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EFFECTS OF EXTRACTION PH ON THE VOLATILE COMPOUNDS FROM PEA PROTEIN ISOLATE: SEMI-QUANTIFICATION METHOD USING HS-SPME-GC-MS

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Highlights:

- An external calibration was performed for compounds involved in “beany” off-flavor
- The method allowed determination of the global volatile compounds profile
- The method allowed semi-quantification in a pea protein isolate
- Acidic pH allowed the extraction of the volatiles initially bound to protein
- Variation coefficient of the HS-SPME technique was 15%, on average

Abstract

HS-SPME-GC-MS is widely used to characterize the profile of volatile compounds despite some bad uses with a lack of information on the precision and repeatability of this technique. This work proposes a method, including a calibration step, to determine the global volatile compounds profile of a pea protein isolate at different pH of extraction. At the same time, nine compounds of interest were semi-quantified: hexanal, nonanal, 2-nonenal, 3-methylbutanal, benzaldehyde, 1-octen-3-ol, 3-octen-2-one, 2-pentylfuran, and 2,5-dimethylpyrazine. The variation coefficient of the method for a single fiber was 15%. Semi-quantification was done by external calibration. The global volatile compounds profile was composed of 39 compounds including 13 aldehydes, 9 alcohols, 13 ketones, and 4 furans. The quantification of the nine compounds of interest at different extraction pHs showed the importance of pH for aroma release from pea protein isolates. For example, hexanal release was found 59% higher with extraction using pH 4.5 than with pH 6.5.

Keywords: volatile compounds, HS-SPME-GC-MS, pea protein isolate, semi-quantification, binding, beany off-flavour

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1 Introduction

With the increasing demand for vegetable protein-based products, more and more studies are conducted on the sensory aspects of these proteins, especially the leguminous ones (Bi et al., 2020; Trikusuma et al., 2020). Actually, leguminous proteins are often negatively described through their bitter, astringent, and vegetable taste (Cowan et al., 1973). Their flavor is described as “beany” or “pea pod”. These sensory attributes can be associated with “green”, “earthy”, “mushroom” attributes, as well as “hay” and “mold” attributes (Vara-Ubol et al., 2004). In regards to these sensory aspects, some consumers poorly accept the leguminous protein-based products (Youssef, 2017). In order to better the flavor of the products, the volatile compounds involved in the off-flavor must be known and quantified. Different studies were conducted toward the development of knowledge on the sensory characteristics of leguminous proteins and on the identification of some aroma compounds responsible for typical “beany” notes (Azarnia et al., 2011; Murat, 2013; Murray et al., 1968, 1976). However, this typical flavor has not been yet fully characterized.

Among the different possible analytical techniques to study the volatile fraction, Headspace Solid Phase Micro-Extraction (HS-SPME) and Gas Chromatography coupled with Mass Spectrophotometry (GC-MS) are now currently used for their convenience and rapidity. However, the lack of information on the repeatability and the difficulty to quantify the compounds with SPME are detrimental to the use of this technic, especially, when used to characterize volatile compounds of a specific matrix. Some recent works have investigated this technic with calibration steps to quantify the compounds (Song et al., 2020; X. Wang et al., 2019). Despite good results, the presented methods were complex, with several extraction steps to obtain all the desired information.

Therefore, the present study aimed to improve the use of SPME-GC-MS by allowing, in one extraction, to detect and identify as many volatile compounds as possible, and to quantify some compounds of interest. The developed method enable expressing the results with great precision. It was applied to a pea protein isolate (PPI) to define the profile in volatile compounds and to quantify target compounds.

Nine compounds responsible for the “beany” off-flavor were selected for semi-quantification in PPI: hexanal, nonanal, 2-nonenal, 3-methylbutanal, benzaldehyde, 1-octen-3-ol, 3-octen-2-one, 2-pentylfuran, and 2,5-dimethylpyrazine. These compounds were selected due to their reported involvement in the “beany” off-flavor (Murat, 2013; Ullrich & Grosch, 1987; Vara-Ubol et al., 2004; Zhang et al., 2020). Additionally, they were selected for their different formation pathways (Frankel, 1983; Gargouri et al., 2008; Marilley & Casey, 2004; Sessa, 1979; Snyder et al., 1988), and their different structures and chemical families. This last parameter was taken into account to determine which compounds were more suitable as calibration compounds.

The targeted compounds were extracted from a PPI, at different pHs. The study of the impact of pH variations on the volatile profile came as a complement to studies focused on the impact of different processes on the flavor (Lan et al., 2019; Zha et al., 2019). Only free compounds could be extracted at neutral pH when the proteins were dispersed in distilled water. Modifying the extraction pH was known to change protein structure and surface hydrophobicity. This could lead to the release or the retention of a given compound (Heng et al., 2004; K. Wang & Arntfield, 2014, 2016). In the present study, three pHs were selected based on data in the literature and on practicality (Heng et al., 2004; Murat, 2013; Youssef, 2017). PH 6.5 was selected as the neutral, non-denaturing pH, corresponding to the pH of the protein matrix; pH 4.5 as a partially denaturing pH, inducing the denaturation of some proteins (7S globulin precipitation) and pH 2.0 as a strong denaturing pH, inducing the denaturation of all the proteins (including 11S globulins).

2 Material and methods

2.1 HS-SPME-GC-MS Method

2.1.1 HS-SPME material

An SPME device containing a 1 cm fused-silica fiber coated with a 50/30 µm thickness of DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane) was used for HS-SPME extraction. This fiber was selected to ensure the best extraction of a diversity of volatile compounds (Azarnia et al., 2011; Barra et al., 2007; Murat, 2013; Singracha et al., 2017; Steenson et al., 2002). The fiber (24 Ga 50/30 µm, for manual holder, 3 pK, 57328-U) was purchased from Sigma and used

with a manual fiber holder. SPME vials (20 mL, VA201) and septum caps (18-mm caps, 8-mm PTFE/Silicon septum, SACA001) were purchased from JASCO France. The extractions were carried out in an electro-thermal magnetic stirrer with a water bath (MS-H-Pro⁺, DLAB) to ensure a homogeneous temperature and constant agitation for the sample and headspace. The fiber was conditioned before analysis by heating it in the gas chromatograph injection port at 270 °C for 30 min, according to the manufacturer's specifications.

