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Comparison of volatile trapping techniques for the comprehensive analysis of food flavourings by Gas Chromatography-Mass Spectrometry

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Trapping volatiles is a convenient way to study aroma compounds but it is important to determine which volatile trapping method is most comprehensive in extracting the most relevant aroma components when investigating complex food products. Awareness of their limitations is also crucial. (Un)targeted metabolomic approaches were used to determine the volatile profiles of two commercial flavourings. Four trapping techniques were tested as was the addition of salt to the mixture. Comprehensiveness and repeatability were compared and SBSE proved particularly suitable for extracting components such as polysulfides, pyrazines and terpene alcohols, and provided an overall broader chemical spectrum. SPME proved to be more suitable in extracting sesquiterpenes and DHS in extracting monoterpenes. Adding salt to the sample had only quantitative effects on volatiles as detected by SPME. These results help clarify the advantages and limitations of different trapping techniques and hence deliver a valuable decision tool for food matrix analysis.

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1. Introduction

Volatile and semi-volatile compounds play a central role in food quality as they are often the fragrant or bioactive compounds that are primarily important in imparting positive sensory attributes. However, such components can also be instrumental in imparting negative sensory attributes through being so-called, ‘off-flavours’. Off-flavours (and taints) are undesirable sensory notes often arising as a result of incorrect preparation methods, natural product degradation or the use of incorrect storage procedures [1]. For many years the more standard techniques used for extracting volatiles and semi-volatiles involved some kind of liquid-liquid extraction. However, a recent trend is a progression towards simplification, miniaturization and minimization of organic (toxic) solvents used in order to be more sustainable and also reduce waste [2,3]. There are now several sorptive-based methods which are faster and avoid the use of organic solvents for the analysis of volatile compounds [4]. These methods commonly use ab- and adsorptive materials in which the volatiles are collected either in or above (headspace) a (liquid) food matrix. Headspace extraction (e.g. SPME: Solid-phase micro extraction; DHS: Dynamic headspace; HSSE: Headspace sorptive extraction) or in-liquid extraction (e.g. SBSE: Stir bar sorptive extraction) are the most popular among all the techniques proposed in recent years [5].

Fig. 1 shows a schematic set up of the four trapping techniques used in this study. Stir bar sorptive extraction in solution (SBSE) and in headspace (HSSE), are based on the trapping of volatiles onto a polymer coated on a magnetic stir bar [6]. Both SBSE and HSSE are techniques that were developed 20 years ago and have shown great capacity for the static sorptive extraction at (ultra-)trace levels of non-polar to medium-polar solutes with volatile to semi-volatile characteristics in complex food systems [5]. The stir bar can be either placed in the liquid sample or in the so called headspace above (Fig. 1). The latter requires that the volatiles are

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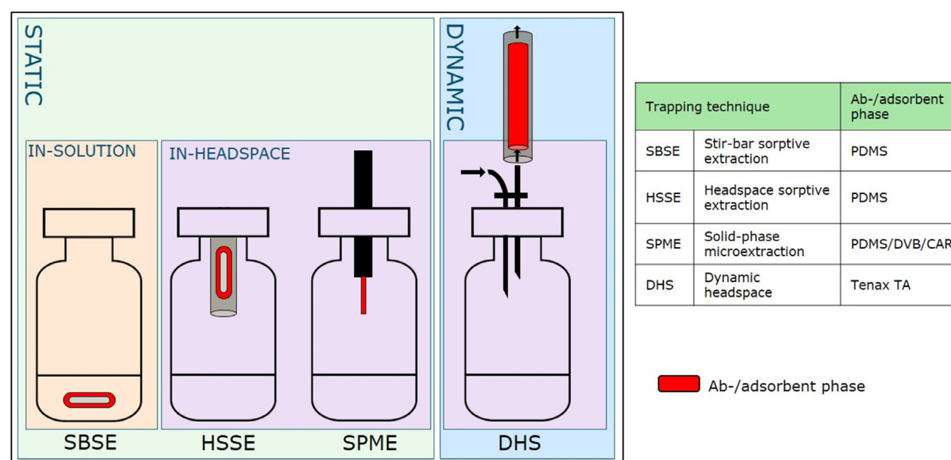

Fig. 1. A schematic representation of the four volatile trapping techniques used in this study. The ab-/adsorbent coatings, used specifically in this study, are highlighted in red. We can differentiate between static in-solution, static in-headspace and dynamic in-headspace approaches. SBSE: Stir-bar sorptive extraction; HSSE: Headspace sorptive extraction; SPME: Solid-phase microextraction; DHS: Dynamic headspace system.

driven out of the sample material into the headspace. On the other hand, SPME has become a more widely used headspace technique to analyse volatiles arising from many types of food sample [7], due to particularly its easy automation and the wider variety of ab-/adsorbent polymers available. SPME uses a small fused-silica fiber that is coated with one or more polymers to trap the volatiles. The other commonly used method to trap volatiles in foodstuffs is done by a dynamic headspace system (DHS). Its main difference compared to static techniques is that DHS traps volatiles through flushing the sample with a flow of gas. This helps accumulate the analytes more efficiently in the adsorbent phase which can be directly connected to the vial through a needle. DHS also provides a wide variety of adsorbents. All four techniques have their own advantages and disadvantages and hence the specific research question should determine which is optimal. In respect to sensitivity and selectivity, for instance, the availability of different coating polymers and coating volumes can influence the performance of the technique for certain compounds. With SBSE, as compared to SPME, more sorptive phase volume is used (24µL and 0.5µL, respectively) and consequently, higher sensitivities can be achieved [8]. On the other hand, SPME fibres are currently available in several combinations of different types of coating while for SBSE and HSSE the coatings are limited to two (PDMS and EG-Silicone). Consequently, the selectivity of the technique for certain compounds differs depending on the coating used. Additionally, static and dynamic modes have shown different chemical profiles of the same food samples [9]. DHS does not depend on the equilibrium between the gas and liquid phase. Therefore, DHS is able to concentrate the analytes at ultra-trace levels and thus sensitivity also improves [10]. The goal of this study was to compare the four trapping techniques for comprehensiveness and repeatability by analysing two process flavours with high diversity in its chemical composition.

