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Analysis of volatile thiols in alcoholic beverages by simultaneous derivatization/extraction and liquid chromatography-high resolution mass spectrometry

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Running title: Volatile thiols in alcoholic beverages by derivatization and LC-HRMS

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ABSTRACT

A simultaneous derivatization/extraction method followed by liquid chromatography-electrospray-high resolution mass spectrometry for the determination of volatile thiols in hydroalcoholic matrixes was optimized and used to identify and quantify volatile thiols in wine and beer samples. The method was evaluated in terms of sensitivity, precision, accuracy and selectivity. The experimental LOQs of eleven thiols tested ranged between 0.01 ng/L and 10 ng/L. Intra-day relative standard deviation (RSD) was in general lower than 10% and inter-day RSD ranged between 10% and 30%. Recovery in the model and real matrixes ranged from 45% to 129%. The method was then applied for the analysis of four white wines and six beers. Five out of the eleven reference thiols were identified and quantified in the samples analysed. The non-target approach, carried out by monitoring the diagnostic ion at *m/z* 275.9922 $[C_{13}H_{10}ONSe]^+$ in the fragmentation spectrum, allowed detecting, in the same samples, fourteen non-target thiols.

Keywords: wine, beer, thiols, derivatization, high resolution mass spectrometry (HRMS), HRMS/MS, Orbitrap.

1. INTRODUCTION

Volatile sulfur compounds are major contributors to several food and beverage aromas (McGorrin, 2011; Vermuelen Gijs & Collin, 2005). Due to their extremely low odor thresholds, they have a significant sensory impact even at very low concentrations. In particular, volatile thiols are well known to be powerful odorants in beverages such as wine (Tominaga, Baltenweck-Guyot, Peyrot des Gachons & Dubourdieu, 2000; Kotseridis & Baumes, 2000) and beer (Vermeulen, Lejeune, Tran & Collin, 2006; Hugues, 2009). Among wine varietal thiols, 4-mercapto-4-methyl-pentan-2-one, 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA), are responsible for box tree, exotic fruit and grapefruit aromas, respectively, in wines made from Sauvignon Blanc, Colombard, Scheurebe, Merlot and Cabernet Sauvignon cultivars, among others (Tominaga et al., 1999; Schneider, Charrier, Razungles & Baumes; 2006; Kotseridis & Baumes, 2000; Guth, 1997; Darriet, Tominaga, Lavigne, Boidron & Dubourdieu; 1995; Bouchilloux, Darriet, enry, Lavigne-Cruege & Dubourdieu, 1998). Other thiols such as 2-furanmethanethiol (2FMT), 2-methyl-3-furanthiol (2M3FT), and benzenemethanethiol, have been associated with the empyreumatic notes of aged wines (Blanchard, Tominaga & Dubourdieu, 2001; Tominaga, Blanchard, Darriet & Dubourdieu, 2000; Tominaga, Guimbertau & Dubourdieu, 2003). Furthermore, several polyfunctional thiols have been reported in beer, usually associated with sensory defects. In particular, 3-methyl-2-buten-1-thiol (3MBT) imparts the well-known lightstruck and “skunky” off flavors (Huvaere, Andersen, Skibsted, Heyerick & Keukeleirea, 2005; Goldstein, Rader & Murakami, 1993; Lermusieau & Collin, 2003), while 4-mercapto-4-methyl-2-pentanone (Cosser, Murray & Holzapfel, 1980) and 3-mercapto-3-methylbutyl-formate (Schieberle, 1991) induce a “ribes, catty” odor. Recently, 3-mercapto-2-methylbutanol and 2-mercapto-3-methylbutanol were associated with onion-like notes in beer (Vermeulen et al., 2006). Moreover, 2M3FT (Lermusieau, M. Bulens & Collin, 2001) and 3MH

61 are often present in fresh lager beers, although their sensory impact seems of little relevance
62 (Vermeulen et al., 2006).
63 Finally, highly volatile thiols, such as hydrogen sulfide (H_2S), methanethiol, ethanethiol and
64 propanethiol, have been identified as responsible for putrefaction, garlic, onion or rotten egg-
65 like notes, in beer (Hugues, 2009) and in wine, as reviewed by Mestres et al. (2000).
66 The analytical assay of volatile thiols in food and beverages is particularly difficult due to the
67 complexity of the matrixes, together with the typically low concentrations and high reactivity
68 of the thiols. The most widely used analytical methods are based on the liquid–liquid
69 extraction of thiols from a wine or beer matrix, followed by derivatization with *p*-
70 hydroxymercurybenzoate (Vermeulen et al., 2006; Tominaga, Murat & Dubourdieu, 1998) or
71 by reversible covalent chromatography (Schneider, Kotseridis, Ray, Augier & Baumes, 2003).
72 More recently, 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr) has been used to derivatize thiols
73 on solid-phase microextraction (SPME) fibers (Mateo-Vivaracho, Cacho & Ferreira, 2007) or in
74 the wine matrix, followed by isolation of the derivatives either via solid-phase extraction (SPE)
75 (Mateo-Vivaracho, Cacho & Ferreira, 2008) or SPE followed by SPME (Rodríguez-Bencomo,
76 Schneider, Lepoutre & Rigou, 2009). The best quantification performance achieved with these
77 methods uses stable isotopic dilution, which involves the synthesis of deuterated thiols.
78 These procedures require relatively large sample amounts, they are laborious and time-
79 consuming, and some of them involve several sample-manipulation steps during which volatile
80 thiols can be lost or undergo degradation.
81 In order to devise a simple, reliable, selective and sensitive method for the analysis of volatile
82 thiols at trace levels in hydroalcoholic matrices such as wine and beer, a simultaneous
83 derivatization/extraction method followed by liquid chromatography-electrospray-high
84 resolution mass spectrometry (LC-ESI-HRMS) was developed using ebselen as the
85 derivatization reagent. This choice was based on recent reports of its high selectivity, fast

reaction and high derivatization yield for some biological thiols (Xu, Zhang, Tang, Laskin, Roach & Chen, 2010), and volatile thiols in lipid matrixes (Vichi, Cortés-Francisco & Caixach, 2013). HRMS was chosen to provide the highest chemical selectivity . The analytical conditions were optimized in model systems and real wine and beer samples, both spiked with eleven representative volatile thiols. The method was first evaluated in terms of sensitivity, precision, accuracy and selectivity, and then applied to real samples. Moreover, a non-target approach based on the formation of a diagnostic product ion was evaluated using real wine and beer samples.

