Martin Biendl, Laura Hoferer and Christina Schmidt

Single-run liquid chromatography-tandem mass spectrometry method for the simultaneous determination of selected free and bound polyfunctional thiols in hop samples

The polyfunctional thiols 4-methyl-4-sulfanylpentan-2-one (4MSP), 3-sulfanyl-4-methylpentan-1-ol (3S4MP), and 3-sulfanylhexan-1-ol (3SH) are among the best-known volatile hop compounds, contributing to the aroma profile of beer even at extremely low concentrations. They occur in hops not only as free aroma-active molecules, but also in bound form. At a conversion yield below 1 % the free forms can be released from such S-conjugates in the brewing process, mainly during fermentation. A novel method is presented for the rapid and concurrent analysis of free thiols in hops together with the precursors 3-S-cysteinyl-hexan-1-ol (Cys-3SH), and 3-S-glutathionyl-hexan-1-ol (G-3SH). Before determination by liquid chromatography tandem mass spectrometry (LC-MS/MS) within one run, the free forms are derivatized with 4,4´-dithiodipyridine (DTDP) during sample preparation. This allows for the simultaneous quantification of such derivatives as well as the S-conjugates, with a repeatability precision of less than 15 % (relative standard deviation). Similar straightforward approaches for the concurrent analysis of free thiols and their precursors have been already published for wine, tea and beer but not for hops. So, the presented method now enables such a simple single-run LC-MS/MS analysis to be also applied in hop research, and even in industry for routine purposes.

Descriptors: Hops, 4-methyl-4-sulfanylpentan-2-one, 3-sulfanyl-4-methylpentan-1-ol, 3-sulfanylhexan-1-ol, 3-S-cysteinyl-hexan-1-ol, 3-S-glutathionyl-hexan-1-ol

1 Introduction

Polyfunctional thiols contain a reactive sulfhydryl (-SH) group in addition to another functional moiety such as alcohol or ketone. Here, these natural compounds are simply called thiols. In beer they originate primarily from hops, while malt plays only a minor role [1]. Of the more than 40 different thiols found in hops, the key compounds are 4MSP, 3S4MP, and 3SH (also referred to as 4MMP, 3M4MP, 3MH, and synonymous with these terms) (Fig. 1). Their presence depends on the variety. A recently published study reports maximum levels of 38 µg/kg 4MSP, 96 µg/kg 3S4MP, and 16 µg/kg 3SH found in a total of 250 samples from 97 different varieties from 12 hop growing countries [2]. Even at concentrations in the low ng/L range, these compounds can contribute to characteristic fruity-tropical aroma impressions in beer (Table 1).

Highly volatile thiols occur in hops not only as free aroma-active molecules, but also in bound form. Such precursors exist as S-conjugates to cysteine or related peptides containing cysteine (e.g. glutathione). For 3SH, the odorless precursors can even exceed concentrations of 10 mg/kg, compared to only a few µg/kg of free thiols. S-conjugated 3S4MP have rarely been detected so far, and only in a very limited number of hop varieties. The bound form of 4MSP occurs more frequently, though its levels are in the low µg/kg range and thus comparable to those of the free molecules [1].

In 2017, Roland et al. analyzed 14 different hop varieties and identified the same thiol precursors that had been discovered in winemaking already twenty years earlier [3]. These include 3-S-cysteinyl-hexan-1-ol (Cys-3SH) and 3-S-glutathionyl-hexan-1-ol (G-3SH) (Fig. 2), which were first identified in hops in 2012 [4] and 2016 [5], respectively. In the study published 2017, both 3SH precursors were present in all investigated cultivars from various growing countries with maximal contents of 1,905 µg/kg Cys-3SH, and 20,678 µg/kg G-3SH [3]. If at all, the cysteinylated or glutathionylated forms of 4MSP, 4-S-cysteinyl-4-methylpentan-2-one (Cys-4MSP) or 4-S-glutathionyl-4-methylpentan-2-one (G-4MSP), could only be detected at trace amounts below 0.1 µg/kg. Precursors of 3S4MP were not considered in this study.

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Thiols bound to cysteine or glutathione in this way can be cleaved by yeast into free molecules during winemaking or brewing, thereby contributing to the aroma of wine and beer. However, it was found that such releases occur only to a very limited extent and depend on various process conditions, especially the yeast strain. In a brewery, the conversion yield of the corresponding biochemical reaction is typically below 1 % [1].