Process flavours, also referred to as 'reaction flavours', are composed of a complex mixture of ingredients that are thermally processed under controlled conditions during manufacture. They are important flavouring ingredients regularly added to improve taste and enhance specific sensory attributes in savoury food products, such as soups, snacks, sauces or ready-to-eat meals [11]. One of the most prominent group of process flavours now used as flavouring ingredients are based on yeast autolysates and extracts [12]. They enhance flavour by imparting cheesy, meaty or savoury notes, and can be used in meat substitutes for vegetarian food applications. Two examples of commercial process flavours are Maxagusto™ (DSM Food Specialties, the Netherlands) and Flavour Yeast Extract (Hubei Angel Yeast Co. Ltd, China). By varying the yeast strain, the yeast processing conditions, such as temperature and time, and the combination with other basic ingredients, different savoury notes can be obtained to create a wide palette of high quality taste and aroma supplements [13–15]. Studying which volatile compounds might contribute to the aroma and taste of process flavours is of great importance for food formulation and flavour studies. The reactions that lead to the formation of these volatiles during the production of process flavours are mainly related to lipid oxidation, Maillard reactions and thermal degradation of sugars, proteins, ribonucleotides, pigments and vitamins [16]. Of particular interest are Maillard reactions, which occur between a nitrogen-containing compound and a reducing sugar [17]. When catalysed by high temperatures, a cascade of chemical reactions are triggered to potentially form a vast range of volatile aroma-related compounds. These compounds are chemically diverse and display many different physico-chemical properties (for an overview see [11]). Consequently, characterizing the volatile composition of process flavours will give us insight into strategies for improving flavour quality, and approaches for investigating the link of certain volatiles to desirable sensory attributes.

This study aims to detect the highest number of volatile compounds present in process flavours in a reproducible manner. We have focused on the most novel sorption-based techniques (SBSE, HSSE, SPME and DHS) as they do not need the use of any (toxic) organic solvents and they are fast, easy to manipulate and cost-effective, among many other reasons [5]. We hypothesized that, from all sorptive techniques, SBSE will cover a higher range of volatiles as it is in direct contact with the liquid and thus, more polar and less volatile compounds will (additionally) be trapped as compared with the most commonly used techniques (SPME and DHS).

To test our hypothesis, we have developed and compared four volatile trapping techniques coupled to GCMS in order to study which technique gives the most comprehensive overview of the volatile composition of two different process flavours (Maxagusto G28 and Maxagusto S99). Repeatability of the techniques was also tested. Furthermore, the use of salt in sample preparation was also tested as some salts are known to release more volatiles into the gas phase.

2. Materials and Methods

2.1. Food materials

Two yeast extract-based process flavours from the series Maxagusto were obtained from DSM Food Specialties (Delft, the Netherlands). These are commercially available as Maxagusto G28 and Maxagusto S99 (https://www.dsm.com). Maxagusto G28 is an aromatic, pungent garlic flavouring reported to have a distinct fresh, fried garlic profile. Maxagusto S99, in contrast, has a natural roasted spice base for Asian-type recipes with the flavour of cooked vegetables (https://www.dsm.com). Both were obtained as 50 g dry powder in sealed foil bags. After the samples were aliquoted, they were stored at -80°C until analysis.

Prior to analysis, Maxagusto samples were suspended in tap water at a concentration of 2 mg/mL. This concentration is equivalent to the dosage level commonly used in the food application. Once the powders were fully suspended, they were sonicated for 10 min to break up any remaining aggregates or small solid particles. For the method involving salt (Section 3.4), a saturating amount of salt at a final concentration of 5 M (for $CaCl_2$) and 6 M (for NaCl) was added after the Maxagusto samples had been dissolved. The vials were stirred and immediately closed after the addition of salt. Screw-cap glass vials (10 mL and 20 mL) with silicone/PTFE septa (Supelco, PA, USA) were used.

2.2. Chemicals

A range of reference chemicals were used: an *n*-Alkane (C_8-C_{22}) series was purchased from Sigma-Aldrich (Milwaukee, WI, USA). The standards used for metabolite identification were: Benzaldehyde, Diallyl-disulfide, Dimethyl-disulfide, 2,5-Dimethyl-pyrazine, Dimethyl-trisulfide, *p*-Eugenol, Furfural, Hexanal, D-Limonene, Methyl-propyl-trisulfide, Nonanal, Octanal, alpha-Pinene, alpha-Terpineol and alpha-Terpinolene. All compounds were purchased from Sigma-Aldrich. Methanol (Biosolve BV, NL) was used as solvent for the preparation of the standard solutions. Calcium chloride ($CaCl_2$) and sodium chloride (NaCl) were purchased from Sigma-Aldrich and Honeywell-Fluka (Seelze, Germany), respectively.

2.3. Trapping techniques

Four different trapping techniques for volatile compounds were tested in this study. For all four, samples were analysed using the same GCMS instrument with the same settings by thermally desorbing them using a multi-purpose sampling robot (MPS-2, Gerstel, Mülheim, Germany). Prior to the comparison of these four methods an extensive series of preliminary trials was performed for each in order to determine which specific set of parameters and settings gave the best results. Different combinations of adsorbent types, extraction times, temperature regimes, etc. were tested as described in Supplementary Table S1. The best result was considered to be those conditions which gave the most comprehensive overview of the components present. The optimal procedure for each method is described below.

2.3.1. Stir bar sorptive extraction in solution (SBSE) and in headspace (HSSE)

ForSBSE and HSSE, we used a set of 20 Gerstel 10 mm x 0.5 mm PDMS stir bars (Gerstel, Germany). Prior to sampling, the stir bars were conditioned for 60 min at 260°C under a continuous stream of helium gas (grade 5.0) in empty glass tubes. After conditioning, they were individually stored in clean closed screw-cap glass vials until use. Clean tweezers or a magnetic bar were used for handling the stir bars. Each sample had its individual and traceable stir bar. For the preliminary tests shown in Supplementary Table S1, PDMS MonoTrap (Monolithic Material Sorptive Extraction (MMSE), GL Sciences, Japan) and EG-Silicone stir bars (Gerstel, Germany) were also tested and as the results did not show a comparable or better coverage of volatiles as PDMS stir bars, we did not proceed further with these approaches.

The following methodology was applied for the sample series: A volume of 3 mL sample solution (2 mg/mL) in a 20 mL glass vial was used for volatile trapping, which translates into a phase ratio of 125 (sample volume/PDMS volume, thereby 3000 µL/ 24 µL = 125). The use of a small phase ratio is fundamental for an uniform extraction of a wide range of compounds. For SBSE analysis, the stir-bar was placed either in the Maxagusto solution or positioned in the headspace above the sample using a glass insert (Gerstel, Mülheim, Germany) for HSSE (Fig. 1). In the case of $SBSE^2$ analysis [18], two stir bars were placed in the same vial, one for SBSE and one for HSSE (Fig. 4). After inserting the stir bars, vials were immediately closed and incubated in a water bath at 60°C for 10 min. To extract volatiles, samples were placed on a multipoint magnetic stirring plate (Thermo Scientific Variomag, Waltham, USA) at room temperature (RT), 450 rpm for a further 80 min. After the full extraction time (90 min, 60°C+RT), the stir bars were removed from the samples using tweezers or a magnetic bar, rinsed for 2-3 seconds in ultraclean water, dried with a clean tissue and put into empty clean glass liners for desorption. The analyses were done right after sampling. Volatiles were first desorbed from the stir bars in a Thermal Desorption Unit (TDU, Gerstel) connected to the Cooled Injection System (CIS) of the GCMS. Desorption in the TDU was done in splitless mode at a temperature of 30°C for 0.5 min, and a ramp of 120°C/min to reach a final temperature of 250°C (with a 5 min hold) using helium (Grade 5.0) as carrier gas. The desorbed volatiles were focused in the CIS on a glass liner packed with Tenax TA at -10°C which was then flushed for 0.2 min at -10°C with helium flow of 35 mL/min (solvent vent mode). The volatiles in the CIS were transferred to the analytical column by rapidly firing the trap from -10°C to 250°C with an increase of 720°C/min after which the temperature was held at 250°C for 5 min. A split of 1:5 was used. The desorption and injection was fully automated for all samples using a Gerstel MPS-2 autosampler and operated using Gerstel MAESTRO software version 3.2.

