



Stir bar sorptive extraction of aroma compounds in soy sauce: Revealing the chemical diversity

Carmen Diez-Simon^{a,b,1,*}, Charlotte Eichelsheim^{a,d,1}, Doris M. Jacobs^e, Roland Mumm^{b,c}, Robert D. Hall^{a,b,c}

^a Laboratory of Plant Physiology, Wageningen University and Research, Droevendaalsesteeg 1, Wageningen 6708 PB, the Netherlands

^b Netherlands Metabolomics Centre, Einsteinweg 55, Leiden 2333 CC, the Netherlands

^c Wageningen Plant Research (Bioscience), Wageningen University and Research, Droevendaalsesteeg 1, Wageningen 6708 PB, the Netherlands

^d Laboratory of Food Chemistry, Wageningen University and Research, Bornse Weiland 9, Wageningen 6708 WG, the Netherlands

^e Unilever Foods Innovation Centre, Bronland 14, Wageningen 6708 WH, the Netherlands

ARTICLE INFO

Keywords:

Soy sauce
Aroma
Stir bar sorptive extraction (SBSE)
Gas chromatography-mass spectrometry (GC-MS)
GC-olfactometry-MS (GC-O-MS)
Volatiles
Fermentation

ABSTRACT

Fermented soy sauce is used worldwide to enhance the flavour of many dishes. Many types of soy sauce are on the market, and their differences are mostly related to the country of origin, the production process applied and the ratio of ingredients used. Consequently, several aromas, tastes, colours, and textures are obtained. Nowadays, soy sauce can also be produced without microorganisms making the process shorter and cheaper. However, flavour may be lost. We have carried out a comprehensive metabolomics analysis of volatile compounds using stir bar sorptive extraction (SBSE)-GC-MS to relate differences in volatile content to production history and origin. The results revealed major differences between fermented and non-fermented soy sauces, and a list of volatile compounds is reported as being characteristic of each type. This study was able to relate volatiles to the production process using SBSE-GC-MS and to aroma characteristics using GC-O-MS.

1. Introduction

Soy sauce is a very common seasoning used worldwide. It is characterized by its strong umami, salty and smoky flavour, which is used to enhance the overall taste and aroma experience of many types of dishes. Soy sauce originated in China over 2200 years ago during the Western Han dynasty and spread to Japan in the 7th century where it is still today known as *shoyu* (Hosking, 1996). In Japan, soy sauce export was started in 1647 by the Dutch East India Company (Ohyama, 2013). Today, several countries (and industries) are involved in producing soy sauce, with China and The Netherlands being the two largest exporters in the world ("FAOSTAT," 2017). Currently, the annual global consumption is estimated at 10 billion litres (Lee & Khor, 2015), which makes soy sauce a substantial component of the global food seasoning market.

Soy sauce is a black liquid seasoning made essentially from soybeans, wheat (optional) and brine. There are two methods for producing soy sauce; firstly, traditional fermentation (TF) which uses starter cultures of microorganisms (e.g. *Aspergillus oryzae*) and secondly, acid hydrolysis (AH), which is an artificial process using concentrated acid and high

temperatures to break down the raw ingredients and generate various flavour compounds. Although AH is less time-consuming (days compared to months – years (Diez-Simon, Eichelsheim, Mumm, & Hall, 2020)), the product lacks many typical fermentation-derived flavour compounds. For this reason, AH soy sauce is sometimes mixed with TF soy sauce to create a product referred to as 'mixed' or 'blended' soy sauce. Recently, both production procedures have been explained and reviewed in depth, in relation both to the metabolite content as well as their sensory characteristics (Diez-Simon et al., 2020).

Soy sauces can also be classified according to their geographic origin or to the ratios of raw materials used. For instance, the Japanese type is made using equal amounts of soybeans and wheat, whereas Chinese type soy sauce is produced with predominantly soybeans (80%) (Wanakhachornkrai & Lertsiri, 2003). Tamari is a speciality Japanese type soy sauce which is prepared without any or only a small amount of wheat (Luh, 1995). As volatiles are determinant of aroma characteristics, various studies have investigated the volatile profiles of different types of soy sauce (Feng et al., 2014; Lee, Lee, Choi, Hurh, & Kim, 2013; Sun, Jiang, & Zhao, 2010; Zheng, Wu, Huang, Zhou, & Liao, 2013; Lee, Kim,

* Corresponding author at: Laboratory of Plant Physiology, Wageningen University and Research, Droevendaalsesteeg 1, Wageningen 6708 PB, The Netherlands.
E-mail address: carmen.diezsimon@wur.nl (C. Diez-Simon).

¹ Diez-Simon and Eichelsheim share first equal author as they contributed equally to the manuscript.

& Kim, 2019). So far, nearly 300 aroma-related volatile compounds have been identified (Devanthi & Gkatzionis, 2019). Soy sauce fermentation is a complex process which depends on several variables and chemical reactions to produce a wide range of volatile compounds. Both enzymatic, and non-enzymatic reactions lead to the formation of volatiles such as alcohols, aldehydes, acids, esters, furan(one)s, ketones, phenols, pyrazines and sulphur-containing compounds (Nunomura, Sasaki, Asao, & Yokotsuka, 1976), many of which have specific aroma qualities and intensities.