2.1.2 HS-SPME extraction

SPME extraction comprises two steps: the equilibrium step, allowing the volatile compounds to pass through the different matrix phases to the headspace, and the extraction step when the fiber is exposed to the headspace and the compounds are adsorbed. As per the literature and preliminary trials, the two phases were conducted at 40 °C with agitation at 350 rpm in the dark. The equilibrium time was 30 min and the extraction time was 60 min (Azarnia et al., 2011; Barra et al., 2007; Blagden & Gilliland, 2006; Lee et al., 2013; Murat, 2013; Oomah et al., 2007; Sala et al., 2000; Schindler et al., 2012; Singracha et al., 2017; Steenson et al., 2002; K. Wang & Arntfield, 2014).

2.1.3 GC-MS

An HP 6890 Series Gas Chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with an HP 5973 Mass Selective Detector (Agilent Technologies, Palo Alto, CA) (Quadrupole) was used with a DB-WAX column (30m*0.32mm*0.25µm, 123-7032, Agilent, J&W Scientific USA) to analyze the compounds of interest (Murat, 2013; Schindler et al., 2011, 2012; Ullrich & Grosch, 1987). The SPME fiber was desorbed and maintained in the injection port at 250 °C for 5 min. The sample was injected in split mode, with a purge flow of 140 mL/min at 0 min to generate sharp, well-separated peaks on the chromatograph. Helium was used as a carrier gas at 1.4 mL/min with a linear velocity of 43 cm/s. The programmed temperature, selected from preliminary trials, was isothermal at 40 °C for 3 min, raised to 100 °C at a rate of 3 °C/min, and then raised to 230 °C at a rate of 5 °C/min and held for 10 min. The total run time was 59 min (Schindler et al., 2011, 2012). The ionization source and transfer line temperatures were set at 230 °C and 190 °C, respectively. The mass spectra were obtained using a mass selective detector with an electron impact voltage of 70 eV in full scan over the range

m/z 29 to 400. Compounds were identified by comparing their mass spectra with NIST 08 (National Institute of Standards and Technology), Wiley, and INRA libraries, with an integration limit of 50 000 in peak area, allowing the best peak identification. The mass spectra and retention times of the nine compounds of interest were compared with standards.

2.2 Calibration method

2.2.1 Standards

The following standards (selected volatile compounds) were purchased from Sigma-Aldrich: hexanal (98% purity, CAS 66-25-1, ref: 15606-100mL), nonanal (> 98%, CAS 124-19-6, 8.07166.0050), trans-2-nonenal (97%, CAS 18829-56-6, p 2426096), 3-methylbutanal (97%, CAS 590-86-3, 146455-100mL), 1-octen-3-ol (98%, CAS 3391-86-4, 313480/1), 3-octen-2-one (98%, CAS 1669-44-9, W341606-sampleK), 2-pentylfuran (98%, CAS 3777-69-3, W331708-sampleK), benzaldehyde (99%, CAS 100-52-7, B1334-250mL), and 2,5-dimethylpyrazine (98%, CAS 123-32-0, W327204-100g).

2.2.2 Calibration

An experimental plan may require the use of different fibers. Then, it is necessary to calibrate and determine the repeatability of the method and of the different fibers before use. This calibration step provides also the repeatability among the different fibers.

The calibration solution contained 50 ppm of each of the 9 studied compounds: hexanal, nonanal, trans-2-nonenal, 3-methylbutanal, 1-octen-3-ol, 3-octen-2-one, 2-pentylfuran, benzaldehyde, and 2,5-dimethylpyrazine. It was prepared with about 50 mg (exactly weighted) of each compound dissolved in 1 L of distilled water. The solution was covered with aluminum foil and left overnight under homogenization to ensure complete solubilization of the compounds.

A volume of 20 μ L of the calibration solution was added to the extraction vial and diluted with distilled water to obtain a final volume of 2 mL and a final concentration of 0.5 ppm (0.5 mg/L) in the vial. HS-SPME extraction was conducted in triplicate in the same day. The results were compared and the coefficient of variation, in percent, was calculated using equation 1. Two fibers were calibrated with this method, using the same solution, with one-month interval.

$$\text{Eq. 1 : coefficient of variation for hexanal (\%)} = \frac{\sigma_{Ah}}{m_{Ah}} \times 100$$

m_{Ah} = Mean Area of hexanal

σ_{Ah} = Standard deviation Area of hexanal

The service life of the extraction fiber was monitored over time, along the experiment. The fiber deterioration led to the detection of increasing amounts siloxane (a compound originating from the fiber). The amount of siloxane was tracked over a hundred of extractions, with the exposure of the fiber alone in the GC injector (injection of the fiber with no sample extraction). Siloxanes can also appear from column or septum-injector bleeding or septum-vial bleeding. These sources of siloxanes were deemed negligible as no siloxanes were retrieved in column blanks, showing no bleeding from the column or injector septum.

2.3 Semi-quantification method

A quantification method was developed to quantify the compounds of interest, with the same standard solution as described in 2.2.1. Genetally speaking, quantification can be achieved with an internal standard or an external calibration. Naphthalene D8 (98% purity, CAS 1146-65-2, ref: 176044 Sigma) was first used as an internal standard, but it gave poor repeatability. The correlation with concentration varied for each concentration and each compound. Due to these irrelevant results, an external calibration was chosen. The calibration curves of each nine compounds of interest were obtained for concentrations ranging from 0.001 to 2.5 ppm, in distilled water. Each compound was analyzed in the presence of the others in order to take into account the potential competition between compounds in the headspace. For two compounds, hexanal and 3-methylbutanal, the calibration curves were no longer linear above a given concentration. Consequently, the two compounds were run separately to obtain the calibration curves. Limit of detection (LOD) in ppm, limit of quantification (LOQ) in ppm, and linearity were also determined for each compound.

2.4 Application on a pea protein isolate

2.4.1 Sample and preparation

Spray-dried pea protein isolate (85% protein dry matter, composed mainly of globulins) was supplied by Roquette Frères S.A. A 0.2 g PPI sample was weighed directly in a clear 20 mL vial. Liquid was added to obtain a 2 mL suspension at 10% (w/v) and a liquid/gas ratio of 2/18 (v/v). These conditions allowed the complete dispersion of the product under constant agitation without leading to the saturation of the headspace with the aroma compounds (Steenso et al., 2002). Distilled water was used for extraction at neutral pH (6.5), 0.1 M HCl for extraction at pH 4.5, and 1 M HCl (CAS 76647-01-0, Chem-Lab, Belgium) for extraction at pH 2.0. The samples were analyzed in triplicate for each pH.