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

Mass spectrometry grade dichloromethane and methanol (MS SupraSolv®) were purchased from Merck (Darmstadt, Germany). Water was of ultrapure milli-Q grade. Ammonium formate was from Sigma-Aldrich (St. Louis, MO, USA). Nitrogen (Alphagaz N_2, purity 99.999%, Air Liquid) was used in the Orbitrap-Exactive as nebulization and fragmentation gas.

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one, purity 98%), absolute ethanol (purity 99.8%), ethylenediaminetetraacetic acid (EDTA) (purity 98,5%); 3-mercaptohexyl acetate (3MHA) (purity 98%); 3-mercaptohexanol (3MH) (purity 98%); 3-mercapto-3-methylbutan-1-ol (3MMB) (purity 98%); 2-furanmethanethiol (2FMT) (purity 97%); *p*-mentha-8-thiol-3-one (MT) (purity 98%); 1-hexanethiol (HT) (purity 98%); 4-methoxy-α-toluenethiol (IS, purity 90%) were purchased by Sigma-Aldrich (St Louis, MO, USA). 4-mercapto-4-methylpentan-2-ol (4MMP) (purity 98%), 4-methoxy-2-methyl-2-butanethiol (4MMB) (purity 98%); 3-mercaptohexyl hexanoate (3MHH) (purity 98%); 1-phenylethyl mercaptan (1PEM) (purity 98%) were from Endeavour Speciality Chemicals Ltd (Northants, UK). 3-methyl-2-butene-1-thiol preparation kit was provided by Toronto Research Chemicals Inc. (North York, Canada). Molecular structures of the reference thiols are shown in **Fig. S1 (Supplementary information)**.

111 **2.2. Model wine and model beer**

112 Model wine was prepared according to Labanda et al. (2009), by dissolving 5 g/L of glycerol, 10
113 g/L of glucose, 5 g/L of tartaric acid, 10 mg/L of albumin, 300 mg/L of pectin, 100 mg/L of
114 tannic acid, 150 mg/L of sodium methabisulfite and 120 m/L of absolute ethanol in deionized
115 water. The pH of the model wine was 3.0.

116 Model beer was prepared according to Eagles & Wakeman (2002) with some modifications:
117 1.5g/L of glycerol, 1.5 g/L of maltose 100 mg/L of citric acid, 100 mg/L of albumin, 300 mg/L of
118 pectin, 50 mg/L of tannic acid and 50 mL/L of absolute ethanol in deionized water. The pH of
119 the model beer was 4.2.

120 **2.3. Wine and beer samples**

121 The method was applied to distinct commercial samples of wine and beer provided by local
122 retails. Wine samples were: Albariño (2012) Denomination of Origin (D.O.) Rias Baixas (Spain)
123 (sample W1); Sauvignon Blanc (2012) D.O. Rueda (Spain) (W2); Riesling (2012) D.O. Penedés
124 (Spain) (W3); Sauvignon blanc/gewürztraminer (2012) D.O. Penedès (Spain) (W4). Two lager
125 beer samples exposed to light (B1, B2), one lager (B3), one double-malt (B4), one alcohol free
126 (B5) and one stout (B6) commercial beers were analyzed.

127 **2.4. Derivatization/extraction conditions**

128 The derivatization and extraction conditions were optimized in model and real wine and beer
129 samples spiked with reference thiols at 5 ng/L. The most suitable conditions were determined
130 by comparing absolute peaks areas. Derivatization/extraction conditions were finally fixed as
131 follows: 20 mL of sample were weighed into a screw cap-tube, added with 400 μL of EDTA 30
132 mg/mL and spiked with 4-methoxy-α-toluenethiol (IS) to a final concentration of 10 ng/L. 6 mL
133 of ebselen 0.1 mM in dichloromethane was then added and the mixture was vortex-mixed
134 during 1 min. The sample was maintained under nitrogen atmosphere during this process.

After centrifugation (4000 rpm, 15 min), 1 mL of the dichloromethane phase was collected, dried under nitrogen flow and redissolved in 0.1 mL of methanol.

2.5. High performance liquid chromatography (HPLC)

The HPLC system consisted of a Surveyor MS Plus pump coupled to an Accela Open automatic sampler (Thermo Fisher Scientific, San Jose, California) equipped with 10 μL loop. The chromatographic separation was performed on a Luna C18 (150 × 2.1 mm, 5 μm) analytical column (Phenomenex, Torrance, CA). Elution was performed at a flow rate of 200 μL/min, using water (solvent A) and methanol (solvent B), both containing ammonium formate 10 mM. The solvent gradient changed according to the following conditions: 50% (A)-50% (B) during 2 min, to 100% (B) in 18 min; 100% (B) during 13 min, then to 50% (A)-50% (B) in 1 min, followed by 6 min of maintenance.

2.6. High resolution mass spectrometry (HRMS)

Mass spectrometric analysis was carried out with an Orbitrap-Exactive-HCD (Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray source (H-ESI II). The ionization conditions in positive mode were according to Vichi et al. (2013): spray voltage 3.75 kV, capillary voltage 25 V, skimmer voltage 16 V, tube lens voltage 80 V. Sheath gas flow rate was set at 40 arbitrary units (au), auxiliary gas flow rate was 10 au, capillary temperature was 275 ºC, and heater temperature was 30 ºC. The mass range was set to *m/z* 50-1200. The automatic gain control was used to full fill the C-trap and gain accuracy in mass measurements (high dynamic range mode, $3x10^6$ ions). Maximum injection time was 500 ms. High resolving power defined as R: 50,000 (*m/z* 200, FWHM), 2 HZ, was set. High energy Collision Dissociation (HCD) voltage was fixed at 25 eV. In a single injection, the Orbitrap mass analyzer alternated full scan mode and "All Ion Fragmentation" (AIF) mode at a resolution power of 50,000 (*m/z* 200, FWHM). Mass accuracies better than 2 ppm were achieved for molecular and product ions, always working with external calibration.