The quantification of free and bound thiols is usually based on separate analytical procedures. Bound thiols are mainly analyzed using high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) [5]. In contrast, gas chromatographic (GC) methods are the primary choice for free thiols (which are small, highly volatile molecules) using either sulfur-specific detectors (e.g. flame photometry, chemiluminescence) or mass spectrometry [6, 7]. HPLC-MS methods are applicable in this context only if the volatile free thiols are derivatized during sample preparation, thereby converting them into non-volatile compounds [5, 8]. On this basis, it is even possible to analyze free and bound thiols simultaneously using a single method.

To achieve this, it is necessary to overcome both the fundamentally different volatilities and the challenge posed by the widely varying concentrations of both thiol forms. Such a simultaneous analysis of free thiols and their non-volatile precursors was first published for wine [9]. After all, such an achievement saves time and money. In the meantime, this concurrent analysis could also be developed for beer [10] and tea [11].

The first methods of this kind used 2-phenyl-1,2-benzoselenazol-3(2H)-one (also called ebselen) as derivatization reagent for free thiols [9, 10]. This is an organoselenium compound associated with toxicity and high costs [12]. In 2023, a procedure for simultaneous determination of free and bound thiols in oenological samples was introduced that proposes 4,4'-dithiodipyridine (DTDP) as a safe, readily accessible and more cost-effective alternative [12].

The objective of the work presented here was therefore to establish a simple quantitative analytical technique to simultaneously determine both the most prominent thiol precursors and the most important free forms of hops based on derivatization with DTDP.

The application of DTDP was initially reported for wine analysis already in 2015 [13]. Nine years later, such an approach was adapted to hops [8]. Shortly thereafter, this DTDP based method was slightly modified and used to analyze 4MSP, 3SH, and 3S4MP in a wide range of hop varieties from around the world [2]. Now it could be successfully further developed to enable the chromatographic determination of these free thiols in the form of DTDP derivatives alongside Cys-3SH and G-3SH in a single run. The present work reports on this extension, which will be referred to below shortly as new "simultaneous method". To the best of our knowledge, this marks the first time such a concurrent analysis of free and bound forms is being presented for hops. It was developed to enable routine application in hop quality control for gaining more insights into the thiol potential of different varieties or specific production batches.

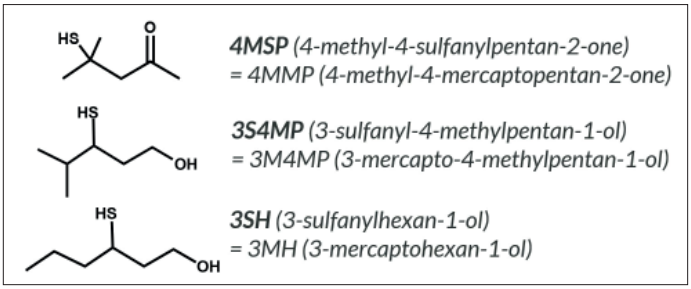

Fig. 1 **Key polyfunctional thiols of hops**

Table 1 **Aroma impressions of free thiols, their threshold concentrations in beer, and their maximal contents found in hops [2]**

Thiol	Aroma impression	Aroma threshold in beer	Maximal content in hops
4MSP	Black currant	1.5 ng/L	38 µg/kg
3SH	Grapefruit	55 ng/L	16 µg/kg
3S4MP	Grapefruit/ Rhubarb	70 ng/L	96 µg/kg

Table 2 **Contents of total α- and β-acids (%, as is) in hop samples of different varieties (all pellet type 90) investigated in this study**

Variety	Origin	Harvest year	Total α-acids [14]	Total β-acids [14]
Cascade	USA	2023	5.4	6.2
Eureka!	USA	2023	17.9	4.5
Calypso	USA	2023	14.3	5.7
Summit	USA	2023	13.3	5.4
Lemondrop	USA	2025	4.5	4.1
Contessa	USA	2025	2.8	8.9
Willamette	USA	2025	3.7	3.3
Chinook	USA	2025	12.4	3.2
Amarillo	USA	2025	9.2	6.6
Alora	USA	2025	7.9	3.8

The results of this new simultaneous method are compared with published data from two studies that use separate approaches to quantify bound or free hop thiols.

The method presented here is an extension of the procedure for quantifying free thiols after derivatization, which was already introduced in a previous publication [2]. Therefore, there is a lot of overlap with this in the experimental part that follows. However, it is necessary here as well to describe all steps of the now successfully expanded determination, along with the precursors, in detail - from sample preparation to all specific mass spectrometric parameters.