2.5 Statistical analysis

The statistical treatment of semi-quantification data was performed using the software Minitab 18 (Minitab, LLC., USA). One-way analysis of variance (ANOVA) was applied for all the compounds after validating the feasibility of the test using variance analysis. Significance was established at $p < 0.05$. ANOVA showing significant differences lead to the use of Tukey's multiple comparison test to group the samples.

3 Results and discussion

3.1 Calibration method, repeatability, and fiber service life

Information on the studied volatile compounds and results of the calibration were presented in Table 1. The manufacturer of fibers (Supelco®, 2020) reported 6.1 to 18.2% variation for intralot variability for fused silica fibers, depending on fiber coating. In accordance with these data, variation coefficients ranging from 0.73 to 17.67%, depending on volatile compounds and fibers were observed. The highest variation coefficient was observed for 2-pentylfuran analyzed using fiber 2. This high value could be attributed to the low water solubility of this compound, which was about 0.041 g/L in water (ChemSpider, 2020; Kim et al., 2019; PubChem, 2020). It could also be attributed to the aging of the calibration solution.

Additionally, the manufacturer (Supelco®, 2020) reported interlot variability from 9.3 to 19.7%. The variation coefficients observed between the two fibers, ranged from 6.2 to 67.9% (table 1). Nevertheless, six compounds among nine showed a variation coefficient lower than the highest value reported by the manufacturer. Nonanal, 2-nonenal, and 2-pentylfuran showed great variation coefficients between the two fibers and were the most unstable compounds. In fact, as reported by the manufacturer, for each fiber, the variation coefficient depended on the compound, with some compounds showing better repeatability than others (Supelco®, 2005).

Variation observed among compounds could be linked to chemical class, solubility and stability over time. For example, alcohols, less prone to chemical changes than other chemical species (Fisher & Scott, 1997; Weerawatanakorn et al., 2015), are generally showing greater repeatability than other compounds. Indeed, depending on their chemical family and structures, flavor compounds can be more susceptible to chemical changes. The presence of active functional groups is a determinant factor. In the study, for intra fiber repeatability, medium-chain aldehydes hexanal and nonanal showed the best repeatability, followed by 1-octen-3-ol and 2,5-dimethylpyrazine. For over time repeatability, 3-methylbutanal and benzaldehyde showed the best stability, and so repeatability, followed by 1-octen-3-ol and 3-octen-2-one. On the contrary, for both aspects, furans and 2-nonenal showed higher variability and should be avoided for calibration purpose.

Another important aspect for analysis was the service life of the SPME fibers. According to the manufacturer, approximately 50 extractions can be performed with an extraction fiber (Sigma Aldrich, 2004). However, the service life depends on the type of extraction, the sample, and the extraction and desorption conditions. A greater number of samples can be handled with softer conditions. To check this, the global amount of siloxane derivate released by the fiber was monitored over 100 extractions and these results were presented in figure 1. No siloxane was detected in the first fiber blank. After few extractions, a small amount of siloxane was already released by the fiber. The amount of siloxane strongly increased after 50 and at 100 extractions, showing an impact of the number of extractions on the integrity of the fiber. The first increase in siloxane after 50 extractions matched with the recommendations of the manufacturer. The increase at 100 extractions showed a decrease in the

precision of the fiber. By monitoring the amount of siloxane in the fiber blanks, it was possible to monitor the service life of the fiber for a particular protocol and sample. The moment the fiber reached its maximal number of precise extractions was also determined. The fiber could also be checked visually, as the darkening of the fiber was correlated with its shelf life.

Good practices to ensure as good precision as possible for the HS-SPME technic are wrapped up in the following lines:

- Each fiber repeatability should be determined prior to use, with a calibration solution containing compounds chosen to be consistent with the analysis to be handled.
- The volatile compounds selected for the calibration solution should be stable compounds, with suitable solubility.
- During the analysis, the fiber service life should be monitored, with a limit of use around 100 extractions.
- Even if it is possible to compare data coming from different fibers by taking into account the variation coefficient between fibers, it is advised to carry out a same experimental run on the same fiber.

3.2 Calibration of the compounds of interest

The results of the external calibration were presented in table 2. The external calibration provided good results as R^2 was above 0.99 for all the calibration curves. The LOD 0, limit of detection with no area integration limit, was less than 0.001 ppm for the majority of compounds. The LOD 50k, limit of detection with a 50 000 area integration limit, depends on the compound, ranging from 0.01 ppm for benzaldehyde to 0.001 ppm for some compounds like hexanal. The LOQ, limit of quantification, also depends on the compound, ranging from 0.05 to 0.005 ppm. From the LOQ, it was concluded that the semi-quantification followed a linear law until the maximum tested concentration except for 1-octen-3-ol. This allowed to semi-quantify the compounds of interest over a satisfying range, corresponding to the concentration of aroma compounds in food products. If the aroma content of the product exceeded the linearity limit, the amount of analyzed sample should be adjusted to be within the linearity range. This method was thus chosen for the semi-quantification of the compounds of interest

in the pea protein isolate. However, this method did not take into account the extraction yield, as internal calibration would have done, and was thus called a semi-quantification method.

3.3 Application of the method on a pea protein isolate

The application of the method on PPI had three different objectives. The method should allow in one extraction the definition of the qualitative profile in volatile compounds and the semi-quantification of compounds of interest. The method should also allow studying the impact of pH on the extraction of volatile compounds.

3.3.1 Volatile compounds profile

The profile of volatile compounds in PPI was presented in table 3. Only the volatile compounds reported as odorant in the literature were kept in the reported profile. Overall, 39 compounds were identified, among which 13 aldehydes, 9 alcohols, 13 ketones and 4 furans. Except for six compounds among 39, the different volatile compounds identified in this study were already reported in pea protein or close leguminous matrices by other authors (table 3). These six newly found compounds were retrieved only at acidic pH extraction. This might explain the absence of these six compounds in other studies for which analyses were generally made at neutral pH. Moreover, except 2,5-dimethylpyrazine, the compounds used for calibration were found in PPI and were relevant for the analysis of pea protein extract.