160 The molecular formulae calculation was performed with Xcalibur 2.1 (Thermo Fisher Scientific,
161 Bremen, Germany). In order to obtain a reliable list of confidence formulae from a mass
162 measurement, heuristic filtering (criteria) were set to generate reliable elemental formulae: C
163 ≥ 13, O ≥ 1, N=1, Se=1, S=1, and RDB ≥ 8.5. The mass peaks considered were single positive
164 charged ions >10^3 area counts.

165 **2.7. Validation**

166 Quantification was performed using an internal standard and by constructing matrix-matched
167 calibration curves in the range 0.01-50 ng/L, except for 3MBT (5-50 ng/L). Matrix-matched
168 calibration curves were made by analyzing blank wine or beer samples spiked with different
169 amounts of reference thiols dissolved in methanol. Spiked matrix solutions were derivatized
170 and extracted as previously described for samples.

171 Linearity within these ranges was evaluated by the correlation coefficient, r.

172 Experimental limits of quantification (LOQ) were determined by the lowest point of the
173 calibration curve, which was assessed in accordance with the established identification criteria:
174 retention time drift <3-fold the standard deviation (SD) of the method, mass accuracy < 2 ppm
175 with real resolution ≥20.000 (full width at half maximum – FWHM) at the mass range of
176 interest, and the presence of isotope ions containing ^{80}Se and ^{78}Se, respectively.

177 The precision, expressed as intra- and inter-day relative standard deviation (RSD) was obtained
178 analysing the same blank wine and beer spiked with thiols at two concentration levels: 1 and
179 20 ng/L. The repeatability was calculated on seven samples analyzed within the same day. The
180 intra-day RSD was calculated in seven samples analysed on the same day, whereas the inter-
181 day RDS was obtained from six samples analysed on different days.

182 The recovery of the extraction and the matrix effect were assessed by comparing peak areas of
183 reference thiols derivatized with ebselen 0.1 mM in dichloromethane with peak areas of thiols
184 after the derivatization/extraction step, using the same ebselen solution. Thiols were dissolved

in 20 mL of: model wine, model beer, white wine, red wine and beer, respectively. Recovery and matrix effect were calculated for eleven reference thiols at two concentration levels: 1 and 20 ng/L.

3. RESULTS AND DISCUSSION

As reported by Xu et al. (2010), Mugesh et al. (2001) and Sarma e al. (2005), the reaction between the SH group of the thiol and the Se-N bond of ebselen yields a selenenyl sulfide derivative through the formation of an Se-S bond **(Fig S2, Supplementary information)**. This fast, selective and efficient reaction enables the derivatization and isolation of volatile thiols from hydroalcoholic matrixes by a simple liquid–liquid extraction, over a minimum time and with minimum sample manipulation. A selective derivatization strategy is the key to stabilizing the free thiol group. The use of ebselen as the derivatization agent allows the reactive thiol group to be protected and the ESI-HRMS responses of the derivative to be increased due to the presence of the easily ionizable aminic nitrogen atom. Moreover, selective chemical tagging of volatile thiol with a molecule containing selenium allowed outstanding detection selectivity. In fact, after derivatization with ebselen, all the thiols showed the characteristic selenium isotopic pattern, as exemplified by 3MHA extracted from spiked wine (1 ng/L) (**Fig. 1a**), which fitted the theoretical isotopic pattern perfectly (**Fig. 1b**). Isotope M+6, which matches the presence of an ^{80}Se atom, presented the strongest signal; followed by M+4, which corresponds to the presence of ^{78}Se. Isotopes M+6 and M+4 were used as quantification and confirmation ions, respectively.

Compliance with the identification criteria for derivatized thiols are reported in **Table 1**. Independently of the concentration and matrix tested, mass accuracy was always better than 2 ppm, with a SD of the mass error of between 0.1 and 0.4 ppm. The real resolution was > 28,000 for all the thiols analyzed.

3.1.1. Optimization of derivatization/extraction conditions

The derivatization and extraction conditions previously optimized for lipid samples (Vichi et al., 2013) were adapted to hydroalcoholic samples by using dichloromethane as the extraction solvent. Distinct solvent volumes and ebselen concentrations were tested to optimize the recovery of derivatized thiols in such matrixes. The sample volume to extraction solvent volume ratio was proportional to the thiol uptake (**Fig. S3a**, supplementary information). Given that above ratios of 2.5-3 the increase in thiol uptake was low, higher volume ratios were not tested. To enable easy collection of a discrete volume of clean sample extract after centrifugation, the final sample volume to dichloromethane volume ratio was fixed at 20:6 mL. No substantial differences were observed in the thiol response using ebselen concentrations of between 0.05 and 0.1 mM for the analysis of beer or wine spiked at 10 ng/L (**Fig. S3b**, supplementary material). However, the highest of these ebselen concentrations was chosen for further analysis to ensure complete derivatization of thiols even at the higher concentrations reported in the literature (Tominaga et al., 2000; Vermuelen et al., 2006).

Prior to the analysis, a concentration step was applied in order to increase the response of the thiols, and dichloromethane was replaced by methanol to improve the chromatography.

Representative thiols containing different functional groups were chosen to develop and evaluate the method. They expressly included primary, secondary and tertiary thiols in order to detect any discrimination effect in their derivatization. The exact mass, the molecular formula and the molecular structure of the selected reference thiols are shown in **Table 1** and **Fig. S1 (Supplementary information)**.

3.2. LC-ESI-HRMS analysis

The chromatograms obtained from the derivatized extract of a white wine spiked with reference thiols at 1 ng/L (IS 10 ng/L; 3MTB 20 ng/L), by selecting the exact mass of each thiol derivative from the ESI+ full-scan analysis, are shown in **Fig. S4 (Supplementary information)**. The chromatographic retention time drifts, expressed as SD and calculated from samples

analyzed in different days, are reported in **Table 1**. As can be observed, HRMS provided high selectivity and sensibility, with almost no noise. HRMS was chosen since by means of accurate mass (AM) measured with high resolving power it provides the best information about the molecular composition of the compounds, thereby allowing us to confirm or tentatively identify their elemental formulae. Moreover, it has been demonstrated in several studies that high resolution is necessary in the analysis of complex samples in order to avoid interference from isobaric compounds and thus the problem of false positives (Kaufmann, 2012).