2.3.2. Headspace solid-phase micro extraction (SPME)

For SPME, a PDMS/DVB/CAR (Polydimethylsiloxane / Divinylbenzene / Carboxen) 50/30 µm diameter, 1 cm length (Supelco, PA, USA) fiber was used. Prior to analysis, the fiber was conditioned as recommended by the manufacturer.

The following methodology was applied for the sample series: A volume of 1 mL sample solution (2 mg/mL) in a 10 mL glass vial was used to trap volatiles. The vials were incubated at 60°C for 10 min with agitation. Subsequently, volatiles were trapped by exposing the fiber to the headspace of the vial for 20 min at 60°C without agitation (Fig. 1). The fiber was then thermally desorbed in the CIS containing an empty glass liner (1 mm ID) with a helium flow of 1 mL/min at 250°C for 2 min onto the GC column, in splitless mode. Trapping and injection was fully automated using a Gerstel MPS-2 autosampler and operated using Gerstel MAESTRO software version 3.2.

2.3.3. Dynamic headspace extraction (DHS)

The trapping of the volatiles was done using the DHS module by Gerstel mounted onto a Gerstel MPS-2 autosampler and operated using Gerstel MAESTRO software version 3.2. A glass tube packed with 60 mg of Tenax TA (Gerstel, Germany) was used as adsorbent. Before sampling, the Tenax tube was conditioned for 60 min at 285°C with a constant flow of helium (Grade 5.0) as carrier gas.

The following methodology was applied for the sample series: A volume of 3 mL sample solution (2 mg/mL) was added to a 20 mL glass vial. The samples were first incubated at 30°C for 10 min, with agitation. For DHS analyses, 30°C was chosen instead of 60°C due to problems with water trapped on the Tenax phase. After incubation, volatiles were collected on the Tenax cartridge by purging the vial with a continuous flow of helium at 30 mL/min for 10 min (Fig. 1). The temperature of the vial was maintained at 30°C while the temperature in the Tenax trap tube set to 20°C. After collection, the Tenax tube was purged with a helium flow of 10 mL/min for 5 min at 28°C in order to remove moisture and remained oxygen. Cartridges were directly desorbed as described in Section 2.3.1 for SBSE and HSSE. The volatiles in the CIS were desorbed in splitless mode for the first 4 min after which a split 1:40 was applied.

2.4. Gas-Chromatography Mass-Spectrometry (GCMS)

For all four trapping techniques, the same instrument and settings were employed. All analyses were conducted on an Agilent GC7890A coupled to a 5975C quadrupole mass spectrometer. The column used was a Zebron ZB-5MSplus with dimensions 30m x 0.25mm x 1.00µm (Phenomenex). The column oven was temperature programmed starting at 45°C for 2 min, then increased at a rate of 5°C/min to 250°C and then maintained at 250°C for 5 min. The carrier gas was helium, at a flow of 1 mL/min. The column effluent was ionised by electron impact at 70 eV, in the scan range m/z 33–500. The interface temperature was 280°C.

2.5. Experimental procedure and data analysis

We analysed eight replicates for both Maxagusto products. The analysis order was kept the same for all the techniques. A series of n-alkanes were analysed at each sequence for calculating retention indices (RI) using a third order polynomial function.

After visual inspection of the GCMS total ion current chromatograms using vendor software, raw data were processed using an untargeted metabolomics approach. Baseline correction and alignment of mass signals ($s/n \geq 3$) were performed using MetAlign software [19]. Mass signals present in ≤ 4 replicates were discarded. Signal redundancy was removed and mass spectra were reconstructed using MSClust [20]. Metabolites were identified by matching the mass spectra and retention indices to authentic reference standards or those in the NIST17 Mass Spectral library (v.2.3).

For statistical analysis, we compared and visualized the main tendencies of the generated data by principal components analysis (PCA) after log 10 transformation and Pareto scaling of the samples using SIMCA 15.0.2. software (Sartorius Stedim Data Analytics AB, Umeå, Sweden). Graphs were also produced using Microsoft Excel 365.

3. Results and discussion

This study was initiated to investigate the potential of four analytical techniques for trapping a range of different classes of volatiles present in process flavours with contrasting chemical compositions. Maxagusto process flavours were chosen for their diversity in chemical groups relevant for defining flavour and aroma profiles. The four techniques were chosen for their simplicity, easy manipulation and robustness in extracting a high range of volatile compounds directly from the headspace or liquid mixture without having to use toxic organic solvents [5]. Each technique was separately pre-optimized for the best trapping conditions in terms of comprehensiveness before the trapping procedures were compared. The parameters that were optimized are summarized in the Supplementary Table S1.

3.1. Untargeted volatile comparison of the four different techniques

Metabolomics aims to characterize comprehensively a broad range of small molecules in a biological sample. Most importantly, it helps to compare accurately the global metabolite profile between groups of samples and thus to identify discriminatory compounds. However, the method of extraction has a major influence on the range of metabolites detected. In this study, we aimed to demonstrate that the use of different trapping techniques delivers distinctly different profiles for volatile compounds of process flavours, both in terms of comprehensiveness and repeatability. The results were first compared in an unbiased way by looking at the complete volatile compound spectrum using a metabolomics approach. Raw GCMS data were processed using an untargeted workflow pipeline and processed data were tested for their repeatability and selectivity. For both Maxagusto types, SBSE revealed the highest number of compounds as compared to the other three trapping techniques (Table 1). SPME also revealed a high number of compounds, while DHS appeared to detect the smallest number of compounds which was not completely unexpected. With DHS, we experienced technical limitations due to water interfering with the trapping of volatiles when a sampling temperature of 60^{0}C was used, even after using very large purging volumes. Hence, 30°C was used and therefore it would be incorrect to compare results from the SPME technique directly with those from DHS. Nevertheless, we have decided to include the DHS findings due to the strong qualitative differences that were observed for certain sensory-relevant chemical groups. The repeatability represented by the coefficient of variation (CV) of all compounds was lower for the SBSE and SPME data compared to that of HSSE and DHS. HSSE appeared to deliver the least repeatable data. Our results confirm published data where SBSE, although not widely used, has demonstrated its effectiveness in trapping predominantly non-polar and semi-polar compounds from liquid food samples [6]. SBSE has also delivered more comprehensive profiles than SPME in liquid matrices, such as wine [21] and coffee [22].

