Draft Manuscript for Review

Fiber depth, column coating and extraction time are major contributors in the headspace solid phase microextraction – gas chromatography analysis of Nordic wild mushrooms

Journal:	*European Food Research and Technology*
Manuscript ID	Draft
Manuscript Type:	Original paper
Date Submitted by the Author:	n/a

Complete List of Authors:	Aisala, Heikki; Turun Yliopisto, Department of Biochemistry/ Food Chemistry and Food Development Linderborg, Kaisa; Turun Yliopisto, Department of Biochemistry, Food Chemistry and Food Development Sandell, Mari; Turun Yliopisto, Functional Foods Forum

Keywords:	mushrooms, SPME, cantharellus cibarius, volatile compounds

SCHOLARONE™
Manuscripts

For Review

Fiber depth, column coating and extraction time are major contributors in the headspace solid phase microextraction – gas chromatography analysis of Nordic wild mushrooms

Heikki Aisala[†,‡,*], Kaisa M. Linderborg[†], and Mari Sandell[‡]

[†] University of Turku, Department of Biochemistry, Food Chemistry and Food Development, FI-20014 Turku, Finland
[‡] University of Turku, Functional Foods Forum, FI-20014 Turku, Finland
*Correspondence: heikki.aisala@utu.fi

For Peer Review

Abstract

The aims of this research were to systematically study how extraction, desorption and gas chromatography (GC) parameters affect the volatiles composition of mushrooms in headspace solid-phase microextraction (HS-SPME) analysis. The study was carried out both with reference compounds and with *Cantharellus cibarius* mushroom sample. The experiments were carried out with full-factorial multivariate designs.

In the desorption studies, high fiber depth in the GC injector port instead of desorption time or temperature was the most important variable for maximizing peak areas. This could be a function related to the temperature gradient inside the injector as well as a faster transfer of volatiles to the column. Out of the tested fibers, divinylbenzene/Carboxen/polydimethylsiloxane (DVB/Car/PDMS) was the most suitable coating for mushroom volatiles. Additionally, extraction time of 45 vs 30 minutes had a high effect, while doubling the sample volume had a minimal effect.

21 volatiles were identified in *Cantharellus cibarius*. Hexanal and 1-octen-3-ol were the most abundant volatile compounds. Overall, aldehydes and unsaturated C_6-C_{10} alcohols and aldehydes were the most abundant compound groups.

This study demonstrated that despite the wide adaptation and history of SPME, fiber injection depth, fiber coating and extraction time are important factors that should still be carefully tested as a part of method development for mushroom-type matrices. Finally, the results of this study show that it is important to look at the extracted gas chromatogram as a whole instead of focusing few peaks of interest.

Keywords: mushrooms; *Cantharellus cibarius*; SPME; volatile compounds

Funding information

This work was supported by the University of Turku Graduate School (UTUGS) and its Doctoral Program in Molecular Life Sciences (DPMLS), Niemi foundation research grant (2015), and by the Academy of Finland (MS252005).

For Peer Review

Introduction

The aroma of food is critical in appraising its sensory properties, and along with visual cues, orthonasal aroma is the first information a consumer receives from food. Aroma is composed of a set of volatile compounds. For them to have an impact on the overall aroma, they have to be present above their threshold concentrations for humans in the food headspace. Finally, the specific proportions of each aroma-contributing volatile compound in the sample headspace define the final impression perceived by chemical senses such as sense of smell [1].

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

45 Traditionally, the analysis of mushroom aroma compounds has focused on isolating the 46 volatile compounds with different steam distillation [2] and solvent extraction [3] 47 protocols, and their identification and quantification with gas chromatography-mass 48 spectrometric methods.

49 However, these solvent extracts do not fully represent the samples as consumers perceive 50 food on a plate. Extracts contain compounds of highly differing volatility, which means 51 that analyzing the extract gives an answer to a different research question regarding aroma 52 than studying the headspace profile of a sample.

53 On the other hand, headspace measurements by themselves, while demonstrating the 54 aroma profile as the consumer perceives it, have an inherent challenge: concentrating the 55 sample is difficult. Thus, the sensitivity of headspace measurements is often too low for 56 proper identification of sample analytes. Headspace solid phase microextraction (HS-57 SPME) has become an established method over the 30 years since its conception [4], and 58 circumvents many of these limitations. It unifies the sample preparation steps of extraction 59 and concentration into one convenient process. However, it should be remembered that the 60 extracted headspace profile with SPME is never fully equitable to the actual headspace 61 profile and is more of an impression created by the used fiber coating [5]. Furthermore, 62 SPME is an equilibrium method and as such, several variables affect the extracted profile. 63 These include – aside from the selection of fiber coating – extraction time, headspace 64 volume, and desorption conditions [6]. Thus, it's important to optimize the SPME method 65 so that the resulting extract is as close to the actual headspace composition as possible.

66 Nordic forests are home to a wide selection of wild edible mushroom species, one of the 67 most popular species among them being *Cantharellus cibarius*. The last in-depth analysis 68 on their volatile compound composition was done 40 years ago, and with solvent

For Peer Review

extraction based methods [2]. Some of the wild mushroom species as well as the cultivated mushroom, *Agaricus bisporus*, have been analyzed in later years using HS-SPME [7–10]. However, the species that have been analyzed in previous research and are found Nordic forests are from locations that have very different climate and lighting conditions compared to Nordic forests, and it is reasonable to assume that this has an effect on the volatiles composition. Furthermore, previous HS-SPME studies on mushrooms left out some important parameters like the liner type and fiber desorption depth, and arrived in opposite conclusions especially regarding the choice of fiber [7, 8, 11]. Finally, the studies on interaction of different parameters has been minimal, as only one article on mushroom volatiles used full factorial composite designs [11], and even recent publications using HS-SPME on other matrices have mainly utilized univariate study designs [12].

Thus, there was a need to explore which parameters would yield the optimum signal for Finnish mushroom species. The aims of this research were to systematically study how extraction, desorption and gas chromatography parameters affect the volatiles composition of mushrooms in headspace solid-phase microextraction (HS-SPME) analysis. This was done to assess 1) which desorption parameters have a systematic effect on peak shapes, 2) if there are major interactions between mushroom volatile compounds and SPME fiber coatings, and 3) whether the column stationary phase has a stronger impact on resolution than pre-columns or focusing effects.

Materials and Methods

Research workflow

The research was carried out in four stages. The rationale behind this workflow was to split the analysis in independent stages so that interacting variables and thus the number of

For Peer Review

samples would be minimized. First, desorption conditions were optimized. Then, the resolving power of two similar columns differing mainly in their film thickness, with or without a preceding retention gap was investigated. After deciding the appropriate column system, the oven program was further refined. Finally, the effects of different variables in the volatiles extraction were studied.