3.3. Validation.

3.3.1. Recovery and matrix effect

In order to estimate the effect of the matrix on the extraction of derivatized thiols, their recovery was assessed in spiked red and white wine, beer, model wine and model beer. The response of thiols after derivatization and extraction in such matrixes was compared with the response obtained by adding the same concentrations of thiols directly to the ebselen solution. **Table 1** reports the recovery of the thiols from each matrix, calculated as the percentage of the peak areas. The extraction of derivatized thiols, evaluated at two concentration levels, can be considered as quantitative in model systems, beer and white wine for all the thiols except 3MBT, which in some cases presented slightly lower recoveries (though always over 45%). In the case of red wine, a moderate, overall decrease of thiol recovery was observed.

3.3.2. Method sensitivity and linearity

Quantification was carried out using an internal standard and by constructing matrix-matched calibration curves using white wine and beer spiked at the concentrations reported in **Table 2**. The lower limit of these ranges was determined by the limit of quantification (LOQ) of each thiol. Linearity within these ranges, evaluated by the correlation coefficient (r) was > 0.98, except for 3MBT in wine (r = 0.9779).

The experimental LOQ was given by the lowest concentration which it was possible to measure according to the established identification criteria: retention time drift, mass accuracy, resolution, and the presence of ions containing the isotopes ^{80}Se and ^{78}Se, as detailed in the Material and methods section.

The LOQ of the eleven reference thiols, assessed in wine and beer matrixes, ranged from 0.01 to 5 and from 0.01 to 10 ng/L, respectively (**Table 2**): slightly higher in beer than in wine. These LOQ values were compared with those obtained by other analytical methods, when available. The LOQ for 3MH and 3MHA in wine (0.01 ng/L) were far below those obtained by other methods: 0.83 and 4.3 ng/L (Rodríguez-Bencomo et al., 2009); 1 and 0.7-5 ng/L (Schneider et al. 2003), 3.1 ng/L (Sarrazin, Shinkaruk, Tominaga, Bennetau, Frérot & Dubordieu , 2007), 20 and 1.9 ng/L (Mateo-Vivaracho et al, 2007); 0.8 and 6 ng/L (Mateo-Vivaracho, 2008). Likewise, the LOQ for 2FMT in white wine (0.01 ng/L) was lower than previous values: 1.4 ng/L (Mateo-Vivaracho et al, 2007), 0.3 ng/L (Mateo-Vivaracho, 2008), 2.2 ng/L (Tominaga & Dubourdieu, 2006) and below the reported odor threshold: 0.4 ng/L (Tominaga et al., 2000).

3MBT showed a LOQ higher than those of the other thiols. As standard 3MBT is not easily available, a commercial preparation kit was used to obtain this compound, for which the reaction yield could not be verified. A low reaction yield during standard preparation would explain the higher LOQ calculated for this compound. As far as we know, no LOQ data are available for 3MBT by other methods, but an odor threshold of from 1 to 35 ng/L was reported for this thiol (Hugues, 2009). Overall, the thiol LOQ are lower than their odor thresholds reported in the literature (Tominaga et al., 2000; Mestres et al., 2000; Hugues, 2009), indicating that the present method is suitable for current purposes.

3.3.3. Method precision

The precision of the method, expressed as intra-day and inter-day relative standard deviation (RSD), was calculated at two concentration levels fixed in the low and mid-range of the

285 calibration curve and in two matrixes: wine and beer (**Table 2**). Intra-day RSD was in general
286 below 10%, while inter-day RSD ranged between 5% and 30%.

287 **3.4. Non-target analysis**

288 Prompt fragmentation corresponding to the S-Se bond indicated in the scheme (**Fig. 1c)** means
289 that thiol structural information cannot be obtained by MS/MS analysis. However, the
290 formation of a diagnostic product ion at *m/z* 275.9922 $[C_{13}H_{10}ONSe]^+$, which corresponds to
291 the ebselen moiety of the derivatives and preserves the typical selenium isotopic pattern, is
292 useful for the screening of non-target thiols via full-scan mode acquisition, with and without
293 HCD fragmentation. The presence of a non-target derivatized thiol may be revealed by the
294 presence of the diagnostic ion in the HCD chromatogram. Thiol identification must be
295 confirmed by the presence in the full-scan chromatogram of the corresponding identification
296 and confirmation ions (isotopes M+6 and M+4) that fit with the restrictions established for
297 their molecular composition.

298 **3.5. Analysis of wine and beer samples**

299 In order to evaluate the suitability of the optimized method for the analysis of real
300 hydroalcoholic beverages, four white wines and six beers were selected for analysis. Five out
301 of the eleven reference thiols studied were identified and quantified in the samples analyzed
302 (**Table 3**). In the same samples, fourteen non-target thiols were detected and quantified by
303 expressing their concentration as ng IS equivalent/L. **Table 3** shows the exact mass and the
304 elemental composition of these thiol derivatives. Some of them were also tentatively
305 identified on the basis of their molecular formula and reports of their occurrence in wine or
306 beer, when available. Both target and non-target thiols were characterized by the diagnostic
307 ion *m/z* 275.9922, according to the restrictions established for their molecular composition,
308 and contained both quantification and confirmation ions (^{80}Se and ^{78} Se, respectively),
309 identified with a mass accuracy < 2 ppm at R > 28,000.