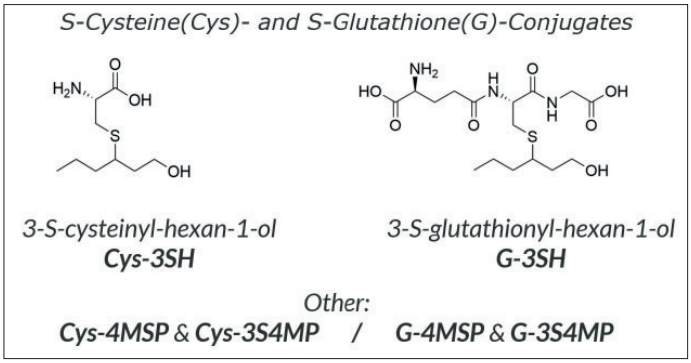

Fig. 2 **Thiol precursors of hops**

2 Materials and methods

2.1 Reagents

The following chemicals were obtained commercially:

LC-MS-grade water and methanol (Chemsolute®, Th. Geyer GmbH & Co. KG, Renningen, Germany); formic acid and ammonium formate (Merck, Darmstadt, Germany); 4,4'-dithiodipyridine (DTDP, 98 %; Acros Organics, Thermo Fisher Scientific); concentrated HCl (37 %) and Na2EDTA (Titriplex III) (Supelco®, Merck, Darmstadt, Germany); 4MSP and 3SH (Merck, Darmstadt, Germany); 3SH(d5), 3S4MP, Cys-3SH, G-3SH (aromaLab, Planegg, Germany).

Preparation of the derivatizing reagent solution:

A 10 mM solution was prepared by sonicating 110 mg DTDP in 10 mL LC-MS water with 50 µL concentrated HCl (37 %), then diluting with water to 50 mL. Aliquots were stored at –20 °C until use.

2.2 Samples

Pellets of crops 2023 or 2025, taken as representative samples during large scale production of at least 1 ton of hop pellets type 90. The samples were packaged under vacuum and then stored cold (< 10 °C). Analysis was always carried out within 3 months after production and begin of cold storage. The varietal identity of the samples was checked by analyzing their contents of alpha- and beta-acids according to method EBC 7.7 of EBC-Analytica [14], see Table 2.

2.3 Sample preparation

20 g pellets (in their original state with a water content of approximately 8 %) were gently milled as recommended in method EBC 7.7 of EBC-Analytica [14]. From this powder, a 0.1 g portion was suspended in 4.16 mL methanol and 5 mL water. After adding 40 µL 3SH(d5) internal standard solution (c = 250 ng/mL), 300 µL Na2EDTA (2.5 %), and 500 µL DTDP solution (10 mM), samples were extracted for 30 min with the help of a shaking device at 225 rpm. An aliquot of the extract was centrifuged for 15 min at 13,500 rpm, and part of the resulting supernatant was analyzed by positive-mode LC-MS/MS. All samples were analyzed in duplicate.

External calibration solutions were prepared accordingly. The concentration ranges for these solutions were 1–200 µg/L for G-3SH, and 1–100 µg/L for Cys-3SH. For the free thiols, this range was at 0.01–1 µg/L, and the internal standard 3SH(d5) was used at a concentration of 1 µg/L (in all cases, measurements as DTDP derivatives; see above).

2.4 HPLC-MS equipment and parameters

ExionLC™ system (SCIEX, Darmstadt, Germany), comprising a binary pump, degasser, autosampler, and thermostated two-column oven, coupled to a 5500+ Q-TRAP mass spectrometer (SCIEX) with an electrospray ionization (ESI) source operated in positive ion mode.

Chromatography used a 100 × 2.0 mm, 2.7 µm EC 100/2 Nucleoshell PFP analytical column with a matching guard column (MACHEREY-NAGEL, Düren, Germany). Mobile phase A was water containing 5 mM ammonium formate and 0.1 % formic acid; mobile phase B was methanol containing 5 mM ammonium formate and 0.1 % formic acid. The column temperature was 40 °C and the injection volume was 10 µL. Isocratic elution at 77 % A and 23 % B was maintained for 28 min.

Sample solution was introduced by HPLC at a flow of 250 µL/min, with the turbo gas maintained at 350 °C and the ion spray voltage at 5500 V. Compound-specific declustering potential and MS/MS parameters were optimized for collision-induced fragmentation of $[M+H]^+$ into target product ions. Collision energy (CE), declustering potential (DP), and entrance potential (EP) settings are listed in table 3. Nitrogen (99.7 % purity) served as the collision gas.