Samples

Standard mixture for desorption studies

Desorption parameters were studied with a reference compound mixture containing seven compounds. 1-octen-3-ol, eugenol and nonanal were bought from Aldrich (St. Louis, MO). 1-hexanol, methional and (R)-(+)-limonene were bought from Sigma (St. Louis, MO). P-xylene was bought from FlukaChemie AG (Buchs, Switzerland). Benzaldehyde was brought from BDH Chemicals (Poole, UK). All reagents were of ≥95% purity. The compounds were selected by consulting earlier literature of relevant mushroom volatiles studies [2, 8, 9, 13], as well as pilot HS-SPME-GC-MS analyses of mushroom samples. Selected compounds also spanned the total chromatographic retention index range on the selected column and represented different compound groups (alcohols, aldehydes, terpenoids, and aromatic hydrocarbons). A master solution of 600 µg/ml concentration for each compound in hexane was made and stored in -20 °C. A fresh 18 µg/ml dilution was prepared at the start of each day from the master solution. This concentration resulted in comparable peak areas with mushroom samples.

For analysis, 3.0 µl of the desorption solution was pipetted in a 5 ml glass vial equipped with a septum cap. The headspace was equilibrated for 10 minutes in ambient temperature and pressure, after which a DVB/CAR/PDMS fiber was placed in the middle of the vial,

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

115 exposed to the headspace and the headspace compounds were extracted for 15 minutes.
116 Finally, the fiber was exposed in the injection port with the desorption conditions dictated
117 by the experimental design.

118 Alkane standard

119 The C_5–C_{20} alkane standard (ASTM-P-0050) from Accustandard (New Haven, CT) was
120 used for building the linear retention index. HPLC grade hexane was used as the solvent. A
121 50 µg/ml solution of the alkane standard was created, and the sample was extracted as
122 described above for the desorption mixture.

123 Additional reference compounds

124 Pentanal, 1-octene, (E)-2-heptenal, 3-octanone, 2-pentylfuran, (E)-2-nonenal, decanal and
125 (E,E)-2,4-decadienal were bought from Sigma Aldrich (St. Louis, MO) and used for
126 identifications in the mushroom sample.

127 Mushroom sample

128 Chanterelle (*Cantharellus cibarius)* was used during the selection of column, optimization
129 of oven programs as well as during assessment of SPME extraction parameters. The
130 chanterelle sample was collected in Pargas, Finland (60.30° N, 22.30° E) in September
131 2015. After arriving to the laboratory within 24 hours after collection, each mushroom was
132 cut to 3–5 pieces of similar size, and blanched in boiling water for two minutes. After
133 cooling down in ambient temperature, the mushrooms were frozen at –20 °C.

134 Before the main analysis, the sample lot was cut, pooled and divided into aliquots. In short,
135 500 g of frozen sample material was cut into 2–7 mm cubes. All material was combined,
136 mixed, spread over a tray and divided into either 10.0 g or 20.0 g sections using the cone

For Peer Review

1
2
3
4 137 quartering method. The individual samples were packaged in re-sealable plastic bags.
5
6 138 After this, the samples were stored at –20 °C for 3 months, and analyzed in two weeks.
7
8
9 139 **SPME fibers**
10
11
12
13 140 Three types of 24 gauge, 1 cm SPME fibers from Supelco (Bellefonte, PA) were used:
14
15 141 50/30 μm divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS), 75μm
16
17 142 Carboxen/polydimethylsiloxane (CAR/PDMS), and 65μm
18
19 143 polydimethylsiloxane/divinylbenzene (PDMS/DVB) coatings. The selected fibers were
20
21 144 typical alternatives used in previous SPME studies on fungal volatiles [7–9, 11, 14] as well
22
23 145 as recent studies on other matrices using SPME [12, 15, 16]. Each fiber was thermally
24
25 146 conditioned in accordance to the manufacturer's specifications before use.
26
27
28
29 147 **Instrumentation**
30
31
32 148 The study was conducted with two gas chromatography instruments, both equipped with
33
34 149 flame ionization detectors. The first and second experiment on desorption parameters
35
36 150 were done with a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Corporation,
37
38 151 Kyoto, Japan). Other studies were done with an HP-6890 series gas chromatograph
39
40 152 (Agilent Technologies, Santa Clara, CA). Both the GC-2010 and HP-6890 had flame
41
42 153 ionization detectors and were equipped with 0.75 mm ID SPME liners from Shimadzu and
43
44 154 Restek Corporation (Bellefonte, PA), respectively.
45
46
47 155 The analytical column for the desorption parameters was a 30 m × 0,25 mm × 0,50 μm
48
49 156 DB-5 capillary column (Agilent J&W, Santa Clara, CA). In the column selection stage, a
50
51 157 30 m × 0.25 mm × 1.00 μm RTX-5 Sil MS column by Restek Corporation (Bellefonte,
52
53 158 PA) was added to comparison. Additionally, the effect of adding a 3 m × 0.25 mm
54
55 159 uncoated fused silica retention gap from Supelco (Bellefonte, PA) was studied. The
56
57
58
59
60

For Peer Review

1
2
3
4 160 retention gap was installed in front of the analytical column with a capillary column butt
5
6 161 connector from Supelco (Bellefonte, PA).
7
8
9 162 The oven temperature was optimized in multiple stages following previous publications [3,
10
11 163 9], and will be outlined in the relevant sections. The carrier gas was helium, with a linear
12
13 164 flow of 40 cm/s for desorption studies and 33 cm/s for the rest of the experiments. The
14
15 165 linear flow velocity was verified with the retention time of injected propane gas.
16
17
18
19 166 **Experiments**
20
21
22 167 Desorption studies
23
24
25 168 Different levels of desorption parameters were compared in three sample sets (Table 1),
26
27 169 each using the same freshly diluted reference standard mixture as a sample. The
28
29 170 experimental design for each experiment was a full-factorial design run in randomized
30
31 171 duplicate. The first experiment contained also three center points for standard deviation
32
33 172 estimation.
34
35
36 173 During desorption studies, the following oven program was used: 40 °C held for 5
37
38 174 minutes, increased at 6 °C/min to 170 °C, and held at final temperature for 5 minutes,
39
40 175 resulting in an approximately 27 minute analysis time.
41
42
43
44 176 Column selection and oven program
45
46
47 177 The column comparison and oven program development aimed to make peaks narrower, to
48
49 178 improve peak symmetry and to improve resolution especially in the crowded retention
50
51 179 index range 950–1050. Thus, both columns with or without the retention gap (thus four
52
53 180 combinations) were tested with three progressively complex oven programs with the
54
55 181 chanterelle sample.
56
57
58
59
60 8