To date, only a single study has analysed and compared TF and AH soy sauces in relation to their volatile differences and aroma properties (Lee, Seo, & Kim, 2006). The authors performed volatile analysis in TF and AH soy sauces using solvent extraction and solid-phase microextraction (SPME) coupled to gas chromatography-mass spectrometry (GC-MS). The TF soy sauces analysed were characterized mainly by alcohols and esters which are formed from microbial activity. In contrast, pyrazines, furans and acids were more abundant in the AH soy sauces, and these are known to be formed during heating processes.

The most common and robust technique to analyse (and identify) volatile aroma compounds is gas chromatography-mass spectrometry (GC-MS). Sorptive-based techniques in combination with GC-MS are effectively used to extract (semi-)volatiles from the complex (food-) matrices such as soy sauce. These have proven to provide a suitable alternative for solvent extraction techniques since for example, they are fast, less labour intensive and more cost-effective (Diez-Simon, Ammerlaan, & et al., 2020; Nogueira, 2015). In soy sauce, solid-phase microextraction (SPME)-GC-MS is now the most widely used approach (Chen, Feng, Cui, Zhao, & Zhao, 2015). However, in recent years, other techniques, such as stir bar sorptive extraction (SBSE), have gained some popularity and have been optimized to cover a broader range of (semi-)volatiles in liquid food materials (David, Ochiai, & Sandra, 2019).

Interestingly, only one study describes the application of SBSE in soy sauce (Lee, Kim, & Kim, 2019). The authors investigated the changes in volatile compounds in a Korean-type soy sauce during the fermentation period. They analysed the volatiles using both SPME and SBSE, and concluded that during long-term fermentation, the levels of most esters, some phenols, benzene and benzene derivatives, lactones, and pyrroles increased, while some alcohols, except for ethanol, and ketones decreased amid fermentation time. However, little is described about the technical comparison of both extraction techniques

The main goal of this study was to characterize comprehensively the volatile profiles of TF and AH soy sauces using an untargeted metabolomics approach. The aim was to relate compositional differences in the volatile profiles to the origin and production history of the samples and hence allow us to make hypotheses on the possible mechanisms behind the formation of volatile compounds and aroma. In order to link aroma to individual volatiles and hence gain insight into their specific contributions to sensory impact, we also initiated the application of GC-Olfactometry-MS to enable the determination of the individual aroma characteristics of key soy sauce volatiles. In the present study, both TF and AH soy sauces were included in order to make a detailed, direct chemical and sensory comparison. A broad range of commercially available products was selected which had originated from different countries and which had been made using different ratios of raw ingredients, processing temperatures and storage histories (aging). The sample metadata is summarized in Tables 1A and 1B.

2. Materials and methods

2.1. Soy sauce materials and chemicals

Twenty contrasting commercial soy sauces were purchased from

Table 1A
Characteristics of the ten contrasting soy sauces selected for the first experiment.

Soy sauce	Type	Abbrev.	Country of origin	Salt content (g/100 mL)	Soybean (content)	Wheat (content)	Other ingredients	Additives	Production details (if known)
Kikkoman natural	TF	KNA	Japan	16.9	Soybeans (n. d.)	Wheat (n. d.)	–	–	Traditional fermentation
Kikkoman low salt	TF	KLS	Japan	9.1	Soybeans (n. d.)	Wheat (n. d.)	Spirit vinegar, alcohol, sugar	–	Traditional fermentation, 43% salt is removed in a special process
Tamari gluten-free (Kikkoman)	TF	TAM	Japan	16.4	Soybeans (n. d.)	–	Spirit vinegar (Brandy)	–	Traditional fermentation using <i>Aspergillus tamarii</i>
Lee Kum Kee Premium Light	TF	LPL	China	17.2	Soybeans (11%)	Wheat flour (n.d.)	Sugar	E631, E627	Traditional fermentation
Lee Kum Kee Double Deluxe	TF	LDD	China	16.7	Soybeans (11%)	Wheat flour (n.d.)	Sugar	–	Traditional fermentation, double fermenting process
Haday superior light	TF	HSL	China	17.3	Soybeans (21.5%)	Wheat (n. d.)	Yeast extract	E631, E627	Traditional fermentation
Healthy Boy Shoyu	TF	HBS	Thailand (Japanese style)	10.2	Soybeans (n. d.)	Wheat flour (n.d.)	Sugar, high fructose syrup, mirin (glucose syrup, alcohol, glutinous rice, alanine)	E631, E627, E640, E211, E150c	Traditional fermentation (>6 months)
Sempio Jin Gold F3	Mixed	SJG	Korea	13.5	Defatted soybean (n. d.)	Wheat (n. d.)	High fructose corn syrup, spirits, liquorice extract, sucralose, yeast extract	E211	Combination of traditional fermented and acid hydrolysed soy sauces
Sempio Jin S	Mixed	SJS	Korea	13.4	Defatted soybeans (n. d.)	Wheat (n. d.)	High fructose corn syrup, spirits, sucralose, yeast extract	E211	Combination of traditional fermented and acid hydrolysed soy sauces
Data Puti	AH	DAP	Philippines	5.2	Soybean extract (35%)	n.d.	–	E150c, E211	Acid hydrolysed soy sauce using soy sauce protein

n.d.: Not defined on the product label or other reliable sources.

ND: Not defined, unclassified soy sauce.

Table 1B
Characteristics of the fifteen contrasting soy sauces selected for the second experiment.

