C. Schmidt, L. Hoferer and M. Biendl

Determination of Variety Dependent “Thiol Impact” Based on LC-MS/MS Analysis of Different Hop Samples Collected Worldwide

Certain volatile thiols are some of the most potent odour-active molecules found in food and beverages including beer. Even contents of a few µg/kg in hops can be high enough to finally result in significant contributions to beer flavour, especially after dry hopping. Most published methods for the determination of free thiols in hops are based on gas chromatographic measurements. However, liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS), as presented in this study, can also serve for their reliable quantification. This method was used to quantify 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH), and 3-mercapto-4-methylpentan-1-ol (3M4MP) in 250 hop samples of 97 different varieties from 12 countries and 5 crop years (2019 – 2023). Thiol contents show high fluctuation ranges not only dependent on the variety but also on growing area, crop year or harvest date. Based on a large number of single results, it is suggested to create a general classification of varieties by assigning three main categories (low/medium/high) for their “thiol impact” in beer brewing.

Descriptors: Free polyfunctional thiols, thiol impact, hops, beer brewing

1 Introduction

Free polyfunctional thiols like 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) as well as 3-mercapto-4-methylpentan-1-ol (3M4MP) are known as very potent and prominent aroma compounds in food and beverages. Chemical structures of the thiols are given in figure 1.

4MMP was identified for the first time in the hop variety US Cascade by Steinhaus et al. in 2006 [1]. In the same year, 4MMP and 3MH were detected as hop-derived aroma compounds in beer by Kishimoto et al. [2], and by Vermeulen et al. [3].

These studies were the beginning of investigations about polyfunctional thiols in hops and beer. Two years later, Kishimoto et al. compared the contents of 4MMP in hop cultivars from different growing regions [4] and demonstrated in a further publication that both, malt and hops, are a source of 3MH precursors [5]. In 2009, Takoi et al. were able to identify 3M4MP for the first time as hop derived and special flavour compound present in the hop cultivar Nelson Sauvin from New Zealand [6–7]. 3M4MP is a structural isomer of 3MH (Fig. 1) with the same molecular mass.

Polyfunctional thiols are also known from fruits and berries imparting blackcurrant-like (4MMP), grapefruit-like (3MH and 3M4MP), and/or rhubarb-like (3MH and 3M4MP) aroma impressions [1,6-7]. The odour threshold values in beer are only about 2 ng/L for 4MMP [2], 55 ng/L for 3MH [2] and 70 ng/L for 3M4MP [6].

Studies of Reglitz and Steinhaus expanded the knowledge. In 2017, they reported on the influence of hop variety, growing region, harvest year, pellet production, and storage on 4MMP concentrations [8].

The group of Sonia Collin not only identified and quantified free polyfunctional thiols in various varieties [9–11] but also their precursors present in both hops and malt as cysteine and glutathione conjugates [11–18]. Roland et al. investigated in 2017 the “thiol potency” as an analytical indicator of thiols release in beers with focus on 14 hop varieties [19]. Recently, Kishimoto et al. published their work about the first identification of disulfide-bonded thiols in malt and hops as possible precursors of free thiols in beer [20]. Current research is also engaged in the interaction of hops and yeast during fermentation. This topic is very complex and influenced, amongst other factors, by the type of hop variety, a broad range of yeast strains or interspecies yeast hybrids as well as different brewing conditions [21–24].

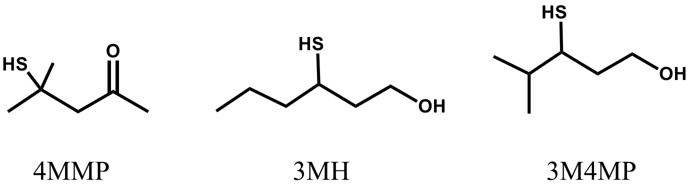

Fig. 1 **Chemical structures of free polyfunctional thiols (4MMP = 4-mercapto-4-methylpentan-2-one, 3MH = 3-mercaptohexan-1-ol, 3M4MP = 3-mercapto-4-methylpentan-1-ol)**

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In the field of free polyfunctional thiol analysis in hops and beer, gas chromatographic (GC) methods dominate. One- or multidimensional GC systems can be coupled with different types of detectors. There are published methods with GC-Flame Photometric Detector (GC-FPD, [6]), GC-Pulsed Flame Photometric Detector (GC-PFPD, [9-11]), GC-Mass Spectrometry (GC-MS, [4]), GC-Tandem Mass Spectrometry (GC-MS/MS, [25–26]) or GC×GC-Time of Flight Mass Spectrometry (GCXGC-TOFMS, [8]) with or without derivatization steps and/or a complex sample preparation.