For Peer Review

1
2
3
4 182 Both tested columns have phenyl substituted polysiloxane stationary phases and thus are
5
6 183 comparable in terms of polarity and other resolving characteristics. The used phase has
7
8 184 been typical in mushroom volatiles analysis [3, 7, 8, 13]. The columns are also of identical
9
10 185 length and inner diameter. The hypothesis was as discussed in the literature [6] that using a
11
12 186 thick-film stationary phase column as well as a retention gap would enhance sample
13
14 187 focusing.
15
16
17 188 After the column selection, the oven program was further refined with the mushroom
18
19 189 sample. The final oven program was the following: 35 °C held for 5 minutes, increased at
20
21 190 5 °C/min to 90 °C, then at 2 °C/min to 100 °C, at 7 °C/min to 260 °C and held at this final
22
23 191 temperature for 2 minutes.
24
25
26 192 Extraction parameters
27
28
29
30 193 Three extraction parameters were optimized: fiber coating (3 types, stated above), sample
31
32 194 mass (2 levels, 10 g and 20 g) and extraction time (2 levels, 30 min and 45 min). The
33
34 195 sample mass levels were based on pilot studies as well as published literature [8, 9] and
35
36 196 extrapolated to the used extraction vial volume. Extraction time points were likewise based
37
38 197 on published literature [11]. The experimental design was a full-factorial model run in
39
40 198 duplicate, resulting in 24 samples. The run order was randomized both for the parameter
41
42 199 combinations and the used sample aliquots.
43
44
45 200 Each sample aliquot was weighed straight from –20 °C storage to a 90 ml glass vial.
46
47 201 Equilibrium time was 30 minutes. Equilibrium and extraction temperature was each time
48
49 202 30 °C and controlled by a water bath surrounding the extraction flask. After SPME
50
51 203 extraction, the volatiles were desorbed from the fiber using the best parameters found
52
53 204 during desorption studies.
54
55
56
57
58
59
60

For Peer Review

1
2
3
4 205 **Peak characteristics criteria**
5
6
7 206 Throughout the study, the following four peak criteria were set as desirable: 1) the highest
8
9 207 possible areas, 2) highest possible signal to noise ratios, 3) lowest possible full widths at
10
11 208 half height, and 4) as symmetrical peaks as possible, i.e. tailing factors as close to 1 as
12
13 209 possible.
14
15 210 Furthermore, the fiber that achieved these with the widest representation of compounds
16
17 211 over the whole retention index range was selected as ideal.
18
19 212
20
21 213 **Identification**
22
23 214 The compounds were identified with reference compounds, by comparing the retention
24
25 215 indices to literature data on the NIST Chemistry WebBook [17], and by gas
26
27 216 chromatography-mass spectrometry analysis. For GC-MS analysis, a GC of the same HP
28
29 217 6890 model as in the main studies was attached to the HP 5973 mass sensitive detector. All
30
31 218 mass spectra were acquired in the electron impact (EI) mode. The mass ranged from 30 to
32
33 219 700 m/z, with a scan rate of 2.23 scans/s. The interface temperature was set to 150 °C, and
34
35 220 other GC analytical conditions were as described above for the extraction parameter study.
36
37 221 The MS peaks were identified with the Wiley 275 mass spectral library as well as with the
38
39 222 in-house mass library created from reference compounds.
40
41 223 **Statistical analysis**
42
43 224 GC chromatograms were integrated and the following key parameters were extracted from
44
45 225 the data: peak areas, signal-to-noise ratios, full widths at half height, and tailing factors.
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

226 Initial evaluation of the effects of desorption parameters on peak areas of example
227 compounds 1-octen-3-ol and nonanal were done with linear regression. The effect of
228 extraction parameters on the total area of peaks was examined with two-way analysis of
229 variance (ANOVA). These analyses were done using SPSS version 23 (SPSS Inc.,
230 Chicago, IL). Limit for statistical significance was 0.05.

231 As unsupervised data analysis, principal component analysis (PCA) was done for
232 desorption studies and extraction parameter datasets with autoscaled data. After discarding
233 variables with minimal variation and strongly collinear peak parameters, the PCA was
234 refined. After this, partial least squares regression discriminant analysis (PLS-DA) was
235 done with either desorption time, temperature and fiber depth (desorption studies), or fiber
236 type, sample mass and extraction time (extraction studies) as Y-variables and peak
237 parameters for each compound as X-variables. All data was autoscaled. PCA and PLS-DA
238 models were created with The Unscrambler X version 10.4 (CAMO Software AS, Oslo,
239 Norway).

240 Results and discussion

241 Desorption conditions

242 First sample set

243 Surprisingly, in the linear regression of 1-octen-3-ol and nonanal, fiber depth alone
244 explained 70 % and 80 % of the variance of the peak areas, respectively. Furthermore,
245 temperature and desorption time were not statistically significant regression predictors for
246 1-octen-3-ol area, and even for nonanal, fiber depth had an order of magnitude higher
247 coefficient in the regression model than the other two parameters. This is also visualized in

For Peer Review

the PCA model (Supplementary information, Figure S1): all peak areas as well as fiber depth have a high negative loading on the first principal component.

The effect is further elucidated on the PLS model (Figure 1), in which first 3 factors explain 83 % of Y-variable variation and 84 % of X-variable variation. Desorption parameters had a minimal effect on peak widths as the relative standard deviation on the first dataset was <3% for all compounds, so peak widths were left out of the displayed model. The peak areas are closely associated with fiber depth, pointing out that increased fiber depth is optimal. Interestingly, longer desorption time seems to correlate with lesser peak tailing. Likewise, higher temperatures correlated with tailing of some compounds. However, looking at data in which Y-variable categories have been split (data not shown), it can be seen that it is mostly the lowest 2 min desorption time which is associated with tailing peaks.

Second sample set

In the PLS model (Figure 2) the first two factors explain over 90 % of variation in the desorption variables and 65 % of variation in peak parameters. Most peak areas are associated with higher temperatures and correlate inversely with peak tailing. Additionally, the widths of 4 of the peaks are associated with longer desorption times. This points to the 240 °C injector temperature and a 3 minute desorption time being optimal values.

Third sample set

In the PLS model (Figure 3), the first two factors explain 75 % of Y-variable and 69 % of peak parameter (X variable) variation. Factor 1 is still associated with all peak areas, with both fiber depth and peak areas having high positive loadings. This reinforces the fact that for fast desorption of analytes, maximal fiber depth is ideal. Factor 2 is associated with

For Peer Review

1
2
3
4 271 desorption time. However, there is no systematic link to peak characteristics; for 4
5
6 272 compounds, longer desorption time associates with wider peaks, while it's the opposite for
7
8 273 the other 3 compounds. Interestingly, longer desorption time seems to be linked to less
9
10 274 tailing peaks on most compounds.

11
12
13 275 The three sample sets thus show that out of the tested variables, optimal desorption
14
15 276 parameters were maximal (60 mm) fiber depth in the holder, 240 °C injector temperature,
16
17 277 and 3 minute desorption time.

18
19
20 278 Out of these variables, using a higher desorption temperature was as recommended in the
21
22 279 literature [6], but the strong effect of fiber depth was surprising. The peak area increase
23
24 280 could be caused by faster access of the volatiles to the beginning of the column. As there's
25
26 281 no need to vaporize any solvents, the main role of the liner is to ensure a high linear
27
28 282 velocity and thus fast desorption of the analytes to the column [18]. The benefits of narrow
29
30 283 bore glass inserts as used in this study have been demonstrated well [19], with still
31
32 284 achieving narrower peaks by using increased flow rates during the desorption. It could be
33
34 285 postulated that setting the fiber closer to the column helps with peak focusing: there is less
35
36 286 time that the desorbed compounds spend in the liner, and thus they will be focused rapidly
37
38 287 at the beginning of a cool column. This kind of effect, paired with the use of a thick-film
39
40 288 column, was also demonstrated by Langenfeld et al [18].