Soy sauce	Type	Repeated in exp. 1	Abbrev.	Country of origin	Salt content (g/100 mL)	Soybean (content)	Wheat (content)	Other ingredients	Additives	Production details (if known)
Kikkoman natural	TF	Yes	KNA	Japan	16.9	Soybeans (n.d.)	Wheat (n.d.)	–	–	Traditional fermentation
Tamari gluten-free (Kikkoman)	TF	Yes	TAM	Japan	16.4	Soybeans (n.d.)	–	Spirit vinegar (Brandy)	–	Traditional fermentation using <i>Aspergillus tamarii</i>
Lee Kum Kee Double Deluxe	TF	Yes	LDD	China	16.7	Soybeans (11%)	Wheat flour (n.d.)	Sugar	–	Traditional fermentation, double fermenting process
Pearl River Bridge	TF	No	PDS	China	21.2	Soybeans (20%)	Wheat flour (n.d.)	Sugar	–	Traditional fermentation, non-GMO, no artificial flavouring added
Pearl River Bridge	TF	No	PLS	China	17.5	Soybeans (29%)	Wheat flour (n.d.)	–	E202 (potassium sorbate)	Traditional fermentation, non-GMO, no artificial flavouring added
Yamasa soy sauce	TF	No	YAM	Japan	16.5	Soybeans (n.d.)	Wheat (n.d.)	Alcohol	–	Brewed
Tai Hua Soy Sauce	TF	No	THD	Singapore	17.4	Soybeans (n.d.)	Wheat flour (n.d.)	Sugar, caramel	E211 (sodium benzoate)	Naturally brewed
Tamari megachef	TF	No	TOB	Thailand	15.1	Soybeans (21%)	–	Rice, glucose fructose syrup	E202 (potassium sorbate)	Traditionally brewed. Gluten-free. No artificial colour added. No MSG added
Wan Ja Shan (less salt)	TF	No	WJS	Taiwan	0.98	Soybeans (12%)	Wheat (n.d.)	Sugar, alcohol, lactic acid, yeast extract	–	100% naturally brewed. No preservatives
Inproba Bio-organic Soja sauce	TF	No	IBO	The Netherlands	19	Soybeans (24%)	Wheat (n.d.)	Alcohol	–	100% natural ingredients. No artificial additives
Chan's soy sauce Yellow Label	ND	No	CYL	The Netherlands	18.9	Soybeans (n.d.)	Wheat flour (n.d.)	Sugar	E150a	n.d.
Maekrua soy sauce Yellow Label	ND	No	MYL	Thailand	18.4	Soybeans (63%)	Wheat flour (20%)	Sugar (3.9%)	E211	n.d.
Sempio Jin S	Mixed	Yes	SJS	Korea	13.4	Defatted soybeans (n.d.)	Wheat (n.d.)	High fructose corn syrup, spirits, sucralose, yeast extract	E211	Combination of traditional fermented and acid hydrolysed soy sauces
Silver Swan soy sauce	AH	No	SSW	Philippines	17	Soybean protein (20%)	Wheat flour (n.d.)	–	E202, E150c	n.d.
Data Puti	AH	Yes	DAP	Philippines	5.2	Soybean extract (35%)	n.d.	–	E150c, E211	Acid hydrolysed soy sauce using soy sauce protein

n.d.: Not defined on the product label or other reliable sources.

ND: Not defined, unclassified soy sauce.

local stores in the Netherlands, or via online outlets. The different brands and types were selected based on the processing method (TF or AH), country of origin, salt content, wheat content and other ingredients or additives (Tables 1A and 1B). Physico-chemical properties of the soy sauces (such as density and pH) were measured and values were constant around 1.14 g/mL and pH 4.6. Samples were kept closed in their own containers at 4 °C, and aliquoted shortly before analysis.

AH soy sauces are often not clearly distinguished as “acid-hydrolysed”. Instead, the term soy protein or soy extract is often stated in the ingredients list, or the use of “brewed” and/or “fermented” in the label is avoided. Many times, they also come in cheaper plastic bottles, and are manufactured in countries such as The Philippines or Thailand where the use of AH is still allowed (China has banned AH soy sauces). Therefore, we hypothesized that soy sauces on the market without any clear labelling of a fermentation process and, instead, stated soy protein were AH soy sauces. In case of mixed soy sauces (SJS and SJG), this was

clearly stated on the product label. In the case of unclassified samples (CYL and MYL, Table 1B), no fermentation process was stated nor the use of soy protein.

Sodium chloride (NaCl) was purchased from Honeywell-Fluka (Seelze, Germany). An n-Alkane (C₈-C₂₂) series for calculating retention indices (RI) was prepared (Sigma Aldrich, St. Louis, MO, USA).

2.2. Sorptive-based techniques

For the determination of the volatile and semi-volatile profiles, two different trapping approaches (SPME and SBSE) were tested (Fig. S1). After extracting the analytes, these were then thermally desorbed and introduced into the GC-MS. The same GC-MS instrument and settings were employed for both trapping techniques, and both were performed using a multi-purpose autosampler (MP-2, Gerstel, Mülheim an der Ruhr, Germany), operated using Gerstel MAESTRO software (version

3.2). Below, the trapping and GC–MS conditions are summarized based on a previous method (Diez-Simon, Ammerlaan, & et al., 2020), with slight optimizations for the soy sauce matrix.