3.3.2 Semi-quantification of the compounds of interest

Using the calibration curves, the nine compounds of interest were semi-quantified in two different PPI samples at three different extraction pHs. The amount of each compound was calculated as in the following example with hexanal. Semi-quantification steps were as following:

(1) Area Hexanal = 7 078 843 A.U.

(2) [Hexanal]_{in the assay} (µg/mL) = (Area Hexanal – b)/a = (7 078 843 – 157 037) / 1 * 10⁷ = 0.69 µg/mL

(3) [Hexanal]_{in the sample} (µg/g) = ([Hexanal]_{in the assay} (µg/mL) * V_{solution} (mL)) / m_{sample} (g)
= (0.69 * 2) / 0.2041 = 6.78 µg/g

(4) $m_{[\text{Hexanal}] \text{ in the sample (n=3)}} = 6.7 \pm 0.2 \mu\text{g of hexanal / g of pea protein isolate}$

Results were presented in Table 4. Due to the lack of quantitative SPME extraction data in the literature, it was not possible to compare the obtained data with others. Eight of the nine compounds selected in this study were found at least in one sample, showing their importance as compound of interest. The only compound not detected in the two samples was the 2,5-dimethylpyrazine, and no other pyrazine derivate compound was detected. However, this pyrazine was kept as a compound of interest, as a representative of the pyrazine family. In fact, many studies are dealing with the importance of pyrazine compounds in leguminous proteins (Murray et al., 1976; Vara-Ubol et al., 2004). In PPI1, 3-methylbutanal was not detected and 3-octen-2-one was not quantified. It could be hypothesized that the two samples of PPI do not have a similar age. Indeed, the 3-methylbutanal is coming from the leucine degradation, which is a slower pathway than lipid oxidation (Marilley & Casey, 2004; Rodríguez-Bernaldo De Quirós et al., 2000). Additionally, it could be noticed that the compounds giving poor repeatability results with calibration solution (2-nonenal, nonanal and 2-pentylfuran) were giving acceptable repeatability when analyzed in the PPI samples.

3.3.3 Impact of extraction pH

Table 3 and table 4 reported the impact of the extraction pH for each compound. To discuss this part of the results, it is important to keep in mind some characteristics of the studied samples. The PPI studied here was composed almost exclusively of globulins. As a reminder, pea globulins are the 7S vicilin and convicilin, trimer proteins and the 11S legumin, composed of six sub-units (Heng et al., 2004; Murat, 2013; Youssef, 2017). The 7S vicilin is made up of 50 kilodaltons (kDa) subunits, with a molecular mass of 150-200 kDa and an isoelectric point at pH 4-5. The 7S convicilin is similar, with a molecular mass around 280 kDa. The 11S legumin has a molecular mass of 360-400 kDa and maintain its structure at pH from 7 to 9 but dissociates at extreme pHs like 2 or 12. For each compound, at least one of the acidic extractions (pH 4.5 or 2.0) led to a higher quantity of compound than the extraction at neutral pH. The impact of the extraction pH was detailed for some chemical classes of compounds in the following lines.

Aldehydes were reported as most commonly bound to proteins. Aldehydes bind to proteins two to five times more than ketones due to two possible binding mechanisms (hydrophobic interaction and covalent binding) compared to only one for ketones (hydrophobic interaction) (K. Wang & Arntfield, 2014, 2016). For the aldehydes, two cases were observed in the study: some aldehydes were detected with extraction at neutral pH and others were detected only with extraction at acidic pH.

For the compounds already detected at neutral pH (hexanal, nonanal, and benzaldehyde), their amount increased significantly at pH 4.5, and a slight decrease was observed at pH 2. These results showed that part of the compounds was initially bound to the protein. At pH 4.5, it could be hypothesized that proteins in the PPI were partially denatured leading to the release of the compounds. This phenomenon should be increased at a more acidic pH. However, Heng et al. (2004) reported that aldehydes were strongly bound to vicilin at pH 7.6 and 3.8 and legumin only at pH 7.6. Thus with the different pH, selective retention or release could take place due to the different proteins in the isolate. At pH 2.0, the protein should be strongly denatured, the loss of structure and the opening of the protein uncovering more binding sites.

The second case was observed with 2-nonenal, which was only detected at acidic pH. Wang & Arntfield, (2014) and Heng et al. (2004) reported that the retention of an aldehyde by a protein increased significantly with the number of carbons of the aldehyde. Thus, as the structure of 2-nonenal was more complex than that of the other studied aldehydes, it could be more retained by the protein and, when present in small amounts in the matrix, could not be detected with an extraction at neutral pH.

These two observations were in concordance with the literature: the change in conformational states of a protein was linked to the change of flavor-binding capacity (Guichard, 2002; Guichard & Langourieux, 2000). It was previously reported that an increase in pH from 3 to 9 led to an increase of flavor compounds retention, explained by a better access to primary and secondary binding sites. This phenomenon was investigated for soy protein isolate (SPI). As pea legumin and vicilin showed great similarities with soy glycinin and β -conglycin (Fischer et al., 2020; Heng et al., 2004), the information available about soy protein might thus be relevant. Guo et al. (2019) reported that at acidic pH (pH

3.0), the SPI became compacted and the interface between subunits tightened. This was leading to the disappearance of the internal binding sites of the protein, causing a decrease in the binding capacity. The acidic denaturation of the protein led to the decrease of the binding capacity, with a weaker binding at pH 3.0 than at pH 6.0 and above. As observed, a diminution of the pH was thus linked to a release of the compounds, also depending on the compound structure and hydrophobicity.

Ketones were known to bind to the proteins via hydrophobic interactions and the pH should have an impact on the release of ketones (K. Wang & Arntfield, 2014, 2016). In the present study, the release of 3-octen-2-one appeared at pH 2.0. However, this impact was observed only at the lowest pH and for one sample. It could be hypothesized that the proteins had already been partially denatured during the manufacturing of the PPI (Youssef, 2017), and that the majority of the ketones had already been released from the protein. The remaining bound part could be released only at pH 2.0. Indeed, the ketones bind much less to the proteins than aldehydes. Heng et al. (2004) reported that vicilin only bound 1 to 17% of the ketones present in the medium (depending on the ketone studied) compared to 75 to 88% for aldehydes. Moreover, they reported no binding for ketones with legumin. Due to the weaker binding capacities of ketones, a slight structural change of the protein could lead to their early release. All these explanations were summed up in a diagram presented in figure 2.

