectives of this work, new insights were generated that underline the contribution of DMSO to the levels of DMS beer. It is therefore suggested that DMSO should be recognized and assessed by the malting- and brewing industry as a DMS precursor. Based on the high water-solubility and stability of DMSO towards chemical alteration technological opportunities for its minimization in the brewing process are limited. Still, an additional CO₂ purging step after yeast multiplication during fermentation seems to be applicable to enhance DMS desorption which in turn would decrease the levels of DMS in beer. Based on the observed accumulation of DMSO in yeast plasma membranes also pre-treatments, like acidic yeast washing prior to pitching may also decrease DMS formation during fermentation.

From the fermentation trials it could also be concluded that the extent of DMS formation is not solely determined by the DMSO concentration in the pitching wort. The natural occurring levels of FAN and especially methionine in malt and wort were shown to play a less meaningful role as potential feedback inhibitors of MSRA during fermentation. Therefore, it is proposed that MetSO, the actual substrate of MSRA, is of higher relevance for the suppression of DMSO reduction and DMS formation during fermentation. However, no data of the levels of MetSO or other related sulfoxides in malt and wort were available from the present or other studies, therefore no evidence for such effects could be provided. Still, it is likely that Met and related, potentially peptide-bound, dialkyl sulfides are also oxidized to the corresponding sulfoxides by similar mechanisms as demonstrated for DMS. Because of their inhibiting effect on DMSO reduction these conditions are probably favorable regarding DMS formation during fermentation. However, prospective research is needed to investigate the effects of sulfoxides on the yeast redox status as well as physiological conditions.

The majority of DMSO was introduced into the brewing process by the malt implying that most auspicious steps to control the levels of DMSO are certainly to be found in the malting process. The yet unexplored findings of the prooxidative behaviour of antioxidant-Mⁿ⁺-systems by means of ROS formation have demonstrated their potential involvement in DMSO formation during the kilning process, for example.

While the prooxidative behaviour of Asco, Cys, GSH and GA towards DMS oxidation was mainly related to H₂O₂ a different mechanism was involved in the prooxidative nature of sulfite-Mⁿ⁺-systems. Evidence was provided for the formation of sulfite radicals, derived from Mⁿ⁺-mediated sulfite oxidation. As sulfite is generally applied during malt kilning it was

concluded that the formation sulfite- and peroxymonosulfate radicals are likely to be formed as well. Because of their exhaustive effect on DMS oxidation they present a highly potential source for DMSO in malt. Furthermore, the abundance of those sulfite-derived radicals opens up questions regarding their reactivity towards other malt constituents that contribute to malt quality. It was concluded therefrom that the application of sulfite as well as the other tested antioxidants cannot be suggested as adjuvants for the control of DMSO in malt, *per se*.

Therefore continued research is needed to characterize the prooxidative behaviour of a bigger variety of antioxidants and M^{n+} present in malt. Knowledge regarding potential synergisms and dose-dependencies together with physical-chemical impact factors are necessary to establish ways to compensate prooxidative effects of antioxidants and to increase their beneficial antioxidative properties.

Besides its formation via antioxidant- M^{n+} -catalyzed autoxidative reactions, H_2O_2 was also detected in barley after germination. It was proposed that the accelerated germination conditions used for the production of malt may induce stress leading to incomplete reduction of O_2 in the respiratory chain and ultimately, to the release of H_2O_2 in the embryo. The high availability of SMM and DMS in these cell compartments indicated that respiration-derived H_2O_2 is one of the most potential primary sources of DMSO in malt. Consequently, it was concluded that a key step for minimizing DMSO in malt is to diminish H_2O_2 formation during malting, essentially before the kilning process, where DMS is formed.

The prooxidative behavior of antioxidant- M^{n+} systems as well as the respiration-derived H_2O_2 formation during barley germination as discovered in this work also points to their potential involvement in other oxidative processes that are crucial for the quality of malt and beer. Coherently, the findings of this work can also be used as fundament for prospective research and may help to control oxidative reactions in food, in general.