Principal components analysis (PCA) was performed to visualise how the volatile composition differed between the four techniques (Fig. 2). Based on all detected volatile compounds the first two PCs show a clear separation of the profiles of the four different techniques for both Maxagusto G28 (Fig. 2A) and S99 (Fig. 2B). The first two PCs explained more than 80% of the total variance for both Maxagusto types respectively. Variation of the samples for each approach was smaller than between techniques indicating good repeatability. The biggest difference was found for SBSE in both sample types (G28 and S99) as PC1 separates these from all other techniques. Furthermore, SPME samples also seem to have a more distinct profile compared to the other methods. Interestingly, despite the different sorbent (PDMS vs Tenax) and means of trapping the volatiles (one being a dynamic and one a static method), there was a strong similarity in the volatile composition from DHS and HSSE. The reasons behind this are not yet clear, however, the variables involved could be related to the type of adsorbent phase used and the dimensions of the coating phase, as well as to the previously mentioned lower temperature used for DHS.

The differences between the techniques in the PCA were reflected by the overlap of compounds visualized by a Venn diagram showing the total number of volatiles detected for both Maxagusto samples (Fig. 2 C and D). In both samples, the subset of compounds commonly detected across all four techniques formed the largest group. In total, 47 compounds were commonly detected in G28 (Fig. 2C) and 53 compounds in S99 (Fig. 2D). However, SBSE also trapped an additional, almost equal number of compounds (39 and 43, respectively) that were unique for this technique while the other techniques uniquely trapped only between 1-12 additional metabolites. Both SPME and SBSE also shared many compounds

Table 1
Number of compounds detected by an untargeted study in Maxagusto samples G28 and S99 using the four techniques. Eight replicates were measured for the statistical analysis. SBSE: Stir-bar sorptive extraction; HSSE: Headspace sorptive extraction; SPME: Solid-phase microextraction; DHS: Dynamic headspace system.

	G28				S99			
	SBSE	HSSE	SPME	DHS	SBSE	HSSE	SPME	DHS
Number of compounds detected	158	73	122	68	164	89	134	76
Number of known compounds	51	31	44	27	59	46	49	36
Coefficient of variation (%)	1.66	18.60	2.99	3.38	3.57	10.35	4.40	7.45

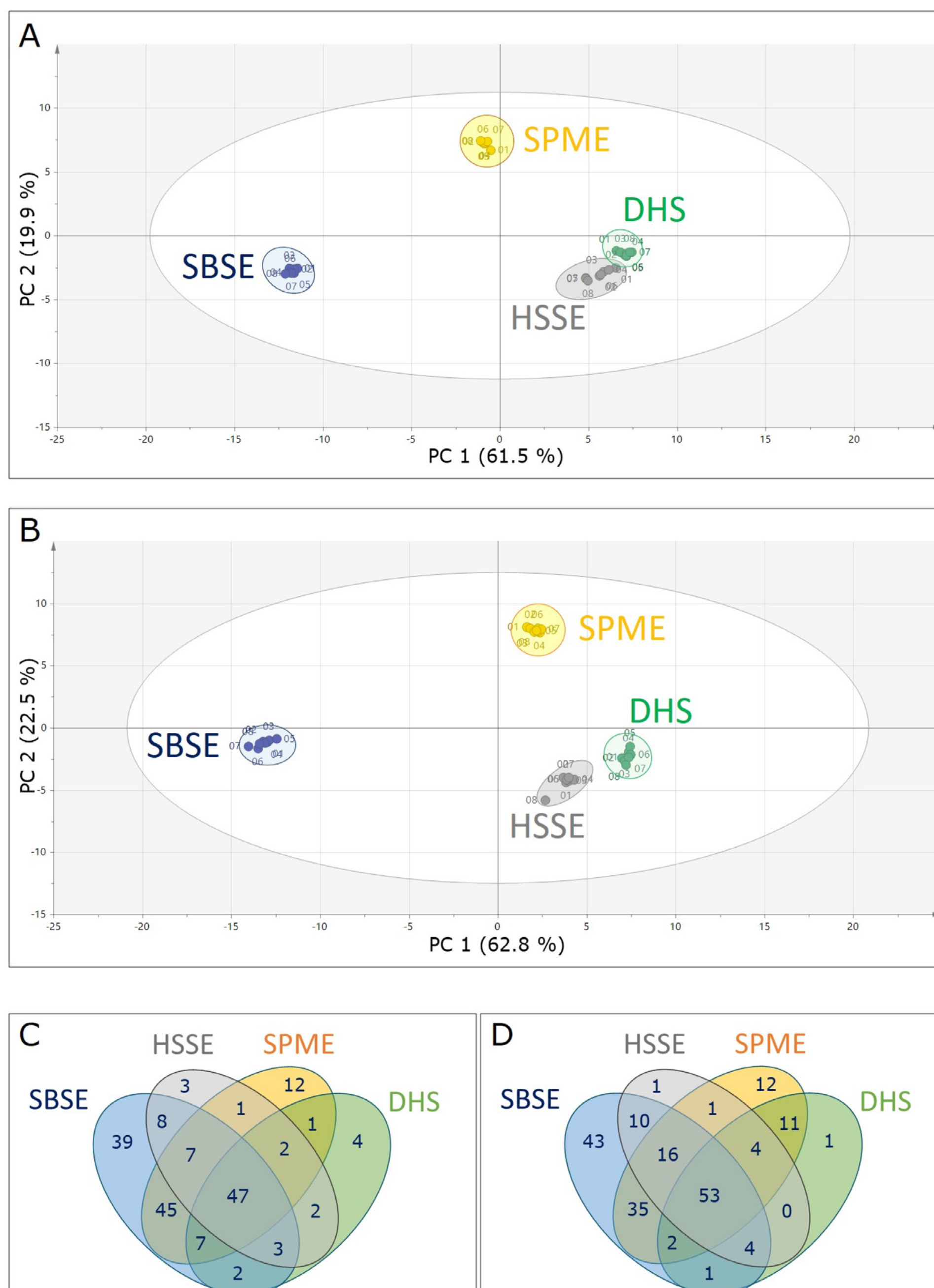

Fig. 2. Principal components analysis (PCA) score plot of the volatile profiles of Maxagusto G28 (A) and S99 (B) using four trapping techniques. Eight replicates for each technique are represented. The first and second PC explain the corresponding percentage of variation shown on each axis.
Venn diagram representing the total number of metabolites detected by SBSE, HSSE, SPME and DHS in Maxagusto samples G28 (C) and S99 (D). SBSE (blue): Stir-bar sorptive extraction; HSSE (grey): Headspace sorptive extraction; SPME (orange): Solid-phase microextraction; DHS (green): Dynamic headspace. Compounds considered at least present in 5 out of the 8 replicates.

that DHS and HSSE could not trap. As a conclusion, SBSE followed by SPME were the techniques giving the most comprehensive profiles in terms of numbers. However, qualitative differences should also be considered of importance as detailed further below.