Quantitation used scheduled multiple reaction monitoring (MRM) with preoptimized fragmentation parameters and reference-compound retention times. Data were acquired in Analyst 1.7.3, and peaks were integrated in SCIEX OS 1.7 (SCIEX, Darmstadt, Germany).

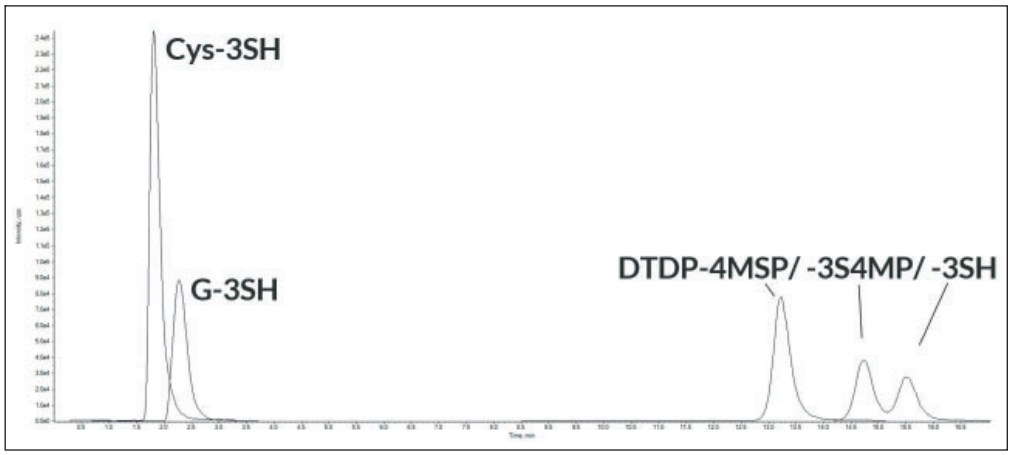

Fig. 3 **HPLC chromatogram of pure standards for all thiol forms analyzed by the new simultaneous method**

All concentrations reported in this study are mean values derived from duplicate determinations and refer to the original hop pellet samples containing approximately 8 % water.

3 Results and discussion

Figure 3 presents the HPLC chromatogram of pure synthetic standards for the examined thiol forms at concentrations of 100 µg/L (Cys-3SH, G-3SH) and 1 µg/L (4MSP, 3SH, 3S4MP, always measured as DTDP derivatives), respectively. Under the conditions described in the method section, the precursors are eluting very early at about 2 minutes. Their peak resolution is clear, while the three DTDP derivatives, eluting much later, are even almost baseline separated. Since 3SH and 3S4MP (and their DTDP derivatives as well) have identical molar mass, it is necessary to separate them efficiently by chromatography to enable reliable quantification by means of mass spectrometry.

Table 4 shows the statistical data of the new method. For the free thiols, limits of detection (LOD) and of quantification (LOQ) were always 0.5 µg/kg and 2 µg/kg. They are defined as the concentrations which yield a peak with a signal-to-noise ratio (S/N) of at least 3 or 10 in samples missing the respective analyte (like e.g. in the cultivar UK East Kent Golding, see [2]). Since no hop sample was free of the two 3SH precursors, their LOD and LOQ could not be determined exactly in this way. These limits were only roughly estimated at approximately 100 µg/kg (LOD) and 300 µg/kg (LOQ), respectively. This derivation is based solely on extrapolation of the according S/N (3 or 10) immediately before and after the actual analyte peaks in samples containing low concentrations of Cys-3SH or G-3SH (like e.g. in the cultivar US Summit; see below). At different spike levels, the recovery rates of the three free thiols as well as of both 3SH precursors are between 91 % and 135 %. For all free thiols, the relative standard deviation (RSD) between four repeated analyses was always below 15 %, and for Cys-3SH and G-3SH it was always below 10 %.

As far as the assessment of reproducibility is concerned, there are no collaborative trials for thiols. Limited comparability with other laboratories can only be established on basis of values that have already been published. To gain such insights, results from the new simultaneous method are first compared with analytical data recently presented for US Cascade hop pellets (2023 harvest). These were used in pilot-scale brewing trials that investigated the release of free thiols from their precursors [15]. Using separate analysis methods for free or bound thiols, in this sample the same parameters were quantified we are covering with the simultaneous procedure. Consequently, we also analyzed a pellet of the same provenance and from the identical crop year. The comparison of analytical data is shown in table 5.