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Appendix

List of publications

Publications in international peer-reviewed journals related to this thesis

- A. **Baldus, Matthias**; Kunz, Thomas; Methner, Frank-Jürgen
The Analysis of Dimethyl Sulfoxide in Malt, Wort and Beer using Headspace Gas Chromatography combined with Pulsed Flame Photometric Detection (HS-GC-PFPD) – Methodology and Applications. *BrewingScience*, **2013**, 66 (5/6), 154-161.
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- B. **Baldus, Matthias**; Klie, Rüdiger; Biermann, Max; Kreuzschner, Pascal; Hutzler, Mathias; Methner, Frank-Jürgen
On the Behaviour of Dimethyl Sulfoxide in the Brewing Process and its Role as Dimethyl Sulfide Precursor in Beer. *BrewingScience*, **2018**, 71 (1/2), 1–11.
<https://doi.org/10.23763/BrSc18-01baldus>
- C. **Baldus, Matthias**; Methner, Frank-Jürgen
Effect of antioxidants on dimethyl sulphoxide reduction and impact on dimethyl sulphide formation during beer storage. *Journal of the Institute of Brewing*, **2018**.
<https://doi.org/10.1002/jib.531>
- D. **Baldus, Matthias**; Klie, Rüdiger; De, Xi; Methner, Frank-Jürgen
Effect of L-Cysteine and Transition Metal Ions on Dimethyl Sulfide Oxidation. *Journal of Agricultural and Food Chemistry*, **2017**, 65 (10), 2180–2188.
<https://doi.org/10.1021/acs.jafc.6b05472>
- E. **Baldus, Matthias**; Majetschak, Sarah; Hass, Daniel; Klein, Raphael; Kunz, Thomas; Kunicka, Marta Saba; Methner, Frank-Jürgen
Effect of Malt-Derived Potential Antioxidants on Dimethyl Sulfide Oxidation. *Journal of Agricultural and Food Chemistry*, **2018**, 66 (40), 10522-10531.
<https://doi.org/10.1021/acs.jafc.8b02906>
- F. **Baldus, Matthias**; Tsushima, Satoka; Xi, De; Majetschak, Sarah; Methner, Frank-Jürgen
Response surface and kinetic modeling of dimethyl sulfide oxidation – On the origin of dimethyl sulfoxide in malt. *Journal of the American Society of Brewing Chemists*, **2018**, 76:1, 29-37.
<https://doi.org/10.1080/03610470.2017.1403816>

Further publications in other international peer-reviewed journals

Feilner, Roland; Rehmann, Dirk; Methner, Frank-Jürgen; **Baldus, Matthias**; Kunz, Thomas; Scheuren, Hans: Wort Stripping – Thermodynamic Considerations on the Evaporation of Aroma Substances in Continuous Desorption Processes. *BrewingScience – Monatsschrift für Brauwissenschaft*, **2013**, 66, (5/6), 65-74.

Scheuren Hans; **Baldus, Matthias**; Methner, Frank-Jürgen; Dillenburger, Michael: Evaporation behaviour of DMS in an aqueous solution at infinite dilution – a review, In *Journal of the Institute of Brewing*, **2016**; 122, 181–190.

Wietstock, Philip C.; **Baldus, Matthias**; Öhlschläger, Magdalena; Methner, Frank-Jürgen. Hop Constituents Suppress the Formation of 3-Methylbutanal and 2-Furfural in Wort-Like Model Solutions. *Journal of the American Society of Brewing Chemists*, **2017** 75 (1), 41-51.

Publications in non-peer reviewed journals with academic editorial board

Feilner, Roland; Methner, Frank-Jürgen; Rehmann, Dirk; **Baldus, Matthias**; Scheuren, Hans Würzestripping: Thermodynamische Betrachtungen des Ausdampfverhaltens (Teil 1) *Brauwelt*, **2013**, 153 (24), 716-719.

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Feilner, Roland; **Baldus, Matthias**; Kunz, Thomas; Methner, Frank-Jürgen Wort Stripping: Based on Thermal Desorption, Supports the Classic Boiling Process with a More Efficient Evaporation and without Using Additional Thermal Energy. *The Master Brewers Association of the Americas – Technical Quarterly*, **2013**, 50 (1), 15-20.

Baldus, Matthias; Methner, Frank-Jürgen; Die Bedeutung von DMSO im Brauprozess – Theoretischer Hintergrund. *Brauwelt*, **2014**, 36, 1082-1084.

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Baldus, Matthias; Methner; Frank-Jürgen: Significance of DMSO in the brewing process, *Brauwelt International*, 2016 vol. 34, 168-170.

Conference Contributions:

Baldus, Matthias; Feilner, Roland; Kunz, Thomas; Methner, Frank-Jürgen: Evaluation and improvement of the evaporation of unwanted aroma compounds after wort boiling by a thermal desorption process, (Lecture) *3rd International Young Scientists Symposium* Nottingham, Great Britain, **2012**.

Baldus, Matthias: Development of aldehydes affecting the taste during storage of beer factors influencing the mechanisms of their development. Mechanisms of dicarbonyl development during wort and beer production, (Lecture) *Barley, Malt & Beer Union Conference*, Moscow, Russia, **2013**.

Baldus, Matthias: Sulfur-related compounds and their redox properties in beer production, (Lecture) *Barley, Malt & Beer Union Conference*, Moscow, Russia, **2013**.