For the alcohol (1-octen-3-ol), a slight increase was observed at pH 4.5 for one sample. Alcohols might bind to the hydrophobic surface of the protein, by hydrophobic interactions (Guichard & Langourieux, 2000; Guo et al., 2019). Alcohol compounds have very few binding sites and low binding constants compared to other compounds, but are generally released at acidic pH (Guo et al., 2019). However, it has been reported that a small protein denaturation strongly decreases the binding of alcohol to the proteins (Heng et al., 2004). As for the ketones, if the protein already underwent small structural changes, alcohols should have been released previously, explaining little impact of the pH on the alcohols in this study.

It was possible to determine the fraction of compounds that were free or bound in the matrix, as with the following example with hexanal in PPI1: 8.1 µg/g of sample of hexanal were found with the acidic extraction, whereas only 5.1 µg/g of sample were found at neutral pH (a 59% increase with pH). It

could be thus considered that 5.1 µg of hexanal/g of sample were initially free in the matrix and 3.0 µg/g of sample were bound to the matrix. Proteins did not much contribute directly to the flavor, but they influenced the perception of other components in food through the flavor compounds binding (Heng et al., 2004). It was thus important to know the amount of compound bound to the matrix as it represented an “aroma stock” inside the matrix. These compounds could be released later on, during the consumption of the product for example. This could also help to explain some drastic change of odor, as the increased odor of SPI when mixed with acidic juice (Guo et al., 2019).

4 Conclusions

First, the calibration step allowed the validation of the HS-SPME-GC-MS protocol by determining its repeatability (including fiber, protocol and variations due to volatile release), which was about 15%. Variations were observed according to the studied compound. The calibration step was rapid and could be used routinely to determine the repeatability of the protocol and to control the different extraction fibers during the experiment. To achieve the highest precision depending on the studied product, specific and representative compounds should be selected. By monitoring the amount of siloxane retrieved in fiber blanks, it was possible to survey the fiber service life and to determine the maximum number of extractions for a given protocol and food matrix.

The method permit the definition of the global profile in volatile compounds of a PPI, with 39 compounds. At the same time, nine compounds of interest were semi-quantified using external calibration. These semi-quantifications, conducted at different pHs, put in evidence some bonding phenomena of the volatile compounds to the PPI. Some compounds were initially (partially or completely) bound to, or interacted, with the proteins. Different extractions at different pHs were necessary to obtain a complete view of the different compounds, their interaction with the food matrix, and to quantify them.

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Figure captions:

Figure 1: Evolution of the global amount of siloxane derivatives released by the fiber and fiber precision based on the number of extractions. Fiber precision was measured with the percent of error on a chosen compound (hexanal) in the sample.

Figure 2: Diagram explaining the binding of hexanal, 2-nonenal and 3-octen-2-one to the PPI at different pH (inspired by Guo et al. (2019)).