Table 3 Retention times, specific mass transitions and optimized parameters for the new simultaneous method (HPLC-MS/MS)

Compound	Retention time [min]	Mass transition m/z Q1 → Q3	DP [a] [V]	CE [b] [V]	EP [c] [V]
Cys-3SH	1.8	222 → 205 [d] 222 → 83	60 60	13 21	10 10
G-3SH	2.4	408 → 162 [d] 408 → 279	81 81	31 21	10 10
DTDP-4MSP	13.3	242 → 144 [d] 242 → 111	71 71	25 41	4.5 4.5
DTDP-3S4MP	14.8	244 → 144 244 → 111	81 81	23 43	4.5 4.5
DTDP-3SH	15.5	244 → 144 [d] 244 → 111	81 81	23 43	4.5 4.5
DTDP-3SH(d5)	15.3	249 → 144 [d] 249 → 111	81 81	23 43	4.5 4.5

[a] declustering potential [b] collision energy [c] entrance potential [d] quantification transition

Table 4 Statistical data of the new simultaneous method (HPLC-MS/MS)

	LOD [a] (µg/kg)	LOQ [b] (µg/kg)	% Recovery (at µg/kg)	% RSD [c]
4MSP	0.5	2	111 (2) 99 (10)	< 15
3SH	0.5	2	126 (2) 111 (10)	< 15
3S4MP	0.5	2	135 (2) 118 (30)	< 15
Cys-3SH	100 [d]	300 [d]	98 (300 100) (1,000)	< 10
G-3SH	100 [d]	300 [d]	115 (1,000) 91 (10,000)	< 10

[a] limit of detection [b] limit of quantification [c] relative standard deviation [d] roughly estimated

Table 5 Free and bound thiols (µg/kg, as is) in hop pellets of the variety US Cascade

Harvest year	2023	2023	2016
µg/kg	Samia et al. [15]	Simultaneous method	Roland et al. [3]
4MSP	2.03	2.0	2.4
3SH	1.83	3.1	10.5
3S4MP	3.09	2.1	n.a. [a]
Cys-3SH	750	743	456
G-3SH	34,000	16,845	13,498
Cys-4MSP	10	n.a. [a]	n.d. [b]
G-4MSP	n.d. [b]	n.a. [a]	n.d. [b]

[a] not analyzed [b] not detected

Table 6 **Free and bound thiols (µg/kg, as is) in hop pellets of the two varieties US Eureka! and US Calypso**

Variety	Eureka!		Calypso	
Harvest year	**2023**	**2016**	**2023**	**2016**
µg/kg	Simultaneous method	Roland et al. [3]	Simultaneous method	Roland et al. [3]
4MSP	13.3	17.5	n.d. [b]	< 0.1
3SH	3.1	11.0	4.2	15.5
3S4MP	16.9	n.a. [a]	n.d.	n.a. [a]
Cys-3SH	638	326	1,587	1,905
G-3SH	8,508	7,595	10,800	14,421

[a] not analyzed [b] not detected

Table 7 **Free and bound thiols (µg/kg, as is) in hop pellets of different US varieties from harvest years 2023 or 2025 (all results from new simultaneous method)**

Cultivar	Harvest year	4MSP	3SH	3S4MP	Cys-3SH	G-3SH
		µg/kg	µg/kg	µg/kg	µg/kg	µg/kg
Lemondrop	2025	n.d. [a]	< 2	n.d. [a]	309	7,764
Contessa	2025	n.d. [a]	2.9	n.d. [a]	760	19,653
Willamette	2025	n.d. [a]	5.9	< 2	1,532	22,320
Chinook	2025	2.2	3.4	3.1	483	5.730
Amarillo	2025	3.5	5.4	14.5	1,312	17,320
Summit	2023	3.7	n.d. [a]	20.9	< 300	1,683
Alora	2025	6.2	3.6	18.2	1,007	11,225

[a] not detected

US Cascade contains all three thiols in free form. Concentrations of approximately 2–3 µg/kg were found here, which are low but not unusual for this variety. Very good agreement was observed for Cys-3SH (743 vs. 750 µg/kg), whereas for G-3SH our result was considerably lower (16,845 vs. 34,000 µg/kg). However, in the paper of Samia et al. it is emphasized that a particular batch of Cascade pellets has been selected because of their relatively high contents of thiol precursors. Nevertheless, they could not detect G-4MSP and reported Cys-4MSP only at a concentration of 10 µg/kg. These results demonstrate once again that the contribution of 4MSP precursors to beer aroma can be considered negligible, as is explicitly mentioned in this paper. In contrast, 3SH precursors can be present in concentrations more than 1,000 times higher. Consequently, even with conversion yields of less than 1 % during the brewing process, the released form can still make a significant contribution to the aroma of beer [15].