The present work is dealing with free polyfunctional thiols to describe primarily the differences between hop cultivars using LC-MS/MS quantification for 4MMP, 3MH, and 3M4MP after derivatization with 4,4'-dithiodipyridine (4,4'-DTDP) as a reliable and robust methodology. Such a method was currently introduced by Liu and colleagues but for 4MMP and 3MH only [27]. It was initially presented by Capone et al. for free thiol analysis in wine [28]. The large data set we collected can serve as the basis for additional information in variety descriptions by creating different "thiol impact" categories which represent the thiol potential of a certain variety for beer brewing.

Table 1 **Specific mass transitions and optimised parameters for the LC-MS/MS of thiols**

Compound	Mass transitions m/z Q1 → Q3	Retention time [min]	DP[a] [V]	CE[b] [V]	EP[c] [V]
d5-3MH (IntStd)	249.1 → 144.2[d]	16.4	81	23	4.5
	249.1 → 111.1		81	43	4.5
4MMP	242.1 → 143.9[d]	14.3	71	25	4.5
	242.1 → 111.1		71	41	4.5
3M4MP/3MH	244.1 → 144.2[d]	15.8/16.6	81	23	4.5
	244.1 → 111.1		81	43	4.5

[a] Declustering potential. [b] Collision energy. [c] Entrance potential. [d] Quantifier ion. IntStd: Internal standard

Table 2 **Quantitative data from literature for free thiols determined in the hop variety Cascade from different crop years and regions**

Reference	Crop year (region)	4MMP (µg/kg)	3MH (µg/kg)	3M4MP (µg/kg)
[4–5]	2006 (US)	11.1	80.0	n.a.
[9]	2007 (US)	1.2	72.6	6.6
	2008 (US)	0.8	117.1	9.8
[26]	2013 (US)	16.8	n.a.	18.1
[15]	2014 (–)	3.5	12.1	n.a.
[8]	2015 (DE)	2.25	n.a.	n.a.
	2015 (US)	6.79	n.a.	n.a.
[27]	2019 (DE)	0.8	5.6	n.a.
	2019 (US)	1.8	10.0	n.a.
	2020 (DE)	0.8	8.8	n.a.
	2020 (US)	1.8	13.7	n.a.

n.a. = not analysed, (–) no information available

Table 3 **LC-MS/MS quantitative data (hop variety Cascade)**

Region (crop year)	4MMP (µg/kg)	3MH (µg/kg)	3M4MP (µg/kg)	Σ3MH+ 3M4MP
USA (2019)	2.8	3.6	6.3	9.9
USA (2020)	2.4	3.6	10.0	13.6
USA (2021)	3.8	6.6	6.6	13.2
USA (2022)	2.6	3.7	3.4	7.1
USA (2023)	2.5	2.9	5.2	8.1
Germany (2020)	n.d.	5.0	5.1	10.1
Argentina (2023)	n.d.	n.d.	10.3	10.3

n.d. = not detectable

2 Materials and methods

2.1 Reagents

Following chemicals were obtained from commercial sources: water and methanol for LC-MS (Chemsolute®, Th. Geyer GmbH & Co. KG, Renningen, Germany); formic acid and ammonium formate (Merck, Darmstadt, Germany); 4,4'-Dithiodipyridine (4,4'-DTDP, 98 %) Acros Organics (Thermo Fisher Scientific); concentrated HCl (37 %) and Na_2EDTA (Titriplex III) (Supelco®, Merck, Darmstadt, Germany). The aroma compounds 4MMP and 3MH were obtained from Merck (Darmstadt, Germany), the internal standard d5-3MH as well as 3M4MP were purchased from aromaLab (Planegg, Germany).

A solution (10 mM) was prepared by sonicating 110 mg of 4,4'-DTDP in 10 mL LC-MS water and 50 µL concentrated HCl (37 %). After dissolution, water was added to give a final volume of 50 mL. Aliquots of this derivatizing reagent were stored at – 20 °C until usage.