2.2.1. Solid-phase micro extraction (SPME)

A 5 mL aliquot of soy sauce was placed in a 10 mL crimp cap vial and 1.75 g of NaCl (to generate saturated conditions) was added. The vials were incubated at 60 °C for 10 min with agitation (250 rpm) to drive volatiles out of the liquid. Subsequently, volatiles were trapped by exposing the SPME fiber to the headspace above the liquid for 20 min at 60 °C without agitation (Diez-Simon, Ammerlaan, & et al., 2020). The fiber was then thermally desorbed onto the GC column via the cooled injection system (CIS, Gerstel, Mülheim an der Ruhr, Germany) containing a glass liner with a helium flow of 1 mL/min at 280 °C for 2 min, in splitless mode.

2.2.2. Stir bar sorptive extraction (SBSE)

A volume of 9 mL of soy sauce in a 10 mL screw-cap glass vial was used to trap volatiles. Subsequently, a stir bar (Twister® coated with 24 µL PDMS) was immersed in the sample and with continuous stirring at 450 rpm for 60 min. After extraction, the bars were removed from the samples, rinsed for 2–3 s with distilled water, dried with a lint-free tissue and placed inside clean glass liners for thermal desorption. Analyses were started immediately after sampling to prevent any loss of volatiles. Stir bars were desorbed in a thermal desorption unit (TDU, Gerstel, Mülheim an der Ruhr, Germany) in splitless mode using a helium flow of 1 mL/min. The initial temperature was 30 °C (0.5 min hold) and was then heated at a rate of 120 °C/min to a final temperature of 175 °C (5 min hold). Volatile compounds were transferred to the CIS containing a packed sorbent liner (Tenax TA), which was heated from –10 °C at a rate of 720 °C/min to a final temperature of 250 °C (5 min hold) to transfer the volatiles to the analytical column. During this, a split of 1:5 was used.

2.3. Gas Chromatography-Mass spectrometry (GC–MS) conditions

Analyses were conducted on an Agilent GC7890A coupled to a 5975C quadrupole mass spectrometer. The column used was a Zebtron ZB-5MSplus with dimensions 30 m × 0.25 mm × 1.00 µm (Phenomenex). The column oven was programmed starting at 45 °C for 2 min, then increased at a rate of 5 °C/min to 280 °C and then maintained at 280 °C for 5 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. Compound identification was based on the principles explained in Section 2.5.

2.4. GC-Olfactometry-MS analyses (GC-O-MS)

In order to relate the specific aroma characteristics with the individual compounds, a double detector (olfactory detection port, ODP, and MS) was used, by splitting the GC column outlet. Volatile extracts for GC-O-MS analysis were obtained using SPME, employing the sampling procedure described in Section 2.2.1. Analysis was performed on the same GC–MS instrument, but now connected with an ODP2 (Gerstel). A four-port splitter stand was located in the GC oven; two ports were connected to the column outlet and an auxiliary gas outlet; the two remaining ports were connected to the MS and the ODP. The column outlet pressure was 20 kPa. The capillary column (0.1 mm) of the transfer line was kept under constant temperature of 240 °C. An auxiliary gas (helium) flow of 5 mL/min was maintained constantly during analyses.

The analytical conditions were similar to those for GC–MS analysis, with a few modifications. The oven temperature was programmed as follows: 45 °C (2 min), 5 °C/min to 150 °C and then 15 °C/min to 250 °C (5 min). The sniffing procedure for each soy sauce was carried out by at least three individual panellists. A compound was deemed to be

aromatically active if it was perceived by two or more panellists at the same retention time and when it was described using similar odour qualities. Occasionally, aroma was also considered when only perceived by one panellist, but only if a similar attribute was identified at the same retention time in more than one soy sauce. The sensory attributes perceived by panellists at the ODP were linked to specific compounds by matching the retention times and by validating these compounds with aroma-active compounds previously reported in soy sauce. In order to relate these observations to (semi)quantitative differences between the soy sauces, the relative abundances (expressed in Total Ion Current, TIC) of the corresponding compounds after combined data processing were assessed.

2.5. Experimental setup

In the first experiment, a series of 10 soy sauces were analysed in a randomized way (Table 1A). In the second analysis, the sample set included 10 other soy sauces, along with five samples from experiment 1, but which had been re-purchased so that they could be considered as true biological samples (Table 1B).

For both experiments an empty glass vial and a blank (6 M NaCl in water) sample were measured at the beginning of each sequence. Quality control samples (QCs), which were a mix of all soy sauces, were repeatedly analysed along the sequence to test the performance stability. An n-Alkane (C₈–C₂₂) series was analysed at the end of the sequence.

The raw GC–MS data were processed using a nontargeted metabolomics approach. Baseline correction and alignment of all mass signals (with a signal to noise ratio $s/n \geq 3$) were performed using MetAlign software (Lommen, 2009). Signal redundancy was reduced to single representative variables and mass spectra were reconstructed using MSclust (Tikunov, Laptinok, Hall, Bovy, & de Vos, 2012). Metabolites were putatively identified by matching the obtained mass spectra and retention indices (RI) to those in the NIST17 Mass Spectral library (v.2.3), following the criteria for metabolite identification proposed by Sumner et al. (2007). Compounds that did not fit the criteria, were annotated as being non-identified. Retention indices were calculated based on a series of n-alkanes (C₈–C₂₂) using a third order polynomial function.