Figure 1

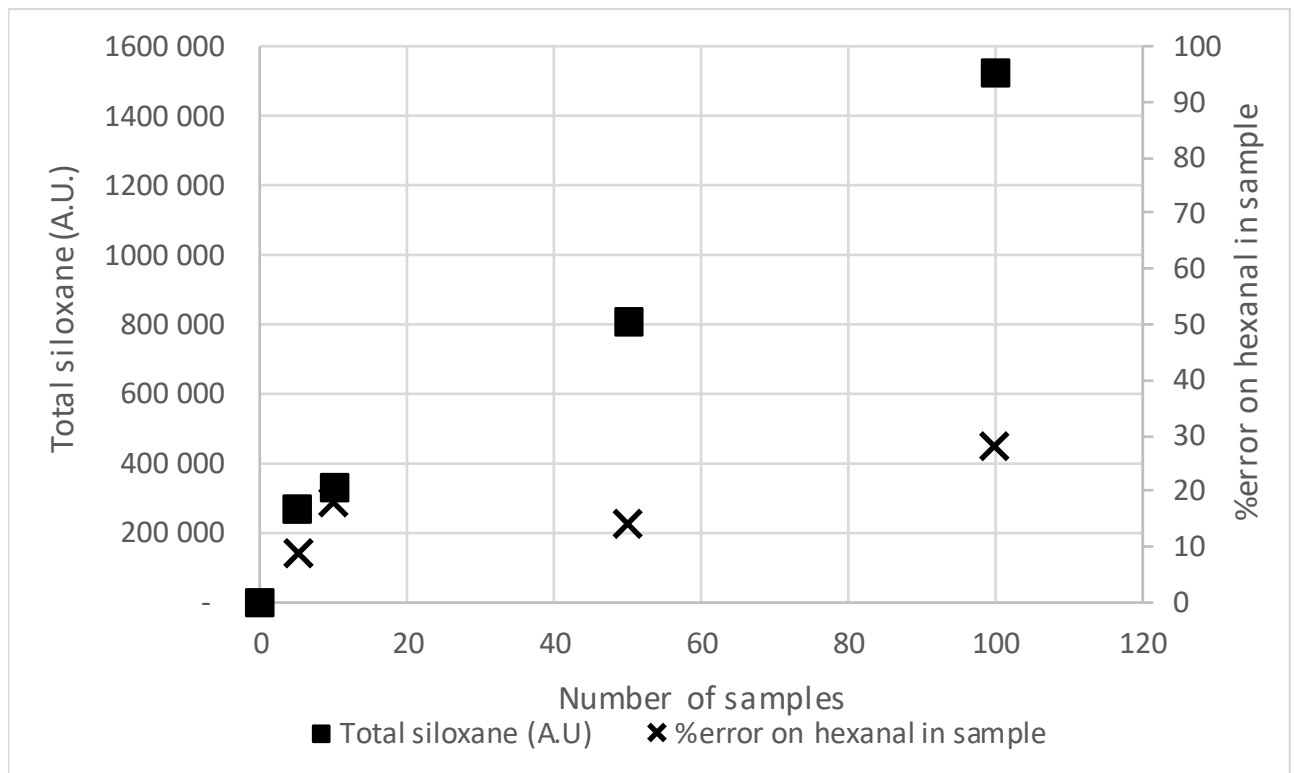


Figure 2

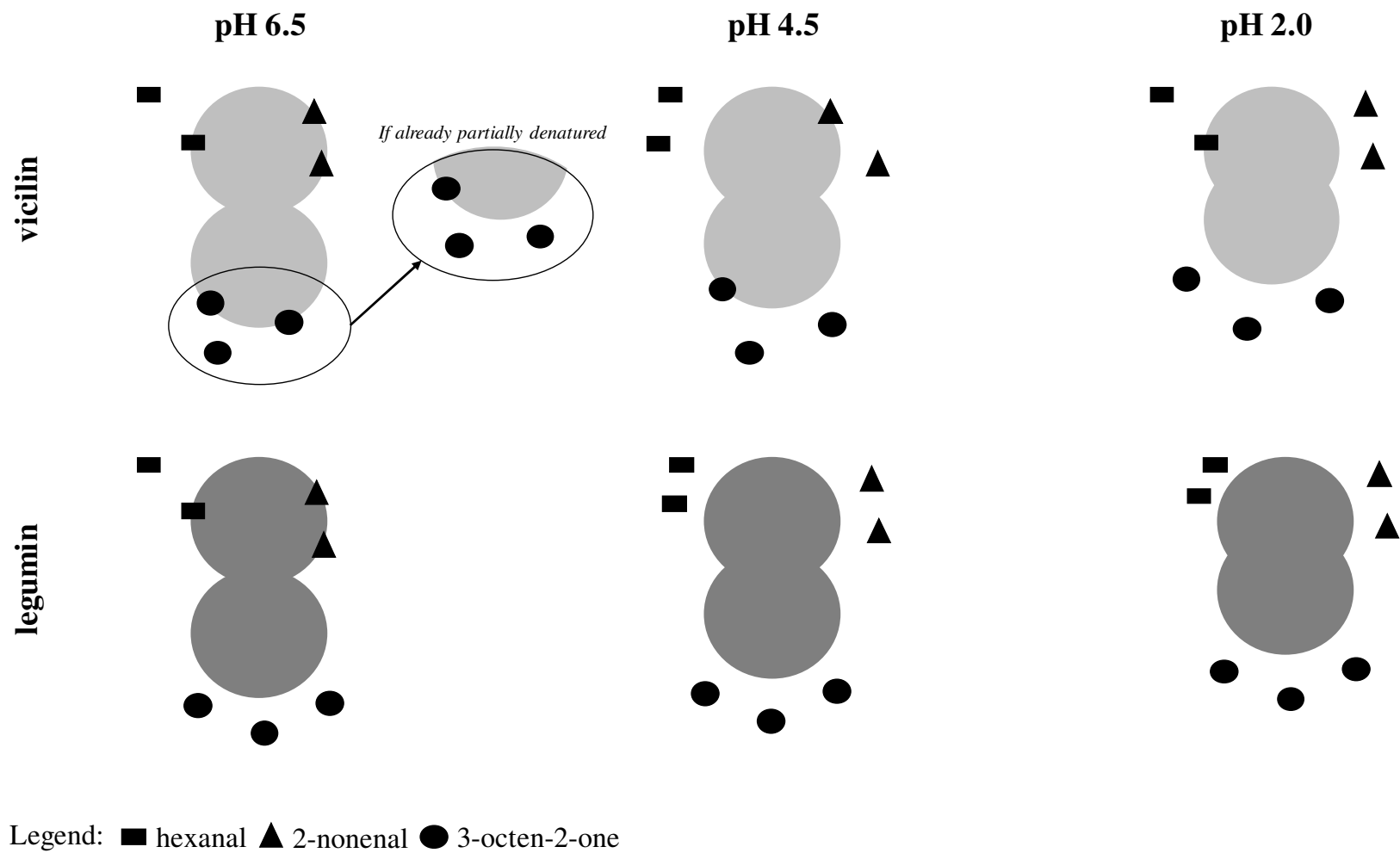


Table 1: Information and chromatographic data on the studied volatile compounds. Variation coefficient for the calibration method of the two SPME fibers, at 0.5 ppm of each compound (n=3).

Chemical family	Compound	CAS	Solubility (g/L) ²	Log P ³	Henry's constant (Pa.m ³ /mole) ⁴	Retention time (min)	Fiber 1		Fiber 2		Variation coefficient between two fibers (%)
							mArea (mean value of 3 samples)	Variation coefficient (%)	mArea (mean value of 3 samples)	Variation coefficient (%)	
Aldehyde	Hexanal	000066-25-1	3.520	1.80	2.13E+01	5.3	4 373 173	6.18	4 207 065	4.30	18.85
	Nonanal	000124-19-6	0.096	3.27	5.00E+01	17.0	9 002 917	2.84	3 846 293	3.71	40.5
	2-nonenal	018829-56-6	0.205	3.06	2.34E+01	22.5	4 774 558	12.80	2 866 753	8.79	28.25
	3-methylbutanal	000590-86-3	11.230	1.23	1.61E+01	2.4	884 178	14.80	806 879	3.53	6.18
	Benzaldehyde	000100-52-7	6.100	1.71	1.36E+00	21.8	4 262 010	14.71	3 512 612	1.28	11.14
Alcohol	1-octen-3-ol	003391-86-4	1.840	2.60	2.34E+00	19.8	9 121 866	10.45	7 425 840	1.11	13.15
Ketone	3-octen-2-one	018402-82-9	1.050	2.29	9.71E+00	17.5	8 515 285	14.01	6 493 417	0.73	16.79
Furan	2-pentylfuran	003777-69-3	0.041	3.87	1.87E+03	10.4	2 340 873	7.64	108 573	17.67	67.9
Pyrazine	2,5-dimethylpyrazine	000123-32-0	31.970	1.03	3.60E-01	13.7	712 528	10.44	652 742	1.50	6.19

² (ChemSpider, 2020; Kim et al., 2019; PubChem, 2020)

³ Calculated by EPI suite 4.1, Log Kow

⁴ Calculated by EPI suite 4.1, Bond Method

Table 2: Calibration curves, LOD, LOQ, linearity of the compounds studied.