2.2 Sampling

During large scale production of hop pellets, samples representing homogeneous pellet batches of at least one ton were taken, packaged under vacuum, and then stored cold (< 10 °C) until analysis. In total 250 samples of 97 different cultivars from 12 countries (Germany, Czech Republic, Slovenia, Poland, United Kingdom, France, Spain, United States of America, Argentina, South Africa, Australia, New Zealand) and 5 crop years (crops 2019 – 2023) were analysed.

2.3 Sample preparation

0.1 g of milled pellets was suspended in 4.16 mL of methanol and 5 mL of water. After addition of 40 µL of internal standard d5-3MH (c = 50 ng/mL), 300 µL Na_2EDTA (2.5 %) and 500 µL of the derivatization reagent 4,4′-dithiodipyridine (4,4′-DTDP, 10 mM solution) the sample was extracted for 30 min with the help of a shaking device (225 rpm). An aliquot of this sample was then centrifuged for 15 min at 13500 rpm and part of the supernatant was analysed using LC-MS/MS in the positive mode. All samples were analysed in duplicate. Detailed information on all hop varieties tested can be taken from the results section.

Table 4A **Examples of hop varieties of different regions with "low thiol impact"**

Variety	Country	4MMP (µg/kg)	3MH (µg/kg)	3M4MP (µg/kg)	Σ3MH+ 3M4MP
East Kent Golding	England	n.d.	n.d.	n.d.	n.d.
Tettnanger	Germany	n.d.	n.d.	n.d.	n.d.
Akoya	Germany	n.d.	<10	n.d.	<10
Perle	Germany	n.d.	<10	n.d.	<10
Saazer	Czech Republic	n.d.	<10	n.d.	<10
Styrian Golding	Slovenia	n.d.	<10	n.d.	<10
Strisselspalt	France	n.d.	<10	n.d.	<10
Lubliner	Poland	n.d.	<10	n.d.	<10
Willamette	USA	n.d.	<10	n.d.	<10
African Queen	South Africa	n.d.	n.d.	<10	<10
Southern Star	South Africa	n.d.	n.d.	<10	<10
Mapuche	Argentina	n.d.	n.d.	<10	<10
Herkules	Germany	n.d.	<10	<10	<10

Table 4B **Further hop varieties with "low thiol impact" for beer brewing**

Variety	Country	Variety	Country
Brewers Gold	Germany	Marynka	Poland
Diamant	Germany	Bramling Cross	England
Hallertau Mittelfrüh	Germany	Challenger	England
Hersbrucker	Germany	First Gold	England
Magnum	Germany	Fuggle	England
Merkur	Germany	Pilgrim	England
Northern Brewer	Germany	Progress	England
Opal	Germany	Target	England
Saphir	Germany	Bitter Gold	USA
Smaragd	Germany	Contessa	USA
Spalter	Germany	Delta	USA
Spalter Select	Germany	Galena	USA
Taurus	Germany	Lemondrop	USA
Titan	Germany	Mount Hood	USA
Tradition	Germany	Nugget	USA
Xantia	Germany	Nugget	Germany
Premiant	Czech Republic	Sorachi Ace	USA
Sladek	Czech Republic	Sultana	USA
Styrian Golding Bobek	Slovenia	Super Galena	USA
Styrian Golding Celeia	Slovenia	Tahoma	USA
Super Styrian Aurora	Slovenia	Vanguard	USA
Aramis	France	Southern Dawn	South Africa
Barbe Rouge	France	Southern Passion	South Africa
Triskel	France	Southern Promise	South Africa