3.2. Targeted volatile compounds trapped by SBSE, HSSE, SPME and DHS

To study the differences between the trapping techniques in more detail, we looked at the number of compounds identified in both G28 and S99 samples (Supplementary Tables S2 and S3, respectively). Compounds were identified based on comparison of the retention index (RI) and the mass spectra of authentic reference standards or from commercial and in-house libraries including NIST. The level of identification given follows the guidelines of the Metabolomics Standards Initiative [23]. All compounds with no or a lower level of identification reliability (levels 3 and 4) were here considered as ‘unknowns' (Supplementary Table S4 and S5). For evaluation, the volatiles detected were divided into the following chemical groups: aromatics, polysulfides, pyrazines, aldehydes, sesquiterpenes/monoterpenes hydrocarbons/alcohols, and ‘others'. Detailed analyses of the groups of identified compounds found in the different trapping profiles revealed that some metabolite classes are better represented by some trapping techniques than others. Polysulfides, pyrazines and aromatics were more abundant in G28, whereas S99 revealed primarily sesquiterpenes and monoterpenes as well as polysulfides and pyrazines. The total number of identified compounds was 55 for G28 and 60 for S99. To visualize the main trend of the identified compounds across the four techniques, a graph was made to show the relative contribution of each compound in each technique (Fig. 3). A noticeable observation was the high contribution of SBSE to trapping pyrazines, as compared to the other techniques. This was observed for both Maxagusto G28 (Fig. 3A) and S99 (Fig. 3B) samples. DHS trapped a considerable amount of monoterpene hydrocarbons whereas sesquiterpene hydrocarbons were more prominent in SPME data than in those of the other techniques. Sesquiterpene alcohols were trapped mostly by SBSE, as well as some other aromatics. The aromatics, aldehydes and the group ‘others' appeared to show the greatest variation between compound regarding trapping across the four techniques. These clear differences are also important when making choices for analysing food samples that may be richer in specific chemical groups of compounds. Or when the purpose of the analysis focuses on characterizing just one class of compounds.

In the following sections some specific observations for the different chemical groups are described.

3.2.1. Polysulfides

Polysulfides are important (savoury) components in food flavour research. They are characterized by having an alliaceous, sulphur, roasted garlic type flavour (http://www.Foodb.ca). In this study, a total of 14 and 9 polysulfides were identified in G28 and S99 samples respectively (Supplementary Fig. S1). Seven were common to both samples. Sulfides or polysulfides (disulfides, trisulfides, etc.) with allyl- and methyl- groups were seen to be the most abundant S-containing compounds. Interestingly, most polysulfides were detected by all the techniques. However, the distributionin the four techniques is not the same for sample G28 and S99 which might be due to the absolute abundance of polysulfides. Polysulfides in G28 were 30 times more abundant than in S99 (Supplementary Fig. S2) and, hence, a slightly different distribution between techniques was observed in both samples. In sample G28, polysulfides with low molecular weight (low RI) were more prominent when using DHS, whereas those with high molecular weight (MW) were more prominent when using SBSE. In the case of diallyl trisulfide (Ps14), highest levels were detected when using SPME. HSSE appeared least successful in trapping polysulfides. Overall, polysulfides with allyl- and methyl- groups were the most abundant sulphur compounds in Maxagusto process flavours and all four techniques could be used for the analysis of this type of aroma compounds. It should be noted that S-containing compounds can be thermally labile and may oxidize to form polysulfides, thus, when analysing by GCMS, artefact formation has been observed [24,25].

3.2.2. Pyrazines and other aromatics

In this study, pyrazines were grouped separately from the aromatic compounds due to their specific relevance to flavour [26]. They confer a roasted, nutty, cocoa, sweet flavour character (http://www.Foodb.ca) and they have been characterized in yeast extract flavourings by SPME-GCMS [27]. A total of 15 and 9 pyrazines were detected in G28 and S99, respectively (Supplementary Fig. S2 A and B). There were two major pyrazines present in both samples: 3-ethyl-2,5-dimethyl pyrazine (P05) and 2,5-dimethyl-3-(3-methylbutyl) pyrazine (P16). Those techniques that trapped pyrazines most effectively were SBSE and SPME. HSSE and DHS did not trap all these compounds. The reason may be related to the temperature used to extract the volatiles: 30^{0}C for DHS, SBSE and HSSE as compared to 60^{0}C in SPME. It is known that some pyrazines can be thermally formed when high temperatures are used [26] and they are more polar compounds. SBSE traps volatiles within the liquid phase, thus potentially making the trapping efficiency higher for more polar and semi-volatile compounds [6].

The other class of compounds better represented when using SBSE is the aromatics. Aromatic heterocyclic compounds can be found in foods, often at low concentrations. However, they can be highly influential to the overall flavour by contributing to aroma complexity of food. Heterocyclic compounds have been strongly linked to ‘roast meat' flavour formation during heating and are important compounds in processed foods and food flavouring [11]. They are formed from many degradation pathways, the most important concerning Maillard reactions between amino acids and sugars. However, some can be formed from lipids or from lipid degradation products [17]. This group of compounds is chemically diverse so the distribution between the techniques for the trapping of these volatiles is more varied (Supplementary Fig. S2). Again, while SBSE appeared the best overall trapping method, other approaches, such as SPME or DHS, were sometimes better for individual molecules suggesting that trapping success is perhaps more structure-dependant within this diverse compound class.

Sulphur-containing heterocycles (1,2-dithiole, Ar08; and 2-vinyl-1,3-dithiine, Ar14) were the most abundant in G28 (Supplementary Figs. S2 C and D). SBSE and DHS were the techniques that trapped the S-containing heterocycles more effectively. On the other hand, thiophenes and N-containing heterocycles, such as pyridines and pyrroles, were better trapped by SBSE with the exception of 3-methyl thiophene (Ar03) which was more prominent in DHS.