Baldus, Matthias; Methner, Frank-Jürgen: Kinetic Investigation on the sulfite mediated reduction of DMSO- Impact on DMS formation during beer storage, (Lecture) *35th Congress of the European Brewing Convention*, Porto, **2015**.

Baldus, Matthias; Satoka Tsushima; Fabian Dierke; Moritz Schneider; Methner, Frank-Jürgen: Abundance and relevance of dimethyl sulfoxide in brewing, (Poster) *35th European Brewing Convention*, Porto, **2015**.

Baldus, Matthias; Methner, Frank-Jürgen: Formation of dimethyl sulfide during beer storage via bisulfite reduction of dimethyl sulfoxide, (Lecture) *Annual Meeting of the Master Brewers Association of the Americas*, Jacksonville, Florida, USA, **2015**.

Öhlschläger, Magdalena; Wietstock, Philip C.; **Baldus, Matthias;** Methner, Frank-Jürgen: Impact of Hop Constituents on the Formation of Staling Aldehydes in Wort-Like Model Solutions, (Poster) *12th International Trends in Brewing Congress*, Gent, Belgium, **2016**.

Baldus, Matthias; Xi, De; Tsushima, Satoka; Methner, Frank-Jürgen: Response surface modelling and kinetics of dimethyl sulfide oxidation in malt model solutions (Poster), *12th International Trends in Brewing Congress*, Gent, Belgium, **2016**.

Baldus, Matthias, Xi, De, Majetschak, Sarah, Methner, Frank-Jürgen: Impact of malt derived pro- and antioxidants on the formation of dimethyl sulfoxide, (Lecture) *World Brewing Congress*, Denver, Colorado, USA, **2016**.

Baldus, Matthias; Xi, De; Majetschak, Sarah; Methner, Frank-Jürgen: Effect of cysteine and transition metals on dimethyl sulphide oxidation, (Lecture) *Studiedag Brouwerij - Mouterij (Symposium Brewing – Malting)*, Hogeschool Gent – Universiteit Gent, Belgium, **2016**.

Baldus, Matthias; Kreuschner, Pascal; Biermann, Max; Methner, Frank-Jürgen: Einfluss der Würzezusammensetzung sowie des Hefestammes auf die Bildung von DMS während der Fermentation, (Lecture) *5. Seminar Hefe und Mikrobiologie*, Freising, Weihenstephan, **2017**.

Baldus, Matthias; Wietstock, Philip; Hass, Daniel; Methner, Frank-Jürgen: Chemical-analytical characterization of the prooxidative behaviour of malt-derived antioxidants, (Lecture), *36th Congress of the European Brewing Convention*, Ljubljana, Slovenia, **2017**.

Baldus, Matthias; Wietstock, Philip C.; Majetschak, Sarah; Hass, Daniel; Methner, Frank-Jürgen. Elucidation of pro-oxidative effects of malt- and beer- derived antioxidants, (Lecture) *Brewing Summit (Meeting of the American Society of Brewing Chemists)*, San Diego, California, USA, **2018**.

Baldus, Matthias; Heukäufer, Florian; Großpietsch, Carla; Methner, Frank-Jürgen: Respiration-derived accumulation of hydrogen peroxide in barley seeds and its impact on malt quality, (Lecture) *Brewing Summit (Meeting of the American Society of Brewing Chemists)*, San Diego, California, USA, **2018**.

Eidesstattliche Erklärung

Hiermit versichere ich, Matthias Baldus, an Eides statt durch meine Unterschrift, dass die vorliegende Dissertation "Redox Behavior of Dimethyl Sulfide and Dimethyl Sulfoxide in Malting and Brewing" in allen Teilen von mir selbständig angefertigt wurde. Alle benutzten Hilfsmittel sind vollständig angegeben.

Vorveröffentlichungen, die in der vorliegenden kumulativen Dissertation verwendet wurden, können dem Anhang entnommen werden und sind in Kapitel 3 aufgelistet. Alle Stellen, die wörtlich aus Veröffentlichungen entnommen wurden, sind als solche kenntlich gemacht und es wurde keine andere als die angegebene Literatur oder sonstige Hilfsmittel verwendet. Nach § 5, Abs. 1, Satz 3, Nr. 5 und 6 Promotionsordnung der Technischen Universität Berlin versichere ich an Eides statt, dass die Darstellung des Eigenanteils in Kapitel 3 der Wahrheit entspricht. Darüber hinaus erkläre ich, dass ich die Promotionsabsicht nie an anderer Stelle angemeldet bzw. ein Promotionseröffnungsverfahren beantragt habe.

Berlin, den 05.10.2018