310 The target thiols 3MH and 3MHA were present in wines at concentrations of from 0.21 to 1.32
311 ng/L and from 0.01 to 0.22 ng/L, respectively; they were most abundant in Sauvignon Blanc, as
312 expected. 3MMB was found in both wine and beer samples, in the range 0.01-0.03 ng/L. 3MBT
313 reached 37.3 and 3.4 ng/L in lager beer samples exposed to light, while it was not detected in
314 other samples. Finally, 2FMT was identified in the stout beer sample, probably due to the use
315 of roasted malt or barely in the production of this product. Anyway, it was at a concentration
316 below the LOQ (<0.1 ng/L).
317 Regarding non-target thiols, those tentatively identified as methyl mercaptopropionate, ethyl
318 mercaptopropionate, methanethiol and mercaptoethanol were the most abundant in wine
319 samples and stout beer, in some cases reaching concentrations of nearly 50 ng/L (expressed as
320 equivalents of IS). Methanethiol was the most abundant non-target thiol in lager beers,
321 ranging between 1.9 and 4.6 ng/L.
322 The importance of HRMS for the correct identification of compounds is demonstrated in **Fig. 2**,
323 which shows the presence in a wine sample of two derivatized thiols with the same nominal
324 mass and quite similar chromatographic retention times, but different molecular formula: the
325 tentatively identified ethyl 3-mercaptopropionate ($C_{18}H_{20}O_3NSSe$, *m/z* 410.0324) (**Fig. 2a**) and
326 3MH ($C_{19}H_{24}O_2SSe$, *m/z* 410.0687) (**Fig. 2b**). The same figure shows the isotopic patterns of
327 both derivatized thiols, and their compliance with the identification criteria.
328
329 In conclusion, the simultaneous derivatization/extraction method followed by ESI-LC-HRMS
330 was optimized for the determination of volatile thiols in hydroalcoholic matrixes and used to
331 identify and quantify volatile thiols in real wine and beer samples. The method was shown to
332 be fit for this purpose by carrying out a validation study to ensure reliable results.
333 Experimental LOQs were between 0.01 and 0.05 ng/L for most of the thiols evaluated, and
334 lower than those available in the literature. Acceptable recoveries where obtained in model

335 and real wine and beer matrixes, as well as satisfactory intra-day and inter-day RSD values. Any
336 positive finding had to satisfy the identification criteria established, based on retention time
337 drift, mass accuracy, real resolution, and the presence of identification and confirmation ions.
338 Five target thiols were identified and quantified in wine and beer samples, while fourteen thiol
339 derivatives were detected by the non-target approach, which were tentatively identified on
340 the basis of their molecular formula.

341

342 **Acknowledgements**

343 This study was supported by the Spanish *Ministerio de Economia y Competitividad* through the
344 Ramón y Cajal program (RYC-2010-07228) and the project AGL 2011-23872.

345

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446

447 **Figure legends**

448 **Figure 1.** (a) Mass spectrum of 3MHA, $[C_{21}H_{26}O_3NSSe]^+$, at 1 ng/L, derivatized and extracted
449 from wine; (b) theoretical isotopic pattern of $[C_{21}H_{26}O_3NSSe]^+$; (c) mass spectrum and
450 molecular structure of diagnostic ion $[C_{13}H_{10}ONSe]^+$, *m/z* 275.9922; R: 50,000 (*m/z* 200,
451 FWHM), mass error tolerance < 2ppm; HCD voltage: 25 eV.

452 **Figure 2.** ESI+ Full scan chromatogram obtained by monitoring the exact mass of a) tentatively
453 identified ethyl 3-mercaptopropionate-ebselen derivative at *m/z* 410.0324, and b) 3MH-
454 ebselen derivative at *m/z* 410.0687, in wine sample W2; both with the corresponding mass
455 spectrum showing identification and confirmation ions: isotopes M+6 and M+4, relative to the
456 presence of isotopes ^{80}Se and ^{78}Se, respectively. R: 50,000 (*m/z* 200, FWHM), mass error
457 tolerance < 2ppm.

458

TABLES

Table 1. Molecular formulae, exact mass, mass accuracy and precision values, real mass resolution, chromatographic retention time and precision values for ebselen-derivatized thiols in positive ESI, setting the R: 50,000 (*m/z* 200, FWHM); recovery (%) of the extraction and standard deviation (SD) calculated real and model wine and beer matriXes and at two concentration levels, by comparison with derivatized thiols in dichloromethane solution (n=3). Peak numbering is according to **Fig. 2** and **S1 (supplementary information)**.

	Compound	Formula $[M+H]^+$	Theoretical *m/z*	Δ^a (ppm) ± SD	RT [c] (min) ±SD	Recovery (%) ±SD									
						1 ng/L					20 ng/L				
						model beer (n=3)	beer (n=3)	model wine (n=3)	white wine (n=3)	red wine (n=3)	model beer (n=3)	beer (n=3)	model wine (n=3)	white wine (n=3)	red wine (n=3)
1	3MMB-Ebs	$C_{18}H_{22}O_2NSSe$	396.0531	1.5 ± 0.3	14.34 ± 0.05	102 ± 14	80 ± 14	99 ± 14	79 ± 17	59 ± 5	91 ± 7	73 ± 13	98 ± 14	75 ± 9	61 ± 9
2	4MMP-Ebs	$C_{19}H_{24}O_2NSSe$	410.0687	1.7 ± 0.3	15.38 ± 0.04	121 ± 3	91 ± 16	104 ± 23	105 ± 18	75 ± 8	120 ± 13	98 ± 15	113 ± 13	107 ± 11	91 ± 13
3	3MH-Ebs	$C_{19}H_{24}O_2NSSe$	410.0687	1.6 ± 0.3	16.35 ± 0.04	129 ± 10	74 ± 11	110 ± 19	107 ± 22	84 ± 9	121 ± 12	81 ± 12	115 ± 17	97 ± 10	80 ± 7
4	4MMB-Ebs	$C_{19}H_{24}O_2NSSe$	410.0687	1.4 ± 0.2	17.45 ± 0.04	114 ± 12	70 ± 21	108 ± 14	93 ±16	73 ± 12	120 ± 14	95 ± 18	110 ± 16	100 ± 12	86 ± 15
5	2FMT-Ebs	$C_{18}H_{16}O_2NSSe$	390.0061	1.3 ± 0.1	16.82 ± 0.02	121 ± 16	83 ± 15	103 ± 12	103 ± 14	83 ± 11	119 ± 15	86 ± 12	100 ±6	92 ± 9	86 ± 18
	IS	$C_{21}H_{20}O_2NSSe$	430.0374	1.3 ± 0.2	18.19 ± 0.02	119 ± 9	84 ± 16	104 ± 23	99 ± 17	66 ± 13	127 ± 14	102 ± 30	120 ± 20	101 ± 13	85 ± 14
6	3MHA-Ebs	$C_{21}H_{26}O_3NSSe$	452.0793	1.5 ± 0.2	18.83 ± 0.02	123 ± 11	94 ± 25	112 ± 18	103 ± 21	80 ±17	111 ± 15	102 ± 24	107 ± 14	98 ± 14	79 ± 13
7	3MBT-Ebs	$C_{18}H_{20}ONSSe$	378.0419	1.6 ± 0.2	18.96 ± 0.04	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	71 ± 21	74 ± 15	70 ± 15	46 ± 11	45 ± 22
8	1PEM-Ebs	$C_{21}H_{20}ONSSe$	414.0425	1.5 ± 0.2	19.11 ± 0.03	109 ± 9	84 ± 18	103 ± 26	96 ± 15	72 ± 14	107 ± 18	97 ± 16	101 ± 14	92 ± 12	80 ± 12
9	MT-Ebs [d]	$C_{23}H_{28}O_2NSSe$	462.1000	1.6 ± 0.2	19.32 ± 0.03	118 ± 12	90 ± 22	107 ± 18	101 ± 23	80 ± 17	121 ± 13	109 ± 27	113 ± 18	102 ± 17	84 ± 15
10	HT-Ebs	$C_{19}H_{24}ONSSe$	394.0738	1.5 ± 0.3	21.44 ± 0.02	89 ± 12	91 ± 24	94 ± 25	79 ± 16	66 ± 15	99 ± 24	112 ± 28	89 ± 15	91 ± 13	87 ± 15
11	3MHH-Ebs	$C_{25}H_{34}O_3NSSe$	508.1419	1.2 ± 0.3	22.12 ± 0.02	104 ± 10	87 ± 25	101 ± 18	94 ± 19	78 ± 17	98 ± 10	106 ± 31	106 ± 17	100 ± 15	88 ± 11