41
42
43 289 In addition to the mechanism postulated above, SPME guidebooks recommend setting the
44
45 290 desorption depth to correspond with the hottest part of the injector [6], which typically
46
47 291 means the center part of the liner. The 95 mm liner in the Shimadzu instrument causes the
48
49 292 SPME fiber to reside in the upper third of the liner, even with the highest depth setting.
50
51 293 However, in the shorter HP/Agilent 78.5 mm liner, the fiber is almost at the center point
52
53 294 with the maximum setting.
54
55
56
57
58
59
60

For Peer Review

Finally, it should be mentioned that the effect size is relatively small. Changing the fiber depth only caused a ~10 % difference in peak areas compared to the general average. As will be pointed out, extraction parameters had a much higher effect on peak parameters.

Column and oven program

The thicker 1.0 µm film in the RTX-5 Sil MS column was clearly beneficial. This is also recommended in literature [6]. This column resolved several convoluted peaks compared to the DB-5 and in general the peaks were also slightly more symmetrical (less fronting and tailing).

In comparison, the retention gap had a minor effect. As a benefit, it did allow a partial, but not baseline-separated resolution of extra peaks at retention time <5 min when combined with the RTX-5 Sil MS column. Additionally, the early eluting compounds had up to 10 % narrower peak widths at half height; however, this trend was reversed at the end of the chromatogram. There was neither a systematic effect to signal-to-noise ratios or peak symmetry. Thus, the retention gap had no marked benefits to the analysis. Selection of an appropriate column and optimization of the oven program yielded larger improvements in resolution. Finally, as creating a column-to-column connection can cause dead volume and leaks in the analysis system, it was concluded that a retention gap with the tested specifications is not needed. In fact, the effect of the 3 m retention gap was comparable to lowering the linear velocity from 40 cm/s to 33 cm/s. With this change, early eluting compounds had up to 20 % higher resolution. Peak areas stayed the same, but early eluting compounds had ~10 % lower S/N and late-eluting compounds up to 20 % higher S/N.

For Peer Review

In the oven program, the largest gains in resolution could be attained by slowing down heating in the 80–120°C temperature range as much as possible. The final program is a good compromise between maintaining small peak widths and resolving peaks over the whole retention index range. The largest challenge in using polysiloxane columns for mushroom volatiles analysis seems to be that the area surrounding 1-octen-3-ol is very challenging to make fully baseline separated for all compounds.

Extraction parameters

21 identified peaks were integrated (Table 2). The retention times were stable across all identified compounds, with standard deviations on peak retention times being 0.3–0.7 s depending on the compound.

As previously reported [7, 8, 11], different SPME fibers had very different extraction profiles (Figures S2-3). The least suited coating for an overall mushroom profile was Carboxen/PDMS. This fiber had very high adsorption for peaks until hexanal, in other words in the retention index range 300–800, but progressively diminishing adsorption for further compounds. Furthermore, these peaks were 10–30 % wider compared to the average width at half height of 2.9 s on the other two fibers. This held true even for peak areas that were smaller compared to other fibers, and they were almost systematically tailing. In fact, the separation for most peaks was too poor to even calculate tailing factors. Therefore, Carboxen/PDMS fiber was left out from further comparison. In general, DVB/Car/PDMS seemed to contain benefits from both fiber coatings and followed the peak average most closely.

Two-way ANOVA on the total area of peaks shows that only fiber type and extraction time are statistically significant variables ($p<0.01$). Neither sample volume nor any of the interaction terms were statistically significant. Furthermore, a model with these two main

For Peer Review

1
2
3
4 341 effects already explained 80 % of the variation in total peak areas. Demonstrating
5
6 342 univariate differences, two-way ANOVA was done also for hexanal and 1-octen-3-ol as an
7
8 343 example. As with the sum of peak areas, only fiber type and extraction time are
9
10
11 344 statistically significant ($p<0.05$) variables. DVB/Car/PDMS fiber had 24% and 18% bigger
12
13 345 peaks, increasing the extraction time from 30 to 45 minutes increased peak areas by 28%
14
15 346 and 36%, but doubling the sample mass actually results in lower signal areas. In fact, the
16
17 347 extraction time was the only extraction variable where one factor level resulted in
18
19
20 348 consistently higher peak areas. This kind of univariate comparison, however, leaves out
21
22 349 essential information on compound level variations.
23
24 350 There is also clear separation between the DVB/PDMS and the DVB/Car/PDMS fibers in
25
26
27 351 multivariate models. In Figure 4, it is shown that factor 1 is mostly associated with
28
29 352 extraction time, while factor 2 associates mainly with fiber type. Factor 3, on the other
30
31 353 hand, associates with sample volume. The first three factors explain 88 % of the variation
32
33
34 354 in peak areas and 78 % of variation in extraction parameters.
35
36 355 While peak area was more closely correlated with DVB/Car/PDMS fibers, samples run
37
38 356 with that fiber had slightly wider peaks (data not included in the model). However, the
39
40
41 357 relative standard deviation for peak widths in the whole dataset was ≤10%, meaning that
42
43 358 on an average peak width at half height being less than 3 seconds, this compromise is of
44
45 359 minimal importance.
46
47
48 360 However, it should be remembered that comparison of absolute peak areas and thus
49
50 361 concentrations of volatile compounds is not alone a sufficient base for studying the aroma
51
52 362 profile of mushrooms. Different volatile compounds have major differences in their odor
53
54
55 363 thresholds. Comparing literature values [20] to compounds identified in the *C. cibarius*
56
57 364 samples, extreme cases would be benzaldehyde and (E,E)-2,4-decadienal, where the
58
59
60

For Peer Review

former has an over three magnitudes higher detection threshold than the latter. However, the peak of benzaldehyde is only 4 times that of (E,E)-2,4-decadienal. Thus, optimizing the extraction efficiency of a seemingly large peak might not influence the actual aroma profile at all, while even minuscule gains to small peaks with potent odor intensity are important. In this study, the Carboxen/divinylbenzene/polydimethylsiloxane and the longer, 45 minute extraction time were associated with higher peak areas of almost all identified compounds, meaning that for unsupervised analysis of odor-contributing volatile compounds, these parameters are the best option.

Based on these results, selecting the Carboxen/divinylbenzene/polydimethylsiloxane fiber is appropriate for mushrooms. Furthermore, major gains could be attained by using the longer 45 minute extraction time. Additionally, keeping the sample amount at 10 g doesn't cause a significant loss in sensitivity. This observation is similar to what was noticed on HS-SPME on virgin olive oils [12].