3.2.3. Terpenes

In plants, terpenoids (monoterpenes and sesquiterpenes) play important highly diverse roles in nature as plant hormones, defence compounds, insect / animal attractants and repellents, etc. [28]. In flavour science, terpenoids are important when formulating new flavouring ingredients as they confer a wide range of aroma characters, such as sweet, herbal, spicy and woody [29]. A total of 6 monoterpene hydrocarbons, 1 monoterpene alcohol, 12 sesquiterpene hydrocarbons and 3 sesquiterpene alcohols were identified by one or more of the four trapping techniques (Supplementary Fig. S3). In sample G28, only 1 terpene (p-Eugenol, Supplementary Table S2) was identified. In S99, the major terpene compound was the sesquiterpene caryophyllene (Supplementary Table S3) but

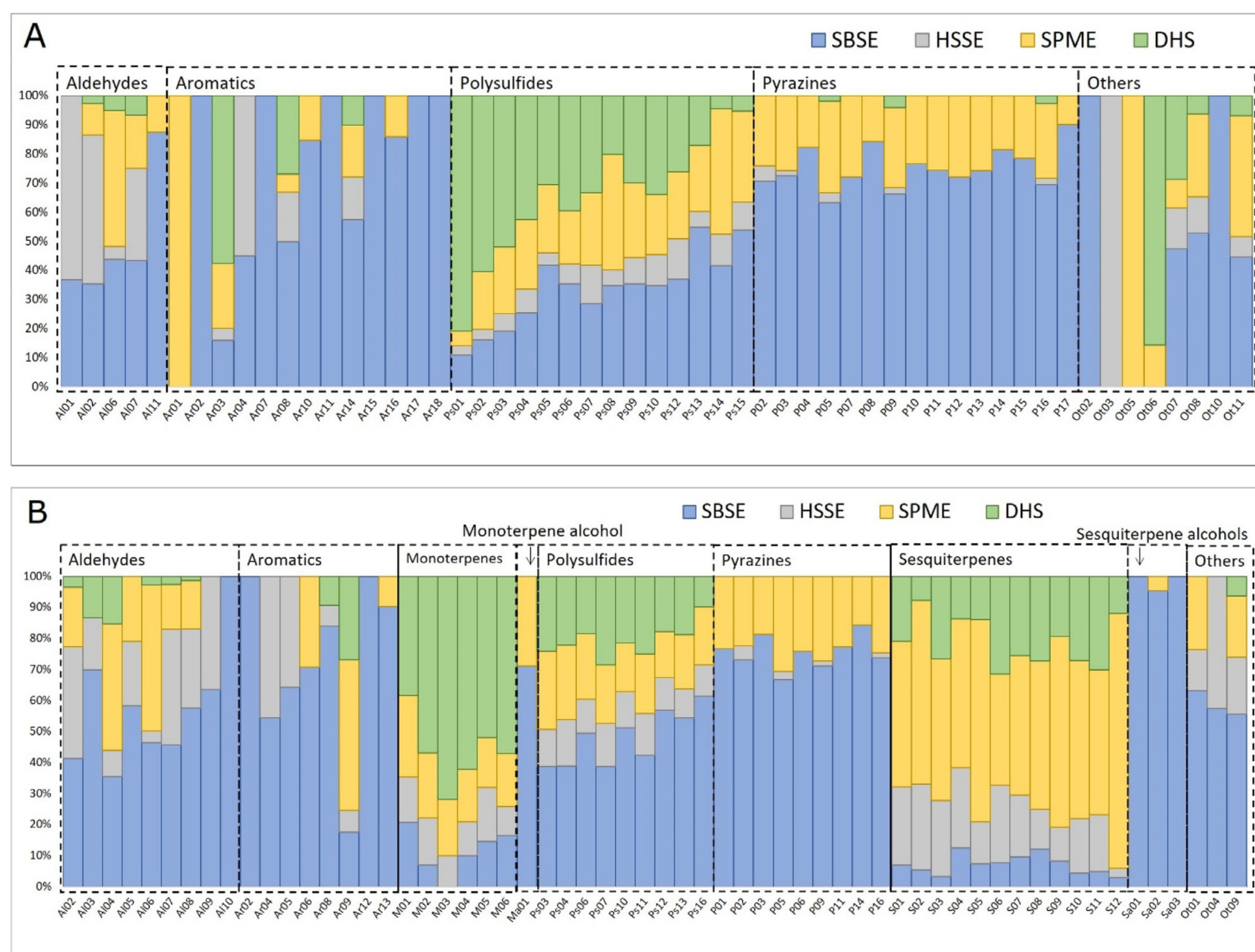

Fig. 3. Relative proportion of identified metabolites trapped for each techniques grouped per compound class. (A) Maxagusto G28 and (B) Maxagusto S99. Metabolite names correspond to metabolites in Supplementary Table S2 and S3.

this has for practical reasons been excluded from Figs. 3 and S3 as its abundance was at least 10 times higher than the others and the peak was saturated. All monoterpene hydrocarbons observed were detected by all the techniques although DHS revealed relatively higher levels than SBSE, HSSE or SPME each of which showed similar results. The same was observed for other terpene compounds, which were better recovered by DHS as compared to SPME [30]. One monoterpene alcohol (Ma01) was clearly visible using SBSE but was not detected when using HSSE and DHS. In the case of sesquiterpene hydrocarbons and sesquiterpene alcohols, all the techniques appeared able to trap all types. SPME was the most effective in trapping sesquiterpene hydrocarbons while SBSE was better (or the only one) able to trap sesquiterpene alcohols than HSSE, SPME or DHS. Based on standard deviation values, SBSE appeared the most repeatable for monoterpenes and sesquiterpenes in sample S99. The high trapping efficiency of SBSE for sesquiterpene alcohols may be due to the high molecular weight (and hence are less volatile) and high polarity of these compounds. This could entail that they are poorly released into the headspace [4]. The SBSE technique has previously been characterized for its effectiveness in trapping semi-polar and polar compounds which SPME and/or DHS are unable to do so [31]. Moreover, modifications of the SBSE technique by e.g. pretreating the stir bars with organic solvents provide interesting potential to broaden the trapping of more polar and less volatile compounds, as compared to normal SBSE, offering improvements for the analysis of food flavourings, including process flavours. Important polar aroma compounds, such as short chain fatty acids (C3-C5), were detected by the modified SBSE method [6,32–34]. However, it also obscures other compounds which co-elute or interfere with the polar metabolites [34]. Overall, all four techniques are able to trap most of the terpenoids observed. SBSE traps a considerable amount of sesquiterpene alcohols that other techniques cannot detect while SPME is able to trap a considerable amount of sesquiterpene hydrocarbons. Interestingly, DHS profiles were rich in monoterpene hydrocarbons making this technique the preferred choice when this group is of specific interest. Their MW is much lower than for sesquiterpenes, potentially making them more suitable for dynamic headspace techniques.