For statistical analysis, we compared and visualized the main tendencies of the generated data by principal components analysis (PCA) after log 10 transformation and unit variance (UV) scaling of the samples using SIMCA 15.0.2. software (Sartorius Stedim Data Analytics AB, Umeå, Sweden). Hierarchical cluster analysis (HCA) and partial least squares-discriminant analysis (PLS-DA) were carried out using SIMCA 15.0.2. software. Graphs were produced using Microsoft Excel 365 and RStudio.

3. Results and discussion

3.1. Comparison between SPME- and SBSE-GC–MS volatile profiles of soy sauce

Soy sauce aroma is characterized by a wide range of volatile compounds which enhance different sensory attributes of the product. Considering the large number of different volatiles present, it is important to develop a technique that allows us to analyse the broadest possible spectrum of volatiles, when we want to relate the chemical profile to aroma attributes and sample origin. We tested two volatile extraction techniques (SPME and SBSE, Fig. S1) to determine which one was able to deliver the broader range of compounds, in a repeatable manner. A nontargeted, MS-based metabolomics workflow revealed a total of 246 volatiles using SPME as compared to 542 for SBSE (Table S1), demonstrating that the overall analyte coverage was considerably higher for SBSE. Similar results had been obtained in a previous investigation of other food flavouring additives (Diez-Simon, Ammerlaan, & et al., 2020). We putatively identified 87 and 114 compounds for SPME and SBSE, respectively (Table S1). The group of

compounds trapped by both techniques was the largest (78). On the other hand, an additional 9 and 36 compounds were uniquely detected by SPME and SBSE, respectively. When comparing the volatile GC–MS profiles of the same soy sauce extracted by SPME and SBSE (e.g. Lee Kum Kee Premium Light, LPL Fig. 1), clear differences with respect to selectivity become apparent. A considerable number of semi-volatile compounds appearing at higher retention times was particularly trapped by SBSE, whereas SPME trapped more volatile compounds eluting earlier. For example, SPME exclusively trapped some highly volatile compounds such as 2-butenal and 2-pentanone, with vapour pressures of 30.0 and 35.4 mmHg at 25 °C respectively (U.S. Environmental Protection Agency, n.d.). SBSE trapped more, less-volatile compounds, including phenylethyl acetate and ethyl cinnamate, which have vapour pressures of 0.03 and 0.003 mmHg at 25 °C respectively (U.S. Environmental Protection Agency, n.d.). Interestingly, other volatiles relevant for flavour, such as quinoxalines (see Section 3.3), were only trapped by SBSE.

We evaluated the repeatability of both SPME and SBSE by analysing 10 technical replicates of the same soy sauce (Kikkoman natural, KNA). Both techniques showed a good repeatability with an average relative standard deviation (RSD) of compound intensities 11% and 13% for SPME and SBSE, respectively.

These findings revealed that SBSE performed well for the analysis of volatile compounds, while extracting an additional number of

compounds as compared to SPME. By this comparison, we are confident that SBSE is a robust method that offers more coverage of volatile compounds compared to SPME. Moreover, the targeted list of volatiles obtained by both SPME and SBSE had significant similarities to what was previously observed for a single Korean-type soy sauce (Lee et al., 2019).

3.2. Relation between volatile profile and production procedure

In the first trial, a set of ten contrasting soy sauces of diverse origin which are routinely available via local and online outlets was used (Table 1A) and subsequently, a second experiment was then performed to validate the findings of the first but using an expanded set of samples including some new samples as well as a number of the same types but from different production batches (Table 1B).

Multivariate PCA based on 542 volatile compounds detected in samples of the first trial showed the distribution of the soy sauces according to their volatile profiles (Fig. 2A). The technical variation in the volatile profiles as derived from the quality control samples and the replicate measurements was small compared to the variation between the different soy sauces (Fig. 2A). The biggest differences were related to the production process, where PC1 (R^2X : 27.6%) separates the TF soy sauces on the right of the score plot from the AH (DAP) and mixed soy sauces (SJG and SJS) on the left. The first two PCs explained ~ 40% of