Volatile compounds in chanterelle

The relative content of identified volatile compounds in chanterelles is displayed in Table 2. Hexanal and 1-octen-3-ol were the most abundant compounds and other compounds were present at an order of magnitude smaller concentrations. Overall, aldehydes and unsaturated C_6-C_{10} alcohols and aldehydes were the most abundant compound groups. Dunkel et al. [1] reviewed the precursors of 226 key food odorants, and according to the review, the precursors of these C_6-C_{10} compounds are fatty acids. Politowicz et al. [10] recently studied the effect of drying conditions on the volatiles in *C. cibarius* and found that 1-octen-3-ol, 1-hexanol and hexanal were the most abundant volatile compounds in freeze-dried chanterelles. De Pinho et al. [8] reported earlier that main compounds in their chanterelle samples were 1-octen-3-ol, 1-octen-3-one and 1-hexanol. Comparing these

For Peer Review

published results to ours shows that the samples in this study had a significantly higher content of hexanal. It should be noted that the sample pretreatment was very different – our samples were cooked and frozen while samples in other studies were not cooked at all and were preserved by different drying processes. It is possible that the cooking step in our sample preparation has resulted in lipid oxidation and thus increased formation of hexanal from other volatile compounds. Furthermore, as Politowicz et al. [10] discuss growth conditions were different between these studies which could explain the difference.

Conclusions

This study points out that fiber type and extraction time are important factors that should be carefully tested as a part of SPME analysis development for mushroom-type matrices. Interestingly, there was no interaction between extraction variables, which could simplify experimental designs. Desorption parameters indeed affected the chromatographic profile, but interestingly fiber depth had the most systematic effect and it should be checked as part of optimization. This was surprising as fiber positioning in the injector is just quickly glanced over in textbooks on the subject [6]. This study also demonstrated that with modern analysis methodology, a thick-film column indeed suffices for focusing volatiles present in mushrooms. The results likewise show the importance of looking at the extracted profile as a whole so that the researcher in charge can make informed decisions on the compromises related to each selected analysis factor. Finally, the identified compounds indicate that fatty acids are the precursors of the majority of volatile compounds in *C. cibarius*.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

For Peer Review

1
2
3
4 412 **Compliance with ethics requirements** This article does not contain any studies with
5
6 413 human or animal subjects.
7
8
9 414
10
11
12
13 415
References

416 1. Dunkel A, Steinhaus M, Kotthoff M, et al (2014) Nature's chemical signatures in human olfaction: a foodborne perspective for future biotechnology. Angew Chem Int Ed Engl 53:7124–7143. doi: 10.1002/anie.201309508

419 2. Pyysalo H, Berg A, Lund H, et al (1976) Identification of Volatile Compounds in Seven Edible Fresh Mushrooms. Acta Chem Scand 30b:235–244. doi: 10.3891/acta.chem.scand.30b-0235

422 3. Grosshauser S, Schieberle P (2013) Characterization of the Key Odorants in Pan-Fried White Mushrooms (Agaricus bisporus L.) by Means of Molecular Sensory Science: Comparison with the Raw Mushroom Tissue. J Agric Food Chem 61:3804–3813. doi: 10.1021/jf4006752

426 4. Pawliszyn J (1997) Solid phase microextraction: theory and practice. Wiley-VCH, New York

428 5. Järvenpää E, Nurmela K (2010) Applicability of SPME techniques for analysis of volatile compounds in complex matrices. First Dice Your Dill Anethum Graveolens - New Methods Tech. Sample Handl.

431 6. Kudlejova L, Risticevic S, Vuckovic D (2012) Solid-Phase Microextraction Method Development. In: Pawliszyn J (ed) Handb. Solid Phase Microextraction. Elsevier, pp 201–249

434 7. Costa R, Tedone L, De Grazia S, et al (2013) Multiple headspace-solid-phase microextraction: An application to quantification of mushroom volatiles. Anal Chim Acta 770:1–6. doi: 10.1016/j.aca.2013.01.041

437 8. de Pinho GP, Ribeiro B, Gonçalves RF, et al (2008) Correlation between the Pattern Volatiles and the Overall Aroma of Wild Edible Mushrooms. J Agric Food Chem 56:1704–1712. doi: 10.1021/jf073181y

440 9. Aprea E, Romano A, Betta E, et al (2015) Volatile compound changes during shelf life of dried Boletus edulis: comparison between SPME-GC-MS and PTR-ToF-MS analysis. J Mass Spectrom 50:56–64. doi: 10.1002/jms.3469

443 10. Politowicz J, Lech K, Sánchez-Rodríguez L, et al (2017) Volatile composition and sensory profile of *Cantharellus cibarius* Fr. as affected by drying method: Aroma profile of fresh and dried *Cantharellus cibarius*. J Sci Food Agric. doi: 10.1002/jsfa.8406

For Peer Review

11. San Román I, Alonso ML, Bartolomé L, et al (2014) Analytical strategies based on multiple headspace extraction for the quantitative analysis of aroma components in mushrooms. Talanta 123:207–217. doi: 10.1016/j.talanta.2014.01.021

12. Perestrelo R, Silva C, Silva P, Câmara JS (2017) Global volatile profile of virgin olive oils flavoured by aromatic/medicinal plants. Food Chem 227:111–121. doi: 10.1016/j.foodchem.2017.01.090

13. Cho IH, Lee SM, Kim SY, et al (2007) Differentiation of Aroma Characteristics of Pine-Mushrooms (*Tricholoma matsutake* Sing.) of Different Grades Using Gas Chromatography−Olfactometry and Sensory Analysis. J Agric Food Chem 55:2323–2328. doi: 10.1021/jf062702z

14. Jeleń H h. (2003) Use of solid phase microextraction (SPME) for profiling fungal volatile metabolites. Lett Appl Microbiol 36:263–267. doi: 10.1046/j.1472-765X.2003.01305.x

15. D'Agostino MF, Sanz J, Sanz ML, et al (2015) Optimization of a Solid-Phase Microextraction method for the Gas Chromatography–Mass Spectrometry analysis of blackberry (Rubus ulmifolius Schott) fruit volatiles. Food Chem 178:10–17. doi: 10.1016/j.foodchem.2015.01.010

16. Soares RD, Welke JE, Nicolli KP, et al (2015) Monitoring the evolution of volatile compounds using gas chromatography during the stages of production of Moscatel sparkling wine. Food Chem 183:291–304. doi: 10.1016/j.foodchem.2015.03.013

17. Stein SE (1997) Retention Indices. NIST Chem. WebBook NIST Stand. Ref. Database 69

18. Langenfeld JJ, Hawthorne SB, Miller DJ (1996) Optimizing split/splitless injection port parameters for solid-phase microextraction. J Chromatogr A 740:139–145. doi: 10.1016/0021-9673(96)00140-9

19. Ilias Y, Bieri S, Christen P, Veuthey J-L (2006) Evaluation of solid-phase microextraction desorption parameters for fast GC analysis of cocaine in coca leaves. J Chromatogr Sci 44:394–398.