3.3. Combining headspace and in-solution trapping ($SBSE^2$)

HSSE seems, in general, the technique least suited for the volatile analysis of Maxagusto process flavours. Nevertheless, for some aldehydes and aromatics it, together with SBSE, could make an important trapping technique when both are combined together. Therefore, a dual stir bar sorptive extraction ($SBSE^2$) was carried out to test the comprehensiveness of combining HSSE and SBSE in one desorption/chromatogram [18]. The analysis was done by placing two stir bars in the same vial, one located in the headspace above the liquid and the second stir bar immersed in the aqueous solution (Fig. 4). After trapping the volatiles, both stir bars were placed in the same desorption glass tube and desorbed together onto the GCMS to deliver a single chromatogram. Results

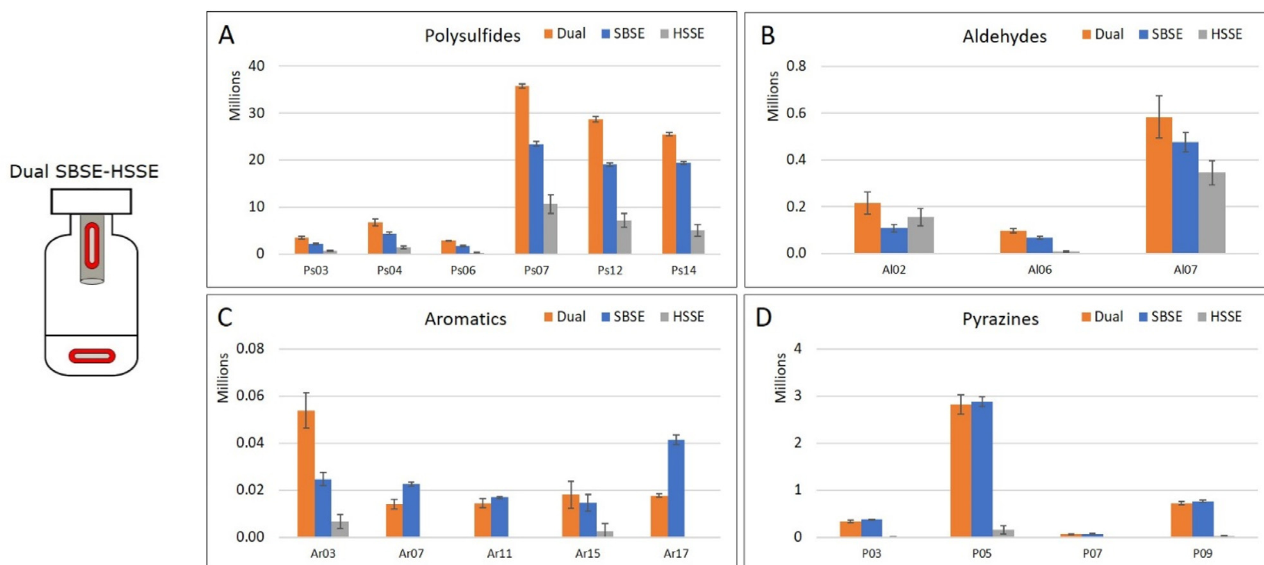

Fig. 4. SBSE^2 sampling. A few distinguished compounds are shown as examples: Polysulfides (A); Aldehydes (B); Aromatics (C); Pyrazines (D). Abundance of those compounds is expressed in Total Ion Current (TIC) * 10^6, for the different trapping techniques (Dual, SBSE and HSSE). Mean and sd values of three replicates are also shown. Metabolite names correspond to metabolites in Supplementary Table S2.

showed that SBSE^2 extraction was able to trap all the volatiles that were extracted by SBSE and HSSE separately. A few examples of polysulfides, aldehydes, aromatics and pyrazines are shown in Fig. 4. Compounds that are mostly (or only) trapped by one mode, such as pyrazines in SBSE, are altogether trapped in the dual mode. However, for the process flavours analysed in this study, the number of compounds trapped only by HSSE was very small, showing no significant additional information. Thus, the combination of both methods, for this type of process flavours, does not bring an improved level of comprehensiveness compared to individual SBSE or HSSE sampling. Nonetheless, performing SBSE^2 was proven to cover the range of volatiles that were more selective for both modes of trapping. Suggesting that a dual combination approach will increase the coverage of volatile metabolites in samples that contain a high diversity of compound classes.

3.4. Effect of salt addition in sample preparation

The addition of salt changes the physico-chemical properties of the sample solution which can sometimes help to release volatiles from the sample matrix into the gas phase when headspace techniques are used. Some salts, like NaCl, have been shown to enhance the release of certain volatile molecules from a liquid into the headspace [2]. In doing so, they increase the sensitivity of the analytical technique. However, in process flavours, this effect might not be wholly beneficial for all chemical groups of interest which are present in the sample.

The total abundance of volatiles was analysed using SPME-GCMS, after samples (Maxagusto G28 and S99) had been prepared with the addition of sodium chloride (NaCl), calcium chloride ($CaCl_2$) or just water. Polysulfide, pyrazine and aromatic groups were more abundant in G28 (Fig. 5A), whereas S99 revealed primarily sesquiterpenes and monoterpenes as well as polysulfides and pyrazines (Fig. 5B). The addition of either NaCl or $CaCl_2$ increased the abundance of most pyrazines, aromatics and aldehydes in the volatile profiles. Likewise, polysulfides were also slightly enhanced. However, the abundance of volatile sesquiterpenes and monoterpenes was observed to decrease when salts were added (Fig. 5). This contrast might be explained by differences in the solubility and polarity between the different classes of compounds. The 'salting-out' effect is proposed to enrich for the more hydrophilic compounds ($\log K_{O/W} < 3$) in the headspace [5]. Pyrazines (and other aromatics) are highly water soluble compounds and, depending on the functional groups attached to them, their surface polarity can also be high. Consequently, their concentration in the headspace can be expected to increase in samples following salt addition. Terpenes, however, are apolar molecules that possess low water solubility. Consequently, their abundance in the headspace decreases when compared to solutions without salt. Monoterpenes are more water soluble than sesquiterpenes because of their lower MW and slightly higher solubility, and this might explain why sesquiterpene abundance in the headspace decreased significantly on the addition of both NaCl and $CaCl_2$ whereas monoterpenes were much less affected (Fig. 5). Polysulfides and aldehydes are also polar and soluble in water, but their solubility and polarity are lower than for pyrazines. Therefore, when salt was added, their abundance increased in both sample types but less dramatically.

A difference between using $CaCl_2$ or NaCl was only observed for pyrazines. Furthermore, we observed the lowest standard deviations (sd) for the pyrazines and the highest for sesquiterpenes (Fig. 5). This may relate to differences in the chemical structure or dynamic range of the compounds. There are no differences in the sd values when comparing samples without salt, with $CaCl_2$ or NaCl.