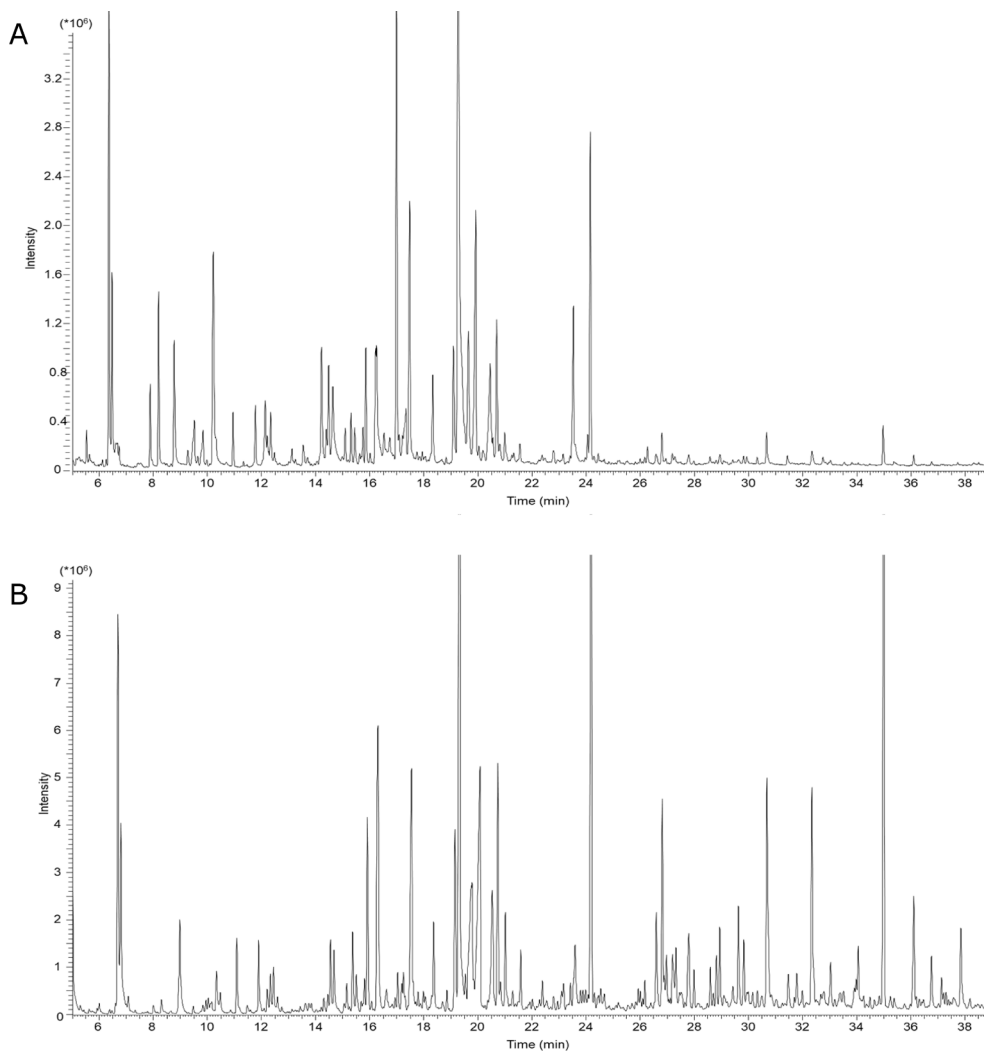


Fig. 1. Examples of representative GC–MS Total Ion Current (TIC) chromatograms of Lee Kum Kee Premium Light (LPL) soy sauce volatiles analysed by SPME (A) and SBSE (B). SBSE reveals more compounds later in the chromatogram, whereas SPME traps more compounds eluting at an earlier stage.