20. Odor Detection Thresholds & References. http://www.leffingwell.com/odorthre.htm. Accessed 10 Aug 2017

For Peer Review

Supplementary Information

Fiber depth, column coating and extraction time are major contributors in the headspace solid phase microextraction – gas chromatography analysis of Nordic wild mushrooms

Heikki Aisala[†,‡,*], Kaisa M. Linderborg[†], and Mari Sandell[‡]

[†] University of Turku, Department of Biochemistry, Food Chemistry and Food Development, FI-20014 Turku, Finland
[‡] University of Turku, Functional Foods Forum, FI-20014 Turku, Finland
*Correspondence: heikki.aisala@utu.fi

For Peer Review

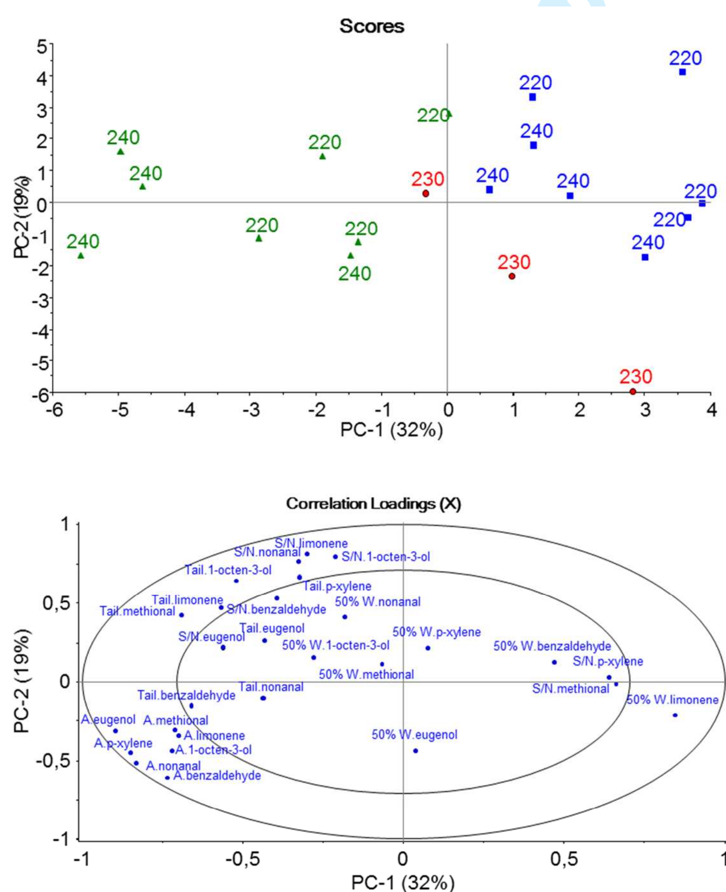

Figure S1. PCA scores (up) and loadings (down) plot of the first desorption data set. All four peak parameters for each of the 7 compounds are included in the model. In the scores plot, data point color refers to fiber depth: blue: 45 mm; red: 50 mm; green: 55 mm. Data point labels are the injector temperatures.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

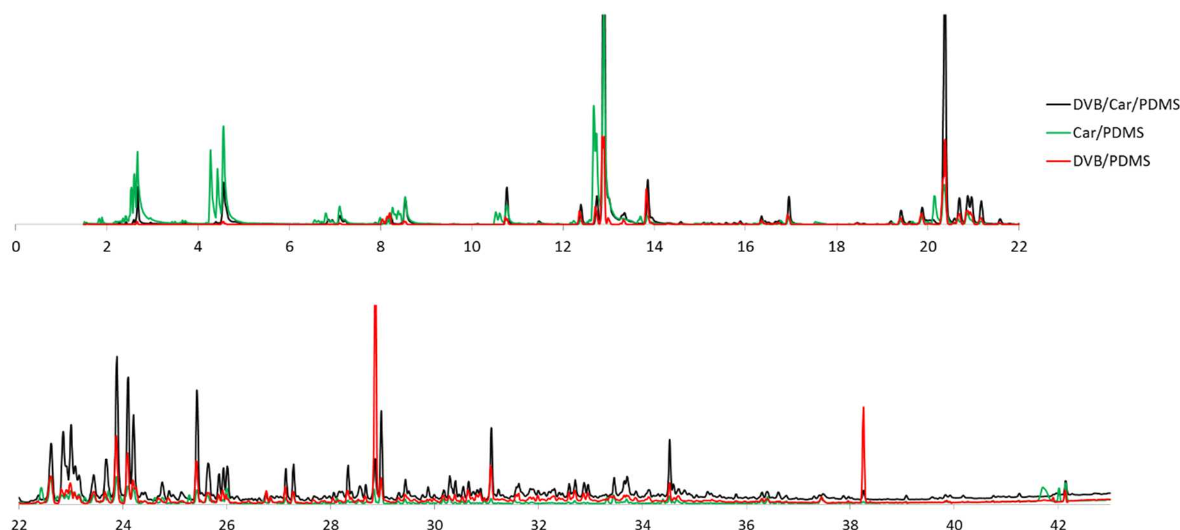

Figure S2. Averaged GC-FID chromatograms for chanterelle mushroom analyzed with three different fibers.

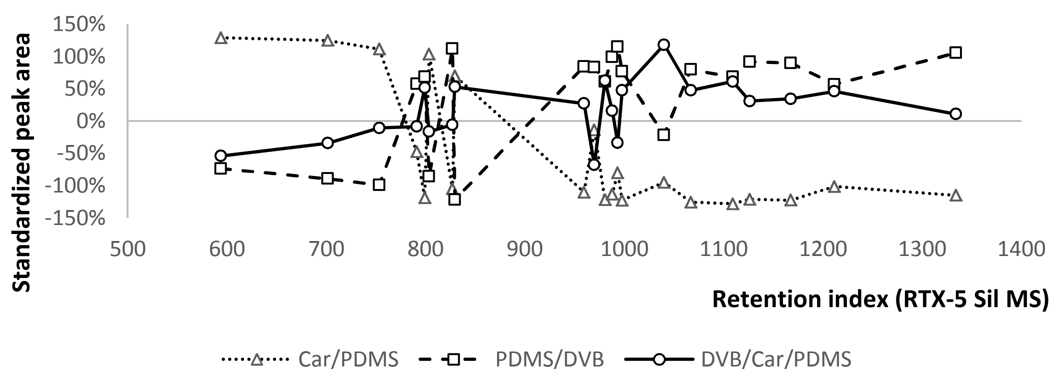

Figure S3. Relative adsorption efficiency of SPME fibers in terms of linear retention index on RTX-5 Sil MS column. Each data point corresponds to the standardized peak area of one compound on 45 minute extraction times and 10 g sample volumes.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