Compound	LOD 0 (ppm)	LOD 50k (ppm)	LOQ (ppm)	Linearity (ppm)	Equation	R ²
Hexanal	< 0.001	< 0.001	0.05	0.05 – 2.5	$y = 1 \cdot 10^7 x + 157\,037$	0.9952
Nonanal	< 0.001	< 0.001	0.01	0.01 – 2.5	$y = 1 \cdot 10^7 x + 123\,911$	0.9942
2-nonenal	< 0.001	< 0.001	0.05	0.05 – 2.5	$y = 1 \cdot 10^7 x - 186\,605$	0.9925
3-methylbutanal	< 0.001	0.01	0.05	0.05 – 2.5	$y = 2 \cdot 10^6 x + 1 \cdot 10^6$	0.9921
Benzaldehyde	0.01	0.01	0.01	0.01 – 2.5	$y = 7 \cdot 10^6 x + 17\,925$	0.9905
1-octen-3-ol	< 0.001	0.005	0.005	0.005 – 1.0	$y = 2 \cdot 10^7 x + 227\,470$	0.9905
3-octen-2-one	< 0.001	0.005	0.005	0.005 – 2.0	$y = 2 \cdot 10^7 x + 531\,187$	0.9950
2-pentylfuran	< 0.001	0.01	0.01	0.01 – 2.5	$y = 3 \cdot 10^6 x - 87\,598$	0.9931
2,5-dimethylpyrazine	0.005	0.05	0.05	0.05 – 2.0	$y = 1 \cdot 10^6 x + 14\,185$	0.9942

LOD : limit of detection, lowest concentration when the compound was detected ; LOD 0: detection of the compounds with no area limit for integration ; LOD 50k: detection of the compound above the integration limit of 50 000, giving an identification quality > 85%; LOQ : limit of semi-quantification, lowest concentration for which the quantity of compound could be calculated within the range of linearity ; Linearity : range of calibration curves where the quantity of compound could be calculated.

Table 3: Profile of volatile compounds for the studied pea protein isolate at different pHs (mean of three repetitions \pm standard deviation).

Chemical family	Compound	CAS	tr (min)	LRI	m_{area}/g of sample			compound already reported in literature
					pH 6.5	pH 4.5	pH 2	
Aldehyde	3-methylbutanal	000590-86-3	2.4	/	n.d.	249 564	272 912 \pm 31 885	1, 2, 7, 8, 9, 10
	Pentanal	000110-62-3	3.1	/	1 805 405 \pm 92 876 ^b	3 969 920 \pm 410 505 ^a	3 871 487 \pm 1 176 475 ^a	1, 2, 4, 5, 6, 7, 9, 10
	Hexanal	000066-25-1	5.3	1078	26 205 328 \pm 752 509^c	41 138 859 \pm 2 634 607^a	34 116 982 \pm 1 122 927^b	All
	2-Hexenal	000505-57-7	9.6	1213	345 986 \pm 9 894 ^b	736 319 \pm 88 963 ^a	296 021 \pm 24 358 ^b	2, 3, 4, 5, 6, 9, 10, 11
	Heptanal	000111-71-7	8.6	1185	1 131 897 \pm 84 028 ^b	2 546 586 \pm 355 942 ^a	2 153 028	2, 3, 4, 5, 6, 9, 10
	2-Heptenal, (E)-	018829-55-5	14.0	1325	357 735 \pm 30 035 ^c	2 006 121 \pm 247 892 ^a	1 510 653 \pm 95 130 ^b	1, 2, 3, 5, 6, 10
	Octanal	000124-13-0	12.7	1293	1 121 496 \pm 70 638 ^b	2 780 324 \pm 343 593 ^a	990 280 \pm 860 436 ^b	3, 5, 6, 8, 9, 10, 11
	2-Octenal, (E)-	002548-87-0	18.3	1432	n.d.	1 404 734 \pm 180 826 ^b	2 175 206 \pm 72 577 ^a	1, 3, 4, 5, 10, 11
	Nonanal	000124-19-6	17.0	1400	5 465 053 \pm 412 073^{ab}	8 175 080 \pm 1 137 161^a	3 275 647 \pm 1 507 864^b	2, 3, 4, 5, 6, 8, 9, 10, 11
	2-Nonenal, (E)-	018829-56-6	22.5	1539	n.d.	501 542 \pm 83 258^b	680 233 \pm 66 018^a	5, 6, 10
	Decanal	000112-31-2	21.3	1507	n.d.	425 076 \pm 77 308	274 347	2, 4, 6, 8, 9, 11
	2-Decenal, (E)-	003913-81-3	26.3	1650	n.d.	n.d.	534 212 \pm 19 500	10, 11
	Benzaldehyde	000100-52-7	21.8	1521	1 511 999 \pm 100 318^b	2 264 672 \pm 331 285^a	1 873 085 \pm 84 585^{ab}	2, 4, 5, 6, 8, 9, 10
	Alcohol	Ethanol	000064-17-5	2.6	/	258 605 \pm 8 888	n.d.	332 070 \pm 84 559
1-Pentanol		000071-41-0	11.5	1262	502 134 \pm 60 348 ^b	674 143 \pm 79 569 ^a	562 929 \pm 38 614 ^{ab}	2, 3, 4, 5, 6, 7, 9, 10
1-Hexanol		000111-27-3	15.8	1370	2 364 526 \pm 55 306 ^{ab}	2 654 065 \pm 204 139 ^a	2 112 372 \pm 90 927 ^b	2, 3, 4, 5, 6, 7, 9, 10, 11
2-Ethyl-1-hexanol,		000104-76-7	21.4	1510	n.d.	1 425 349 \pm 132 803 ^b	2 124 221 \pm 216 848 ^a	2, 6, 8, 10
3-Ethyl-3-hexanol		000597-76-2	20.2	1479	n.d.	322 006 \pm 30 072	n.d.	X
3,4-Dimethyl-3-hexanol		019550-08-4	13.3	1308	n.d.	935 402 \pm 134 020	n.d.	X
1-Heptanol		000111-70-6	20.0	1475	248 472 \pm 9 704 ^a	n.d.	385 807 \pm 105 604 ^a	1, 3, 4, 5, 7, 10
1-Octanol		000111-87-5	24.0	1579	466 763 \pm 35 326 ^a	476 150 \pm 53 645 ^a	584 164 \pm 51 390 ^a	1, 2, 3, 4, 5, 6, 10, 11
1-Octen-3-ol		003391-86-4	19.8	1470	1 031 787 \pm 65 657^b	1 453 934 \pm 149 255^a	1 134 591 \pm 130 594^b	2, 3, 4, 5, 6, 8, 9, 10, 11
Acetone		000067-64-1	1.7	/	341 103 \pm 9 898 ^a	436 096 \pm 90 209 ^a	460 636 \pm 183 006 ^a	5, 6, 9
Ketone	2-Heptanone	000110-43-0	8.5	1182	2 914 251 \pm 719 581 ^a	3 384 973 \pm 1 279 843 ^a	2 398 964 \pm 168 258 ^a	4, 9, 10
	1-Hepten-3-one	002918-13-0	13.0	1301	n.d.	861 709 \pm 178 335	n.d.	X
	3-Methyl-2-heptanone	002371-19-9	2.2	/	n.d.	327 462	n.d.	4
	6-Methyl-5-hepten-2-one	000110-93-0	14.7	1343	282 010 \pm 13 561	293 682	275 776 \pm 21 363	3, 6, 8, 10
	2-Octanone	000111-13-7	12.5	1288	363 899 \pm 14 977 ^b	393 851 \pm 39 817 ^b	475 644 \pm 13 113 ^a	4, 5, 9, 10
	3-Octanone	000106-68-3	11.2	1254	n.d.	262 761	296 389	2, 3, 4, 5, 8, 9
	3-Octen-2-one	018402-82-9	17.5	1412	609 220 \pm 174 395^a	932 755 \pm 130 495^a	692 621 \pm 103 697^a	4, 5, 10
	3,5-Octadien-2-one	030086-02-3	23.9	1576	1 695 929 \pm 164 026 ^a	1 353 879 \pm 228 926 ^{ab}	1 083 916 \pm 31 733 ^b	4, 5, 6, 9, 10
	2,3-Octanedione	000585-25-1	14.4	1335	796 320 \pm 64 074 ^b	1 100 100 \pm 111 882 ^a	1 162 139 \pm 33 550 ^a	4, 10
	2-Nonanone	000821-55-6	16.9	1397	715 439 \pm 71 852 ^a	603 685 \pm 78 975 ^a	611 501 \pm 13 248 ^a	5, 10