In conclusion, the addition of salt increases the abundance of volatiles in the headspace trapped by SPME, with the exception of terpene compounds. Adding salt primarily had more quantitative than qualitative influence on volatile profiles. These results demonstrate that the addition of salt(s), although increasing the abundance of certain compound groups might not necessarily be the method of choice for an untargeted analysis of headspace volatiles in food flavourings. In this study, salt was not used in the final methods due to its negative influence in the abundance of terpenes.

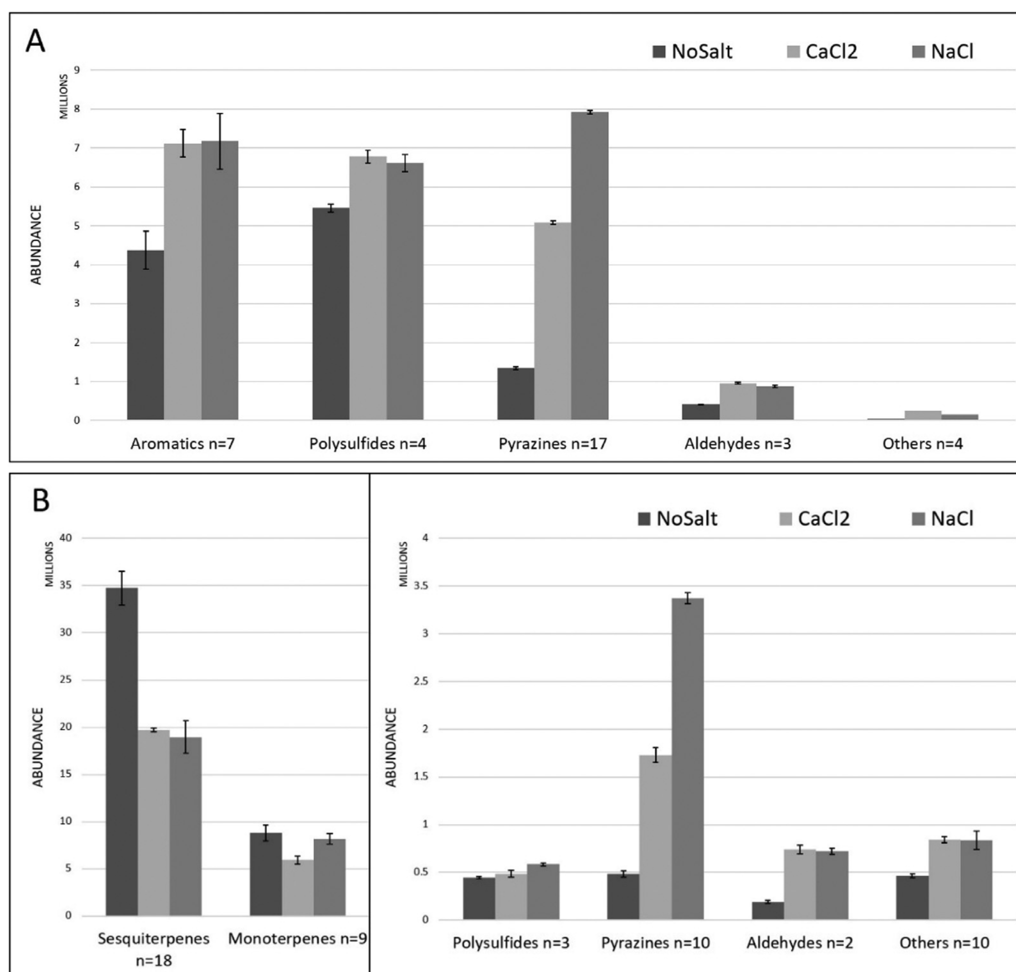

Fig. 5. Effect of salt addition during sample preparation on the relative abundance of different volatile chemical groups, as detected for Maxagusto G28 (A) and S99 (B) respectively, analyzed by SPME-GCMS. Mean and sd of five replicates per treatment are given. Addition of sodium chloride (NaCl; dark grey), calcium chloride ($CaCl_2$; light grey) or just water (NoSalt; black). n = number of identified metabolites found per group.

4. Conclusions

In this study, it was demonstrated that four different trapping approaches, SBSE, HSSE, SPME and DHS indeed provide different volatile profiles for the Maxagusto process flavours used. Under the experimental conditions applied, SBSE proved to be most suitable in extracting volatiles such as polysulfides, pyrazines and terpene alcohols, and generally provided the broadest spectrum of compounds. Moreover, SBSE trapped a significant extra number of compounds absent from the other profiles. This suggests that SBSE would be the most convenient starting point for the comprehensive analysis of volatile compounds in similar food matrices. Moreover, modifications of the SBSE approach provides interesting potential to broaden the trapping of semi-polar and semi-volatiles offering beneficial choices for the analysis of food flavourings, including process flavours. On the other hand, SPME and DHS techniques were the most successful in extracting sesquiterpenes and monoterpenes hydrocarbons, respectively. This entails that, should there be particular interest in this compound class, SPME (and DHS) would (also) be suitable for the analyses. Furthermore, both SPME and SBSE were the most repeatable techniques for generating data on the water soluble process flavours used here.

Few studies have compared directly these kind of trapping techniques for their robustness and comprehensiveness. The comparison of these techniques is crucial when some parameters cannot be maintained constant, as each of the techniques differ in many properties. Here it can be concluded that the extraction method has a significant impact on the volatile profile of process flavours, based on the volatility, solubility and polarity of the compounds targeted. In fact, this is a crucial step when the focus of the analysis is the contribution of these volatiles to the aroma and taste of process flavours. Carefully weighed choices must therefore be made regarding the best combination of analytical procedures to employ. For broadest comprehensiveness more than one protocol might be needed depending on the chemical complexity of the specific samples to be characterised. For this study, a combination of SBSE and SPME would give the best result. However, this entails extra labour and input costs and therefore SBSE would be the individual method of choice.

Compliance with ethical standards

There are no ethical issues associated with this publication

Research involving human and animal participants

This article does not contain any studies with human and/or animal participants performed by any of the authors.

Declaration of Competing Interest

The author declares no potential conflict of interest related to the presented work.

CRediT authorship contribution statement

Carmen Diez-Simon: Conceptualization, Methodology, Formal analysis, Validation, Writing - original draft. **Brenda Ammerlaan:** Resources, Writing - review & editing. **Marco van den Berg:** Resources, Writing - review & editing, Funding acquisition. **John van Duynhoven:** Writing - review & editing. **Doris Jacobs:** Resources, Writing - review & editing, Funding acquisition. **Roland Mumm:** Supervision, Methodology, Writing - review & editing. **Robert D. Hall:** Supervision, Conceptualization, Writing - review & editing, Project administration.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461191.

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