[a]: mean of *n*=6 replicates analysed in different days for blank beer and wine spiked samples considered together, expressed as root mean square error (RMS error) ± standard deviation (SD), both in ppm; [b]: R: real mass resolution; [c]:chromatographic retention time ± SD (*n*=14) ; [d]: MT, mixture of isomers

Table 2. Linearity range calculated in white wine and beer matrix, evaluated by regression coefficient (r); experimental limit of quantification (LOQ) consisting in the lowest concentration satisfying the established confirmation criteria; method repeatability and reproducibility, calculated at two concentration levels, and expressed as intra-day relative standard deviation (RSD) and inter-day RSD, respectively.

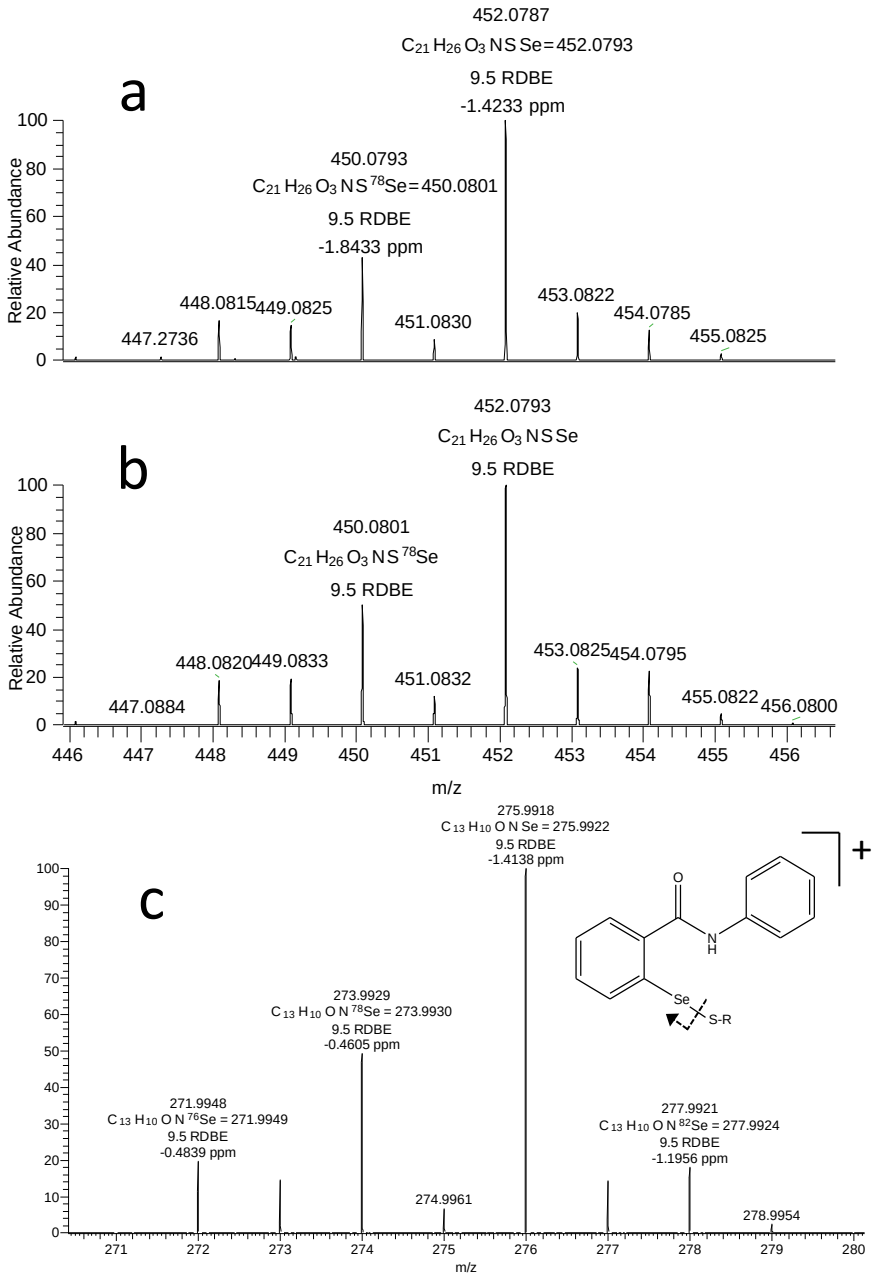
	Compound	Wine							Beer						
		Range (ng/L)	Linearity (r)	LOQ (ng/L)	Intra-day RSD (%) (*n*=7)		Inter-day RSD (%) (*n*=6)		Range (ng/L)	Linearity (r)	LOQ (ng/L)	Intra-day RSD (%) (*n*=7)		Inter-day RSD (%) (*n*=6)	
					1 ng/L	20 ng/L	1 ng/L	20 ng/L				1 ng/L	20 ng/L	1 ng/L	20 ng/L
1	3MMB-Ebs	0.01-50	0.9942	0.01	7	6	18	12	0.01-50	0.9956	0.01	5	4	13	15
2	4MMP-Ebs	0.01-50	0.9896	0.01	4	7	20	10	0.05-50	0.9916	0.05	5	4	10	11
3	3MH-Ebs	0.01-50	0.9961	0.01	5	6	19	12	0.05-50	0.9962	0.05	6	6	16	17
4	4MMB-Ebs	0.01-50	0.9949	0.01	4	9	19	15	0.05-50	0.9856	0.05	6	5	9	6
5	2FMT-Ebs	0.01-50	0.9902	0.01	4	9	12	18	0.1-50	0.9803	0.1	8	6	16	12
6	3MHA-Ebs	0.01-50	0.9955	0.01	8	7	17	11	0.05-50	0.9932	0.05	8	4	8	9
7	3MBT-Ebs	5-50	0.9779	5	-	38	-	52	10-50	0.9937	10	-	7	-	26
8	1PEM-Ebs	0.01-50	0.9865	0.01	9	8	24	17	0.05-50	0.9828	0.05	10	3	11	9
9	MT-Ebs [a]	0.01-50	0.9964	0.01	5	8	18	11	0.05-50	0.9944	0.05	7	5	14	10
10	HT-Ebs	0.01-50	0.9942	0.01	6	23	23	30	0.05-50	0.9973	0.05	14	6	14	12
11	3MHH-Ebs	0.01-50	0.9897	0.01	7	9	22	15	0.05-50	0.9831	0.05	10	6	13	5