Table 5 contains further results for US Cascade from the already mentioned study of Roland et al., which also employed separate methods for the quantification of free and bound thiols [3]. As shown in the last column of this table, here the 4MSP content is 2.4 µg/kg, the 3SH content is relatively high at 10.5 µg/kg, and no data on 3S4MP is presented in this paper. Cys-SH and G-3SH exhibit levels of 456 µg/kg and 13,498 µg/kg, respectively, whereas both 4MSP precursors could not be detected in this sample from harvest 2016.

Further comparative results from the new simultaneous method and that of Roland et al. are presented in table 6. For this purpose, the two US varieties Calypso and Eureka! were selected. According to Roland et al., these contain only traces of 4MSP and relatively high levels of it, respectively [3]. While the results for 4MSP show good agreement, the values for 3SH again differ by a factor of more than 3 like for US Cascade in table 5. Regarding the 3SH precursors, our results were consistently higher for Eureka!, whereas they were lower for Calypso.

However, comparing results from different studies is only possible to a limited extent as the hop samples mostly originate from various harvest years and have been analyzed using widely varying methods. As already known from literature, such analytical comparisons across publications can exhibit significant discrepancies, particularly in the case of thiols. For the cultivar US Cascade alone, for example, the range of variation for 4MSP reported in several papers since 2008 is from 0.8 to 16.8 µg/kg [1-3, 6-8, 15-17], for 3S4MP from 3.1 to 18.1 µg/kg [2, 6, 15, 17], and for 3SH from 1.8 to 117.1 µg/kg [1–3, 6, 8, 15, 18]. As shown in table 5, there are also certain variations among the thiol precursors. Even within the US Cascade variety from the same harvest year (2023), the difference in G-3SH levels exceeds a factor of 2. Potential reasons for this include fluctuations due to growing conditions (such as soil composition or weather) or harvest parameters (timing of harvest, drying process, etc.). For example, Lafontaine et al. have already shown that the concentrations of the precursors of 4MSP and 3SH decreased significantly during the harvest period, while the concentrations of their free forms increased [19]. Furthermore, Uemoto et al. observed this for 3S4MP in several varieties cultivated in Japan [17], the United States, and Germany [20].

Given such fluctuations, it is difficult to derive representative concentrations of free and bound thiols in hop varieties or products based solely on published literature data. Instead, it is advantageous to obtain additional results using simple, rapid routine methods - such as the one presented here.

Finally, table 7 presents further data resulting from the analysis of seven more US hop varieties (pellets, 2023 or 2025 harvest) using the new simultaneous method. This is a comparison of cultivars in which the sum of the three free thiols ranges from barely detectable (Lemondrop) to nearly 30 µg/kg (Alora). As for the 3SH precursors, Summit exhibits the lowest levels (< 300 µg/kg Cys-3SH and 1,683 µg/kg G-3SH), while Willamette shows the highest values (1,532 µg/kg Cys-3SH and 22,320 µg/kg G-3SH). All other varieties (Contessa, Chinook, Amarillo) fall between the ranges mentioned above.

Employing the new simultaneous method, the number of varieties can now be rapidly expanded to gain further insights into

the distribution of free and bound thiols in hop samples. This can also be very useful for research purposes, including the breeding of new cultivars.

4 Conclusion

While simultaneous methods for the concurrent determination of free and bound thiols (in the form of cysteinylated and glutathionylated conjugates) already exist for various other food products, this advancement is now being introduced for hops for the first time. It is based on single-run high-performance liquid chromatography-tandem mass spectrometry analysis after derivatization of free thiols during sample preparation. This new method is quick and easy to perform. The results obtained in this way are within the range of previously published data from other studies that used separate methods to determine free thiols and their precursors (S-conjugates). Provided the necessary equipment is available, such simultaneous determination of free and bound thiols can be employed in routine applications to assess specific insights into the thiol profile of hop samples coming from field, harvest, processing, brewing research, or breeding.

Conflict of interest

The authors declare no conflicts of interest.

CRediT authorship contribution statement

Martin Biendl: conceptualization, writing – original draft, writing – review & editing; Laura Hoferer: formal analysis, investigation, visualization, writing – review & editing; Chritina Schmidt: methodology, investigation, visualization, writing – review & editing.

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