Furan	2-Decanone	000693-54-9	21.1	1502	476 536 ± 76 890 ^a	363 298 ± 42 518 ^a	381 605 ± 3 068 ^a	4, 10
	1-Decen-3-one	056606-79-2	13.0	1301	n.d.	937 292	n.d.	X
	2-Ethylfuran	003208-16-0	2.8	/	711 604 ± 41 780 ^b	860 919 ± 99 991 ^b	2 030 27 ± 88 2250 ^a	1, 2, 6
	2-n-Butyl furan	004466-24-4	6.7	1127	n.d.	329 426 ± 73 257 ^b	628 836 ± 5 984 ^a	X
	2-Pentylfuran	003777-69-3	10.4	1234	14 612 684 ± 1 569 213^b	13 131 839 ± 1 809 401^b	21 303 106 ± 2 213 382^a	2, 3, 9, 10, 11
	cis-2-(2-Pentenyl)furan and trans-2-	070424-13-4	13.2	1306	n.d.	n.d.	739 349 ± 235 952	X

In **bold**: semi-quantified compounds; X = not reported in the literature presented; When no standard deviation was available, the compound was retrieved only in one of the three repetitions; ^{a,b,c} : statistical groups for each compound of each protein extract; LRI = Linear Retention Indices (it was not possible to calculate LRI for compound with a retention time below 3 min due to solvent delay).

Literature: 1: (Azarnia et al., 2011) ; field pea ; 2: (Barra et al., 2007) bean (*Phaseolus vulgaris*) ; 3: (Jakobsen et al., 1998) green peas ; 4: (Murat, 2013) Pea flour and isolate ; 5: (Murray et al., 1976) green peas and pea shells ; 6: (Oomah et al., 2007) bean ; 7: (Ralls et al., 1965) green peas ; 8: (Rodríguez-Bernaldo De Quirós et al., 2000) green beans ; 9: (del Rosario et al., 1984) winged beans and soybeans ; 10: (Schindler et al., 2012) peas ; 11: (Zhang et al., 2020).

Table 4: Semi-quantification of the compounds of interest in two pea protein isolates at different pH (mean of three repetition \pm standard deviation, in μg of compound/g of sample).

Compound	PPI 1			PPI 2		
	pH			pH		
	6.5	4.5	2.0	6.5	4.5	2.0
Hexanal	5.1 ± 0.1^c	8.1 ± 0.5^a	6.7 ± 0.2^b	3.4 ± 0.1^c	5.6 ± 0.4^b	7.3 ± 0.4^a
Nonanal	0.97 ± 0.08^{ab}	1.5 ± 0.2^a	0.6 ± 0.3^b	0.67 ± 0.07^a	0.8 ± 0.2^a	0.6 ± 0.4^a
2-nonenal	n.d.	0.26 ± 0.06^a	0.313 ± 0.008^a	n.d.	0.28 ± 0.02^b	0.56 ± 0.09^a
3-methylbutanal	n.d.	n.q.	n.q.	4.6 ± 0.4^a	5.7 ± 0.8^a	2 ± 1^b
Benzaldehyde	0.41 ± 0.03^b	0.62 ± 0.09^a	0.51 ± 0.02^{ab}	1.6 ± 0.1^b	1.5 ± 0.1^b	2.8 ± 0.3^a
1-octen-3-ol	0.07 ± 0.01^b	0.12 ± 0.01^a	0.08 ± 0.01^b	0.20 ± 0.02^a	0.29 ± 0.06^a	0.27 ± 0.03^a
3-octen-2-one	n.q.	n.q.	n.q.	0.21 ± 0.02^b	0.39 ± 0.09^b	0.7 ± 0.2^a
2-pentylfuran	10 ± 1^b	9 ± 1^b	14 ± 1^a	12.0 ± 0.8^a	11.5 ± 0.7^a	> uloq
2,5-dimethylpyrazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

^{a,b,c} : statistical groups for each compound of each protein extract; n.d. = not detected; n.q. = not quantified; > uloq = above upper limit of quantification.

HS-SPME GC-MS