[a]:MT, sum of isomers

Table 3. Target (in bold) and non-target volatile thiols detected in selected wine and beer samples.

RT [c] (min)	Theoretical *m/z*	derivative formula $[M+H]^+$	thiol formula	Identification or tentative identification	Thiols in wine samples(ng/L)[a]				Thiols in beer samples (ng/L)[b]					
					W1	W2	W3	W4	B1	B2	B3	B4	B5	B6
11.43	354.0061	$C_{15}H_{16}O_2NSSe$	C_2H_6OS	mercaptoethanol[d]	1.2	4.3	6.9	1.9	0.52	0.33	1.0	0.47	0.97	2.27
11.52	366.0061	$C_{16}H_{16}O_2NSSe$	C_3H_6OS		0.10	0.05	0.03	0.17						
11.56	351.9905	$C_{15}H_{14}O_2NSSe$	C_2H_4OS	mercaptoacetaldehyde[d]	0.35	0.19	0.13	0.57	0.10	0.22	0.07	0.09	0.09	0.30
12.33	368.0218	$C_{16}H_{18}O_2NSSe$	C_3H_8OS	mercaptopropanol[d]	0.37	1.42	1.29	0.24	0.06		0.12		0.05	0.30
12.76	426.0273	$C_{18}H_{20}O_4NSSe$	$C_5H_{10}O_3S$	2-hydroxyethyl-3-mercaptopropionate[d]	0.83	0.55	0.80	0.69						
13.02	436.0116	$C_{19}H_{18}O_4NSSe$	$C_6H_8O_3S$						0.06		0.08	0.04	0.04	
13.78	382.0374	$C_{17}H_{20}O_2SSe$	$C_4H_{10}OS$	Mercaptobutanol or mercaptomethylpropanol[d]	0.34	1.68	1.00	0.44	0.02		0.04		0.03	0.01
14.36	396.0531	$C_{18}H_{22}O_2NSSe$	$C_5H_{12}OS$	**3MMB[e]**		**0.03**	**0.01**	**0.02**	**0.16**	**0.20**	**0.21**	**0.08**	**0.13**	**0.23**
14.51	396.0167	$C_{17}H_{18}O_3NSSe$	$C_4H_8O_2S$	methyl-3-mercaptopropionate[d]	6.2	31.3	47.1	15.5	1.9	0.53	4.5	1. 5	2.0	8.2
14.92	323.9956	$C_{14}H_{14}ONSSe$	CH_4S	methanethiol[d]	2.9	6.6	3.0	1.6	4.6	1.9	3.4	3.2	2.0	2.7
15.25	410.0324	$C_{18}H_{20}O_3NSSe$	$C_5H_{10}O_2S$	ethyl 3-mercaptopropionate[d]	1.1	5.4	6.6	1.5	0.23	0.05	1.02	0.15	0.13	1.02
15.66	408.0531	$C_{19}H_{22}O_2SSe$	$C_6H_{12}OS$	4-mercapto-4-methylpentan-2-one[d]		0.10								
15.8	452.0793	$C_{21}H_{26}O_3NSSe$	$C_8H_{16}O_2S$	mercaptohexyl acetate isomer [d]		0.05	0.02	0.01						
16.37	410.0687	$C_{19}H_{24}O_2SSe$	$C_6H_{14}OS$	**3MH [e]**	**0.33**	**1.3**	**0.21**	**0.33**						
16.60	338.0112	$C_{15}H_{16}ONSSe$	C_2H_6S	ethanethiol[d]	0.22	0.48	0.56	0.02	0.01		0.08			
16.83	390.0061	$C_{18}H_{16}O_2NSSe$	C_5H_6OS	**2MFT[e]**										**<LOQ**
16.88	424.0480	$C_{19}H_{22}O_3NSSe$	$C_6H_{12}O_2S$	ethyl 3-mercaptobutyrate/mercaptohexanoic acid[d]	0.03	0.12	0.20	0.04		0.04				
18.89	452.0793	$C_{21}H_{26}O_3NSSe$	$C_8H_{16}O_2S$	**3MHA [e]**	**0.01**	**0.22**		**0.02**						
18.91	378.0419	$C_{18}H_{20}ONSSe$	C_5H_7S	**3MBT[e]**					**37.3**	**3.4**				