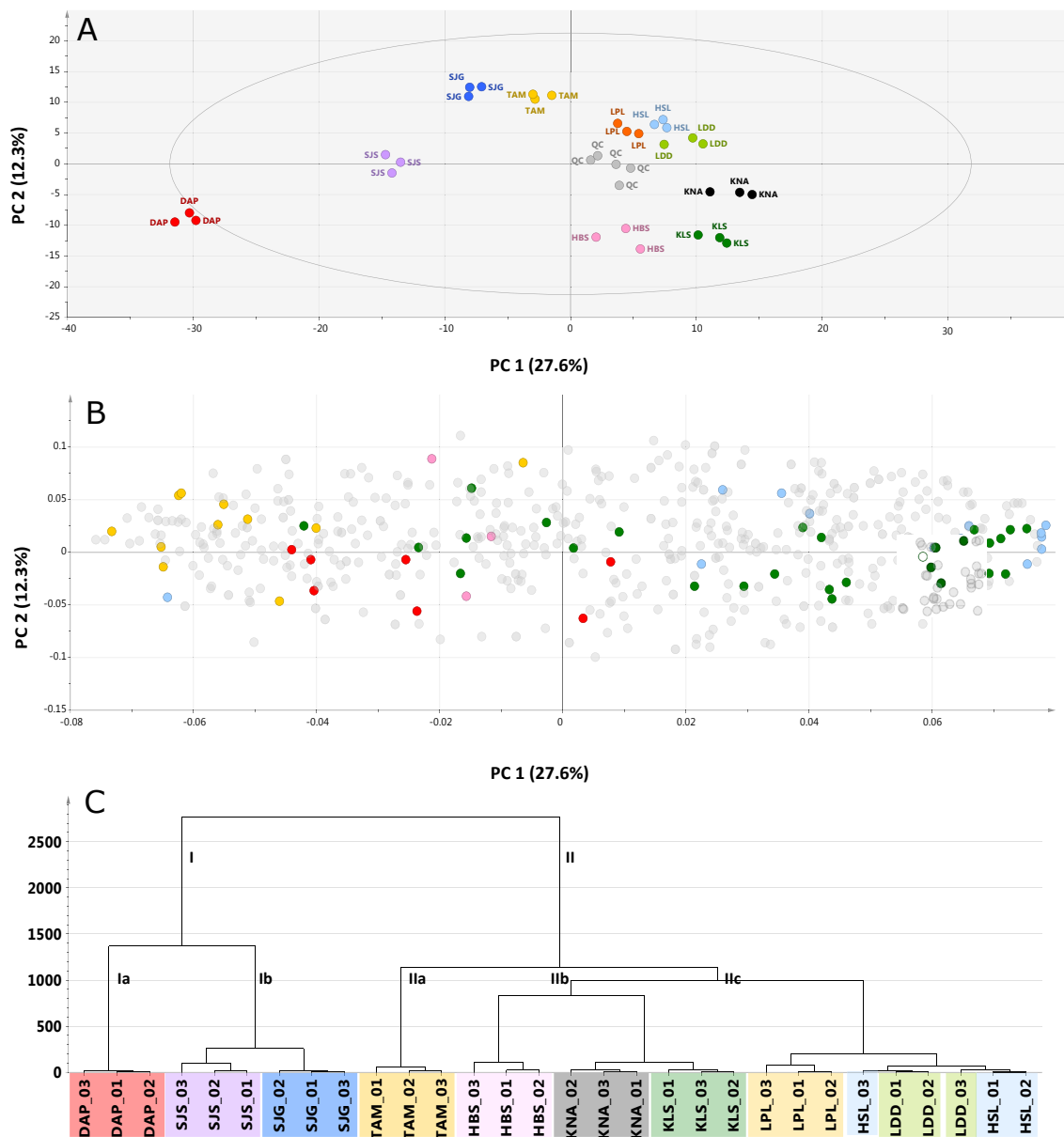


Fig. 2. Principal components analysis (PCA) score plot (A) and loading plot (B) of the 542 volatile compounds of 10 contrasting soy sauces using SBSE-GC-MS in Experiment 1. Three technical replicates are presented for each sample and five quality control samples (QC) are presented in grey. The first and second PC explain the corresponding percentage of variation shown on each axis. Putatively identified volatiles belonging to relevant chemical groups are coloured: acids (red), alcohols (light blue), esters (dark green), pyr(an)ones (pink) and pyrazines (yellow). Unidentified metabolites are coloured grey. (C) Hierarchical cluster analysis (HCA) dendrogram of the volatile profiles of the contrasting soy sauces using SBSE-GC-MS. Groups I and II contain (mixed) acid hydrolysed and fermented soy sauces respectively. Groups Ia and Ib contain exclusively and mixed acid hydrolysed soy sauces respectively. Groups IIa, IIb and IIc contain Tamari (TAM), Japanese type and Chinese type soy sauces respectively. Data Puti (DAP, red); Haday superior light (HSL, light blue); Healthy Boy Shoyu (HBS, pink); Kikkoman less salt (KLS, dark green); Kikkoman natural (KNA, black); Lee Kum Kee Double Deluxe (LDD, lime); Lee Kum Kee Premium Light (LPL, orange); Sempio Jin S (SJS, lavender); Sempio Jin Gold (SJG, dark blue) and Tamari Gluten-Free (TAM, yellow). For a full list of sample codes please see [Tables 1A and 1B](#). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the total variance, while no clear explanation for the distribution along PC 2 was found. Where PCA only depicts the grouping of samples – here in [Fig. 2A](#) and [B](#) based on 40% of the total variance, hierarchical cluster analysis (HCA) depicts associations based on total variance. HCA identified the largest difference between the samples to be related to the production method: chemical hydrolysis (AH, cluster I) versus biological fermentation (TF, cluster II) ([Fig. 2C](#)). However, in the HCA we can also differentiate subgroups within the TF soy sauces. Subgroup IIa contained the gluten-free replicates and the two other subgroups were composed of Japanese-type (IIb) and Chinese-type soy sauce samples (IIc). On the basis of this first analysis, it was concluded that the production method

and the ingredients used result in a clear separation of soy sauce samples based on their volatile profiles.

Looking deeper into the chemical differences, which are potentially causal to the distribution patterns observed in the PCA, revealed some trends. As seen in [Fig. 2B](#), where the identified volatiles are coloured based on chemical class, several identified volatiles, such as pyrazines (yellow), are predominantly co-localizing with the AH sample. On the other hand, esters (dark green) and alcohols (light blue) co-localise on the right side of the PCA with the TF samples. In a previous study using solvent extraction and SPME GC-MS, a high presence of esters and alcohols was detected in TF soy sauces which was attributed to microbial

fermentation (Lee et al., 2006). Pyrazines were more dominant in AH soy sauces and were linked to the long heating procedures used for acid hydrolysis, triggering the increased formation of Maillard reaction products. As our analyses are based on an untargeted metabolomics approach, we can now also detect many other still unidentified compounds which also appear to be specific for the different types of soy sauces. A number of these (in grey) are at the extremes of the axes of the PCA loading plot (Fig. 2B). Further focus to identify these compounds should follow as these can also be potentially relevant for defining the specific chemical nature of different soy sauces and be of relevance to aroma differences.

3.3. Validation of the contrasts between samples

To corroborate the findings and differences described in the first analysis, a second experiment was designed. Here, additional soy sauce brands of similar production procedures, complementary to those previously used, were analysed alongside a number of brands repeated from the first experiment (Table 1B). Soy sauce brands that had unclassified product labels were also selected in order to investigate their volatile distribution and gain more insight into their likely production background. PCA was again performed on the GC-MS data generated (Fig. S2). The results showed good complementarity with those previously obtained, since, once again, the samples with comparable production procedures showed the same distribution pattern.