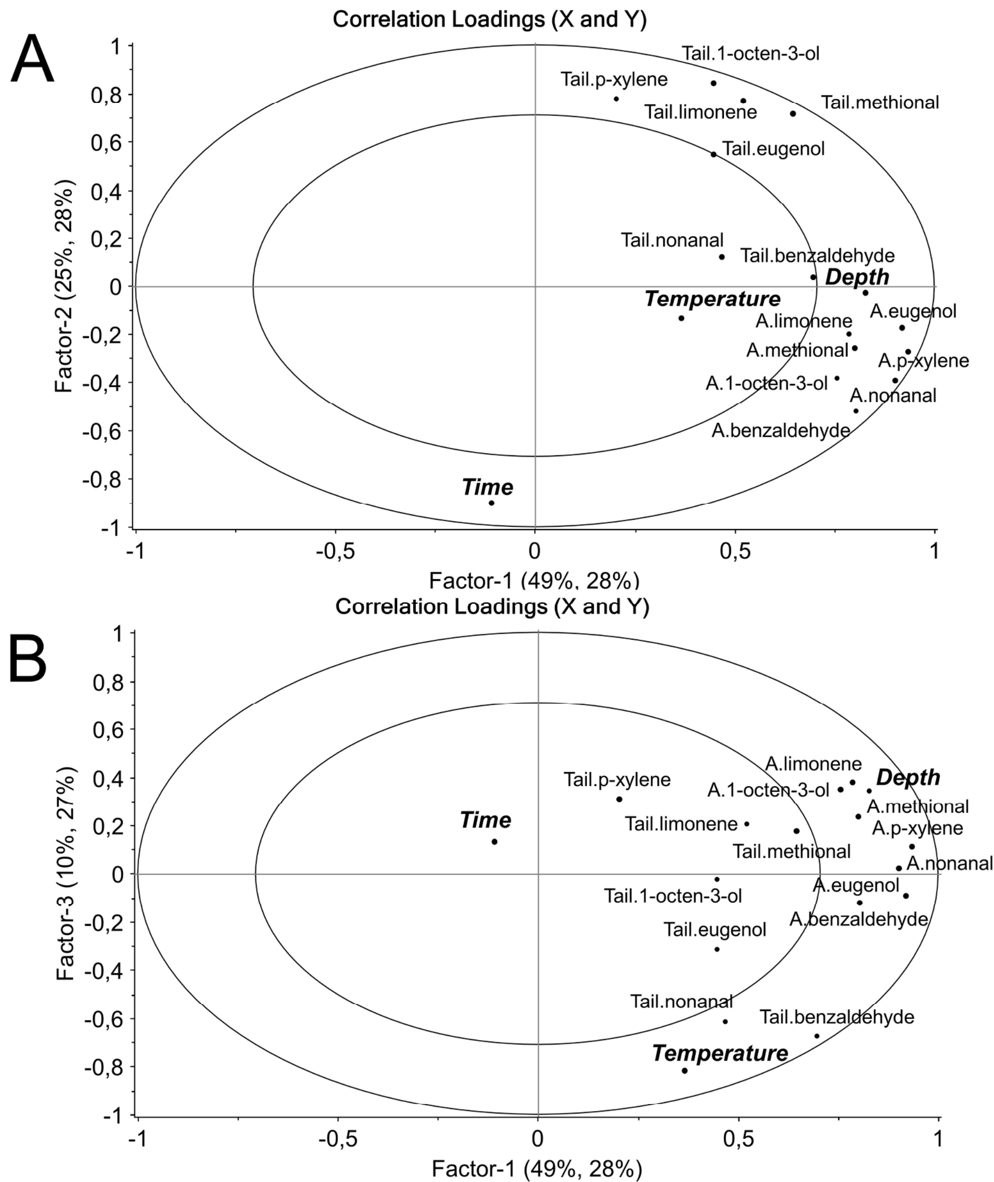

Figure 1. PLS plot of factors 1 and 2 (A) and 1 and 3 (B) the first desorption study. This model includes peak areas and tailing factors which were found to account for the largest data variance in the PCA model.

102x123mm (600 x 600 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

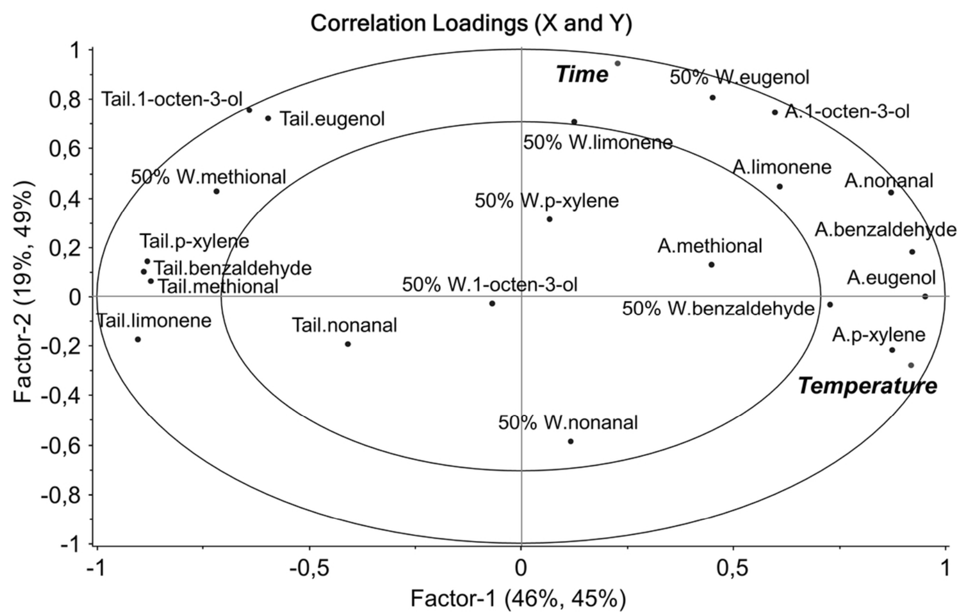

Figure 2. PLS model of the second desorption study.

55x34mm (600 x 600 DPI)

Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

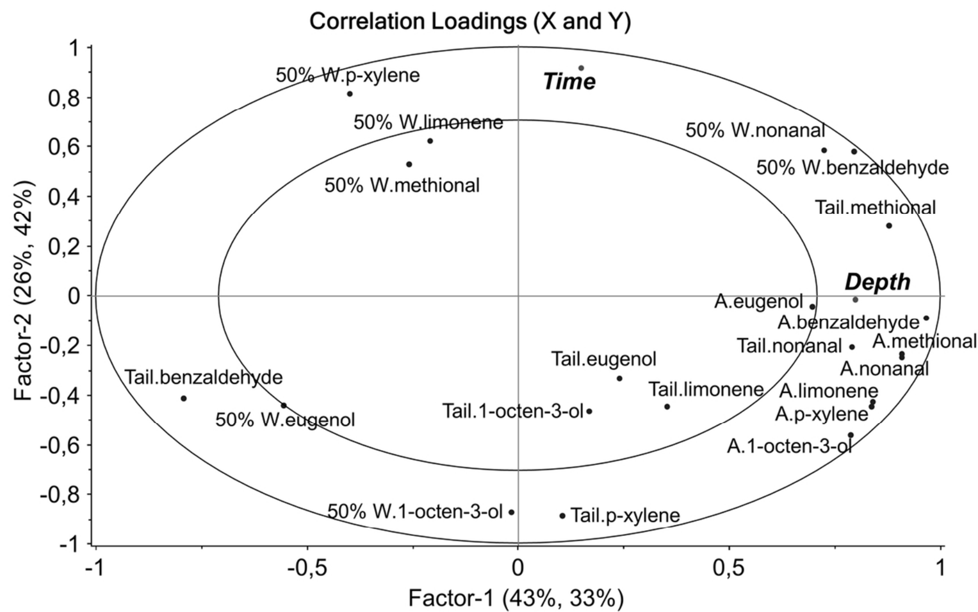

Figure 3. PLS plot of the third desorption study. This model includes peak areas, full widths at half height and tailing factors.