2.4 Sample analysis with liquid chromatography – tandem mass spectrometry (LC-MS/MS)

The ExionLC™ system, consisting of a binary pump, a degasser, an auto-sampler and a thermostatted column oven with capacity for two analytical columns (Sciex, Darmstadt, Germany), was coupled with a 5500+ Q-TRAP mass spectrometer (Sciex, Darmstadt, Germany) equipped with an electrospray ionisation (ESI) source running in the positive ion mode. Samples were introduced by HPLC at a solvent flow of 250 µl/min, which required the use of turbo gas at a temperature of 350 °C. The ion spray voltage was set to 5500 V, the declustering potential and the MS/MS parameters were optimised for each substance to induce fragmentation of the pseudo molecular ion $[M-H]^+$ to the corresponding target product ions after collision-induced dissociation. The collision energy (CE), the declustering potential (DP) as well as the entrance potential (EP) were set as given in table 1. Nitrogen with purity of 99.7 % was used as the collision gas. The quantitation was done using the scheduled multiple reaction monitoring (MRM) mode of the instrument with the fragmentation parameters optimised prior to analysis and the retention times of the corresponding reference compounds. Data processing was performed by using Analyst software version 1.7.3 and data integration was done by SCIEX OS software version 1.7 (Sciex, Darmstadt, Germany). For chromatography, an analytical column (100 x 2.0 mm, 2.7 µm EC 100/2 Nucleoshell PFP, Macherey-Nagel, Düren, Germany) equipped with a guard column of the same type (Macherey-Nagel, Düren, Germany) served as the stationary phase. 5 mM ammonium formate containing 0.1 % formic acid in water was used as solvent A and methanol with 5 mM ammonium formate and 0.1 % formic acid as solvent B. The temperature of the column oven was set at 40 °C. The injection volume was 10 µl. Chromatography was performed using isocratic conditions with 77 % of solvent A and 23 % of solvent B for 28 min. Quantification was done by external calibration in a range between 0.01 and 1 µg/kg with d5-3MH (c = 0.2 µg/kg) as internal standard after the derivatization as described in the sample preparation chapter.

Table 5A **Examples of hop varieties with "medium thiol impact" for beer brewing**

Variety	Country	4MMP (µg/kg)	3MH (µg/kg)	3M4MP (µg/kg)	Σ3MH+ 3M4MP
Cascade	Germany	n.d.	<10	<10	>10
Cascade	Argentina	n.d.	n.d.	>10	>10
Gaucho	Argentina	n.d.	n.d.	>10	>10
Huell Melon	Germany	n.d.	>10	n.d.	>10
Centennial	USA	n.d.	>10	n.d.	>10
Mistral	France	n.d.	<10	<10	>10
Ella	Australia	<10	n.d.	<10	<10
Azacca	USA	<10	<10	<10	<10
Motueka	New Zealand	<10	<10	<10	<10

3 Results and discussion

The LC-MS/MS technique was used to quantify the thiols 4MMP, 3MH as well as 3M4MP in various hop pellet samples. The limit of quantification (LOQ) for 4MMP, 3MH, and 3M4MP was 2.0 µg/kg, and the limit of detection (LOD) was always 0.50 µg/kg. The LOD and LOQ are defined as the concentrations which yield a peak with a signal-to-noise ratio (S/N) of 3 and 10 respectively. The LOD is higher than described in the previous work by Liu et al. [24] but sufficient to differentiate the investigated cultivars. At spike levels of 2.0 and 10.0 µg/kg, the recovery rates of 4MMP were 111 % and 99 %. At the same spike levels, the recovery rates of 3MH were 126 % and 111 %. And at spike levels of 2.0 and 30.0 µg/kg, the recovery rates of 3M4MP were 135 % and 118 %. For all three thiols, the relative standard deviation between four repeated analyses was lower than 15 %.

As mentioned in the introduction part, there are various studies reporting different quantitative data on free thiols. To demonstrate the wide range of concentrations published for one single hop variety, some results on the cultivar Cascade are presented in table 2.

As shown in table 2, large differences can be observed. For example, the overall range for 4MMP is between 0.8 and 16.8 µg/kg, and for 3MH from 10.0 to 117.1 µg/kg in Cascade only grown in the USA. Amongst others, such large fluctuation ranges may come from different analytical methods used in the several studies.

Table 5B **Further hop varieties with "medium thiol impact" for beer brewing**

Variety	Country
Ariana	Germany
Apollo	USA
Apollo	Spain
Bravo	USA
Cluster	USA
Chinook	USA
Columbus	USA
El Dorado	USA
Helios	USA
Lotus	USA

Table 3 shows the results on the variety Cascade based on the LC-MS/MS method as presented in this paper.

Here, the fluctuation ranges are lower as compared to table 2. But even when using an identical method there are still some considerable differences within the same growing country, most likely dependent on soil, weather conditions during the growing season, harvest date, kilning procedure, etc [29–30]. Therefore, we finally decided to use all single concentration data of the large number of investigated samples just for creating a general "thiol impact" classification. For this purpose, for each variety only the maximum quantitative values were considered representing its highest potential of free thiols for beer brewing. Thus, only the results of the sample from crop 2020 and 2021 served for assigning the "thiol impact" category of US Cascade. When grown in USA, this cultivar always contains the three types of thiols, but 4MMP is missed in the samples from Germany and Argentina thus exhibiting less thiol potential and resulting in a lower "thiol impact" classification.