[a]: W1: albariño, W2: sauvignon blanc, W3: Riesling, W4: sauvignon blanc/gewürtztraminer; [b]: B1: lager beer exposed to light, B2: lager beer exposed to light; B3: lager beer3; B4: double malt beer; B5: alcohol free beer, B6: stout beer; [c]: retention time; [d]: tentative identification on the basis of molecular formula; quantified as ng/L of IS; [e]: identified by comparison with authentic reference compound by using matrix-matched calibration curve.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C21 H26 O3 N S Se = 452.0793
9.5 RDBE
-1.4233 ppm
450.0793
C21 H26 O3 N S 78Se = 450.0801
9.5 RDBE
-1.8433 ppm
448.0815
449.0825
447.2736
451.0830
453.0822
454.0785
455.0825
Relative Abundance
b
452.0793
C21 H26 O3 N S Se
9.5 RDBE
450.0801
C21 H26 O3 N S 78Se
9.5 RDBE
448.0820
449.0833
447.0884
451.0832
453.0825
454.0795
455.0822
456.0800
Relative Abundance
m/z
c
275.9918
C 13 H10 O N Se = 275.9922
9.5 RDBE
-1.4138 ppm
273.9929
C 13 H10 O N 78Se = 273.9930
9.5 RDBE
-0.4605 ppm
271.9948
C 13 H10 O N 76Se = 271.9949
9.5 RDBE
-0.4839 ppm
274.9961
277.9921
C 13 H10 O N 82Se = 277.9924
9.5 RDBE
-1.1956 ppm
278.9954
O
N
H
Se
S-R
+
m/z

Fig. 1

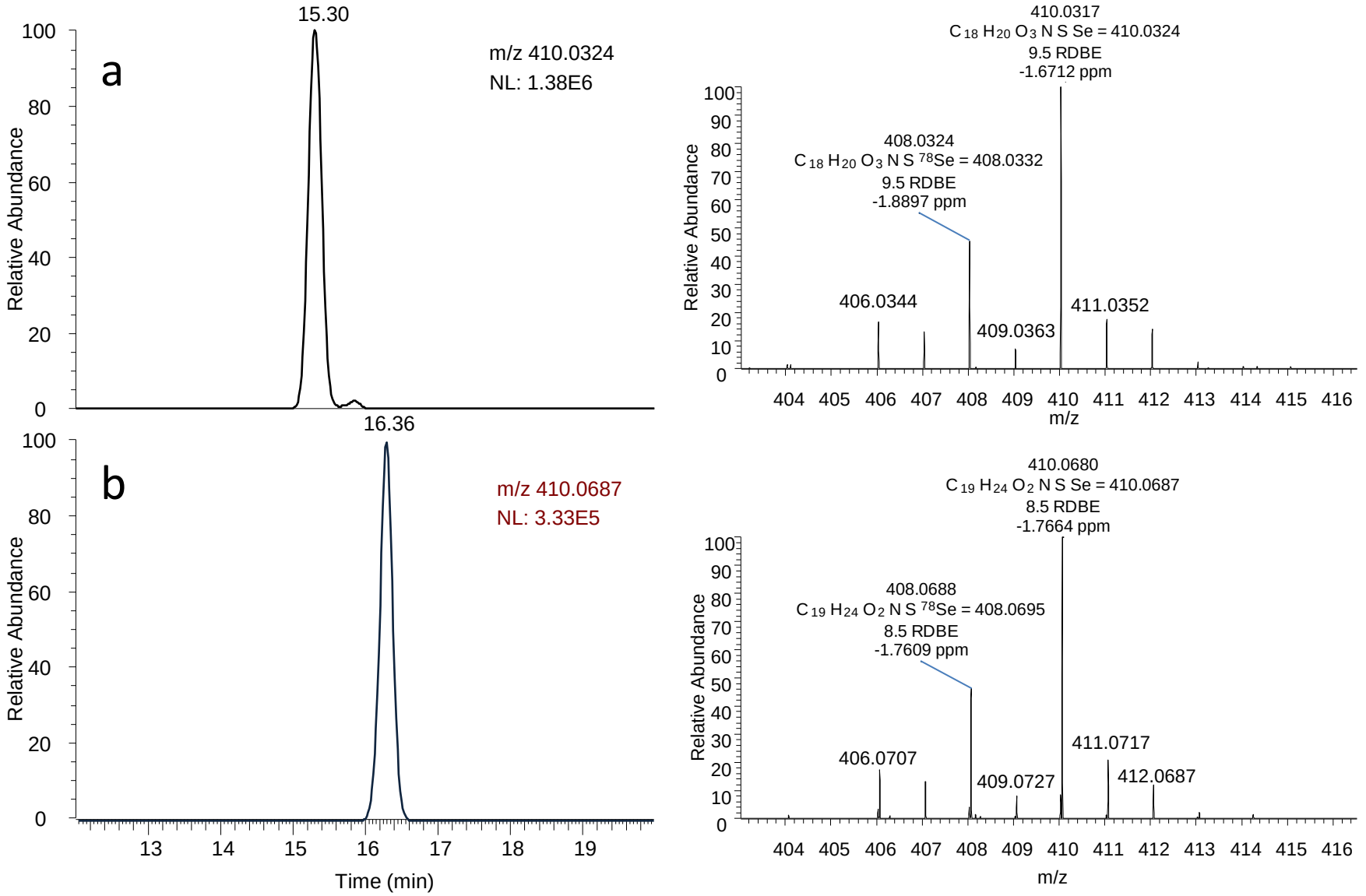
a
15.30
m/z 410.0324
NL: 1.38E6
Relative Abundance
b
16.36
m/z 410.0687
NL: 3.33E5
Relative Abundance
Time (min)
410.0317
C 18 H 20 O 3 N S Se = 410.0324
9.5 RDBE
-1.6712 ppm
408.0324
C 18 H 20 O 3 N S 78Se = 408.0332
9.5 RDBE
-1.8897 ppm
406.0344
409.0363
411.0352
m/z
410.0680
C 19 H 24 O 2 N S Se = 410.0687
8.5 RDBE
-1.7664 ppm
408.0688
C 19 H 24 O 2 N S 78Se = 408.0695
8.5 RDBE
-1.7609 ppm
406.0707
409.0727
411.0717
412.0687
m/z

Fig. 3