56x35mm (600 x 600 DPI)

Review

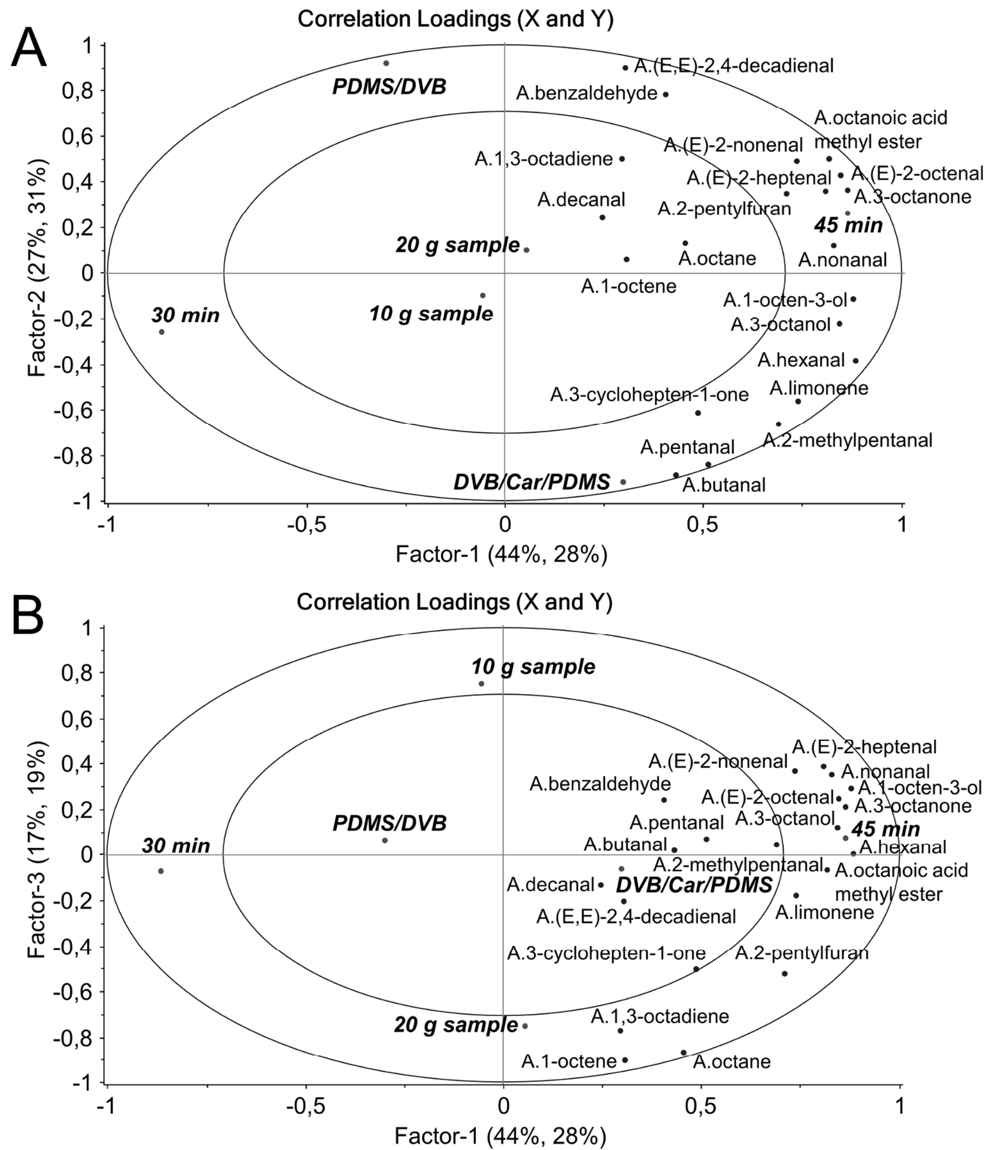

Figure 4. PLS correlation loadings plots of the variables in the extraction parameter experiment using peak areas. A: factors 1 and 2, B: factors 1 and 3.

103x119mm (600 x 600 DPI)

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

1 **Table 1. Experimental designs in the desorption studies.**

Experiment	1[a]			2[a]		3[b]	
Variable	Low level	High level	Center point	Low level	High level	Low level	High level
Desorption time (min)	2	5	3.5	3	5	3	5
Temperature (°C)	220	240	230	230	240	240	
Fiber depth in the injector (mm)	45	55	50	60[c]		50	60[c]

[a] Using Shimadzu GC-2010 Plus
[b] Using HP-6890
[c] maximum setting for the fiber holder

2

For Peer Review

Table 2. Identified compounds, relative areas for each tested fiber and volatile compound distribution in the *Cantharellus cibarius* sample.

#	LRI (calc)	LRI (ref [b])	Compound	Relative areas for each fiber [(a)] Car/PDMS	PDMS/DVB	DVB/Car/PDMS	Relative content (%) [c]
1	594	595	butanal	310	6	36	2.7
2	702	**702**	pentanal	213	34	81	2.5
3	754	758	2-methylpentanal	147	66	99	2.8
4	791	**792**	1-octene	78	102	114	0.8
5	799	**800**	octane	69	109	115	1.4
6	804	800	hexanal	119	83	103	28.7
7	827	827	1,3-octadiene	51	135	102	1.9
8	830	826	3-cyclohepten-1-one	143	43	125	0.8
9	960	**960**	(E)-2-heptenal	60	118	112	1.4
10	970	**972**	benzaldehyde	77	129	89	1.6
11	981	**983**	1-octen-3-ol	49	109	129	20.4
12	988	**989**	3-octanone	55	119	114	2.2
13	994	**994**	2-pentylfuran	63	117	111	1.8
14	998	**996**	3-octanol	49	106	132	1.7
15	1040	**1041**	limonene	43	95	148	0.6
16	1067	1060	(E)-2-octenal	45	125	116	1.2
17	1110	**1111**	nonanal	27	124	130	0.9
18	1127	**1126**	octanoic acid methyl ester	71	116	106	0.2
19	1168	**1168**	(E)-2-nonenal	27	137	118	0.2
20	1212	**1212**	decanal	26	134	122	0.2
21	1334	**1334**	(E,E)-2,4-decadienal	17	159	103	0.4
			Sum of areas	1738	2168	2304	74.1
					Unidentified peaks		26.9

(a) relative to the average area (=100) of each compound for all sample runs
(b) linear retention index; bolded values for reference compounds, others from NIST database [17]
(c) using the DVB/Car/PDMS fiber, 10 g sample size and 45 minute extraction time