The aroma characterisation of 3MH and 3M4MP as well as their odour threshold concentrations are known to be very similar [2,6]. Therefore, their concentrations were summed up for assigning the "thiol impact" category. But the LC-MS/MS methodology presented in this work allows for the quantification of both thiols after chromatographic separation together with 4MMP.

Reglitz et al. investigated the fate of 4MMP in beer production after static dry hopping [31] and they report on a transfer rate of approximately 70 %. We adopted similar transfer rates also for 3MH and 3M4MP. If their sum in hops is lower than 10 µg/kg, even at hop dosages up to 1000 g/hL, the resulting amounts in beer are unlikely to exceed the odour threshold concentrations of 3MH and 3M4MP. Therefore, their limit of classification for the thiol impact was set at 10 µg/kg. Because the odour threshold concentration of 4MMP is much lower, the classification for this thiol further differentiates between not detected (n.d.), and below or above 10 µg/kg.

Hops in the "low thiol impact" category are free of 4MMP and have no or only low contents of 3MH and/or 3M4MP (in total < 10 µg/kg). The complete list of hop cultivars with "low thiol impact" is presented in table 4 (Part A and B).

Overall, more than half of the varieties analysed on free thiols by LC-MS/MS belong to this group, including all the classic aroma hops. It contains cultivars from Europe (Germany, Czech Republic, Slovenia, France, Poland, UK), the United States, South Africa and Argentina. In addition to the sum of 3MH plus 3M4MP, individual results of the isomers are presented in table 4A for 13 hop varieties from different parts of the world. Some varieties contain no thiols at all. Others like Akoya, Perle, Saazer, Styrian Golding, Strisselspalt, Lubliner and Willamette show only low contents of 3MH but no 3M4MP. Whereas for cultivars from South Africa (African Queen and Southern Star) as well as for the cultivar Mapuche from

Argentina only 3M4MP was detectable but no 3MH. The German variety Herkules contains low amounts of both isomers, but their sum is still below 10 µg/kg. In table 4B further hop varieties with "low thiol impact" are listed.

Hop varieties with "medium thiol impact" are presented in table 5 (Part A and B). This category includes cultivars with either no 4MMP but the sum of 3MH plus 3M4MP being higher than 10 µg/kg or both 4MMP as well as the sum of 3MH plus 3M4MP are below 10 µg/kg. Hop cultivars with 3MH only are Huell Melon from Germany and Centennial from USA. Varieties with 3M4MP only are Gaucho and Cascade, both from Argentina. Ella from Australia also contains no 3MH. For all other varieties of table 5A both isomers, 3MH and 3M4MP, were detectable. Table 5B presents further hop varieties with "medium thiol impact".

Hop cultivars with "high thiol impact" as the last category are presented in table 6. They all contain 4MMP and the sum of 3MH plus 3M4MP is always higher than 10 µg/kg.

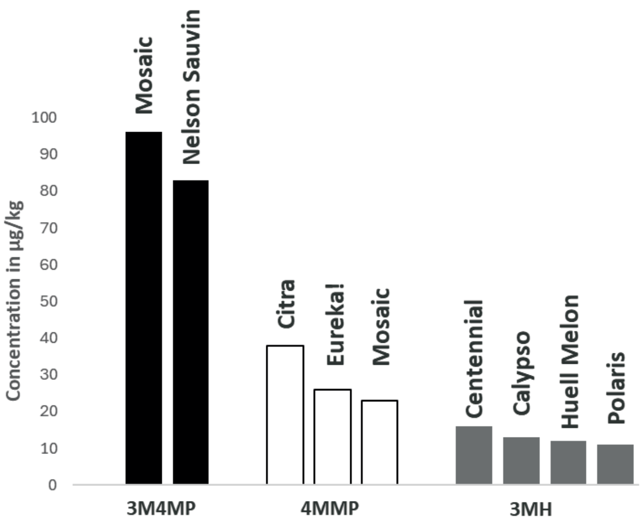

Fig. 2 **Concentrations of 3M4MP (black), 4MMP (white), and 3MH (grey) for selected hop varieties**

The cultivars Nelson Sauvin grown in New Zealand and Galaxy from Australia contain the isomer 3M4MP but no 3MH. This is also the case for Callista, Eureka! and Solero grown in Germany as well as Altus and Summit from United States. Polaris from Germany is the only variety in this group with 3MH but without 3M4MP. For all remaining cultivars both isomers were always detectable together with 4MMP. All varieties presented in table 6 are well-known regarding their dry hopping potential. This confirms the assigned "thiol impact" classification based on the presented LC-MS/MS method.

Table 6 **Examples of hop varieties with "high thiol impact" for beer brewing**

Variety	Country	4MMP (µg/kg)	3MH (µg/kg)	3M4MP (µg/kg)	Σ3MH+ 3M4MP
Callista	Germany	<10	n.d.	>10	>10
Hallertau Blanc	Germany	<10	<10	>10	>10
Mandarina Bavaria	Germany	<10	<10	>10	>10
Polaris	Germany	<10	>10	n.d.	>10
Solero	Germany	<10	n.d.	>10	>10
Tango	Germany	<10	<10	>10	>10
Alora	USA	<10	<10	>10	>10
Altus	USA	<10	n.d.	>10	>10
Amarillo	USA	<10	<10	>10	>10
Calypso	USA	<10	>10	<10	>10
Cascade	USA	<10	<10	<10	>10
Crystal	USA	<10	<10	>10	>10
Idaho7	USA	<10	<10	>10	>10
Summit	USA	<10	n.d.	>10	>10
Citra	USA	>10	<10	>10	>10
Eureka!	USA	>10	<10	>10	>10
Eureka!	Spain	>10	<10	>10	>10
Eureka!	Germany	>10	n.d.	>10	>10
Mosaic	USA	>10	<10	>10	>10
Simcoe	USA	>10	<10	<10	>10
Strata	USA	>10	<10	>10	>10
Galaxy	Australia	>10	n.d.	>10	>10
Nelson Sauvin	New Zealand	>10	n.d.	>10	>10

When looking at all three categories (tables 4 – 6), only for the variety Cascade the "thiol impact" is clearly influenced by the growing country. "High thiol impact" can be expected from US Cascade but "medium thiol impact" when it comes from Germany or Argentina. Whereas in case of the variety Eureka!, growing in USA, Germany or Spain always results in "high thiol impact". Two more cultivars also remain in the same category when grown either in USA or Europe. These are Apollo with "medium thiol impact", and Nugget with "low thiol impact".

Finally, figure 2 shows maximum contents of the three thiols measured by the LC-MS/MS method. Overall, every single level falls below 100 µg/kg. By far the two highest concentrations are reached for 3M4MP in the varieties Mosaic (96 µg/kg), and Nelson Sauvin (83 µg/kg). All other 3M4MP levels as well as all contents of 4MMP and 3MH are always below 50 µg/kg. However, just three cultivars show more than 20 µg/kg 4MMP (Citra (38 µg/kg), Eureka! (26 µg/kg), Mosaic (23 µg/kg)). Although 3MH was detectable in most varieties (see tables 4 – 6), only 4 of them show levels above 10 µg/kg. These are Centennial with 16 µg/kg, Calypso with 13 µg/kg, Huell Melon with 12 µg/kg, and Polaris with 11 µg/kg.

Overall, 3-MH seems to be the least selective criterium to judge the impact of free hop thiols on beer flavour. Maybe it has been overestimated in the past due to analytical challenges. Because of identical molar masses, it is crucial to guarantee its peak separation from 3M4MP even when MS detection is applied. Moreover, contrary to 4MMP and 3M4MP, the thiol 3MH is known to be present in malt in form of precursors as another source for its content in beer. But 3MH keeps important for judging the thiol potential of hops, especially under consideration of possible releases from precursors and synergistic sensory effects.

4 Conclusion and outlook

In total 97 different hop cultivars were analysed with the help of a reliable LC-MS/MS method. On the one hand, more than half of the varieties investigated belong to the group with "low thiol impact" where 4MMP was missing and no or only low contents of 3MH or 3M4MP were detectable (< 10 µg/kg). This group includes all the classic aroma hops. On the other hand, well-known cultivars giving unique flavour impressions (citrusy, fruity, etc.) could be clearly classified as "high thiol impact" with the sum of 3MH and 3M4MP being always above 10 µg/kg, and 4MMP detectable in all cases. All other cultivars are in between, thus exhibiting "medium thiol impact". Such a classification can be useful for variety description, e.g. in data sheets, to get some more information on the expectable sensory impression resulting from dry hopping. The extension of data is always possible to complete the worldwide hop spectrum (more than 300 varieties) and to finally include new breeding lines as soon as released. After the successful implementation of a methodology for the most important free polyfunctional thiols in hops, method development for thiol precursors from hops is under progress.

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