To compare directly the results of the two experiments in one dataset, a heatmap of the merged data is shown in Fig. 3. This heatmap shows a two-dimensional hierarchical clustering, combined with a spatial heatmap representing the variation of metabolite intensities (log transformed data). The samples are divided into three main clusters. Group I comprised the AH soy sauces DAP and SSW (Ia), and the mixed Sempio soy sauces (Ib, SJS and SJG). Interestingly, unclassified soy sauces (CYL and MYL, in blue) grouped together with a number of other soy sauces labelled as being “naturally brewed” or “traditionally fermented” (PDS, PLS, THD and TOB), thus forming group II. That these soy sauces clustered separately from the other TF (group III) suggests differences in their production procedure and perhaps e.g. the use of higher

temperatures. High temperature treatments could trigger the degradation of some of the metabolites common in yeast fermentation and, simultaneously, may trigger the formation of volatiles that have a similar chemistry of those in AH/mixed soy sauces. Moreover, PDS, PLS and TOB had a common description in their label relating to the non-use of artificial flavourings. It may therefore be that these soy sauces may have had a common (natural) supplement. Lastly, the third cluster (group III) comprised TF soy sauces used. Within this cluster, subgroups are formed, which are again related to the country of origin / ratios of ingredients used. Subgroup IIIa consists of the gluten-free alternative (TAM), as was also seen in the first experiment. The absence of wheat in tamari soy sauce is known to result in a low abundance of alcohols, since these are formed from the carbohydrate degradation during yeast fermentation (Harada et al., 2018; Lee et al., 2013). However, as seen in Fig. S3A, alcohols such as 2-methyl-1-propanol are still present in low amounts in tamari, perhaps due to the carbohydrates present in the soybeans (~30%), and the addition of spirit vinegar lowers the pH, which is favourable for yeast activity. From the heatmap, specific volatiles that are highly correlated with Tamari soy sauce are highlighted in box number 1 (Fig. 3). Most of these compounds remain unidentified (See Fig. S3B for an example) but can nevertheless be of great interest as potential markers for tamari soy sauce, although the other tamari-type (TOB) lacked several of these volatiles. Subgroup IIIb consisted of the low-salt soy sauces KLS, WJS and HBS. Both KLS and WJS are the low-salt alternatives from the brand Kikkoman and Wan Ja Shan, and show high degrees of similarity in their volatile profiles. Interestingly, HBS soy sauce was not labelled as being “low-salt soy sauce”, even though it has the lowest salt content of all TF soy sauces (Table S1A and 1B). Moreover, all three had alcohol and sugar added as additional ingredients (Tables 1A and 1B). This demonstrates that salt content in soy sauce changes the volatile profile. The profiles of these brands were characterised by a number of compounds as highlighted in Box 2 (Fig. 3). The most dominant class of compounds from this selection was the acetals. Acetals have been previously detected in TF soy sauces at low amounts (Feng et al., 2017), but here, in the low-salt soy sauces some of the acetals are increased (Fig. S3C, D and E). The soy sauces KLS, WJS and HBS contained added ethanol, resulting in a higher ethanol



Fig. 3. Heatmap based on HCA of twenty soy sauces analysed by SBSE-GC-MS (x-axis) showing the distribution of the different 594 volatile and semi-volatile compounds on the y-axis. Upper bar colour codes: AH soy sauces (black); mixed soy sauces (red); unclassified soy sauces (blue); and TF soy sauces (green). Compounds that are strongly correlated/associated with groups IIIa and IIIb are highlighted in Boxes 1 and 2 respectively. For a full list of sample codes please see Tables 1A and 1B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

percentage as compared to the regular TF ones. For instance, Kikkoman soy sauce (KNA) has 2.5% ethanol whereas the low-salt version of the same brand (KLS) has 3.5% (Shurtleff & Aoyagi, 2012). Interestingly, comparable studies on Chinese soy sauce aroma type liquors containing 40–55% ethanol, revealed acetal presence at high concentrations (Fan & Qian, 2006; Fan, Shen, & Xu, 2011). This suggests that ethanol content may be a major factor influencing acetal formation in soy sauce.

The next subgroup (IIIc) is comprised of LDD, LPL and HSL soy sauces. These three were produced in China, where they use a ratio of soybeans to wheat of 80:20. Lastly, subgroup IIId, contained the soy sauces KNA, YAM and IBO which are characterized as being Japanese-type. IBO, however, was manufactured in The Netherlands but was expected to be a Japanese-type soy sauce, using an equal ratio of soybeans and wheat. As seen in Fig. 3, Chinese- and Japanese-types contained various qualitative and quantitative differences in their volatile profiles. However this data is not further discussed here as both types have been studied and extensively compared before for their aroma profiles (Feng, Su, et al., 2015). These authors compared twenty-seven commercial soy sauces from both origins and found similar volatiles occurring in both although intensities differed. In addition, Sun et al. (2010) studied the volatile composition of twelve Chinese soy sauces and observed major differences between them. This was attributed to the raw materials collected from local areas and the different microorganisms employed by manufacturers.

In this study, the biggest differences in volatile profiles were found between TF and AH soy sauces. In order to find potential markers for the two main groups, a PLS-DA analysis was performed by including AH soy sauces in one class, and TF in the other class (thus - excluding soy sauces from group II). The resulting metabolites, including both identified and non-identified, with the highest regression coefficients were selected as being the best potential predictors to differentiate between the two classes. Volatiles such as esters, and ketones were highly (or only) present in TF soy sauces (Table 2)- for example, butanedioic acid diethyl ester (Fig. S4A), was found predominantly in TF soy sauces. Again, it was previously reported albeit from a single study that the presence of esters is related to fermentation (Lee et al., 2006). However, we also detected compounds that have not been described before in TF soy sauce. For instance, the two ketones 5(or 4)-methyl-2-hexanone were considerably more abundant in TF soy sauces (Fig. S4B). These ketones were also present in mixed soy sauces (SJS and SJG). Their presence in one of the soy sauces in group II (THD) suggests that this sample was also at least partly of TF origin. Moreover, many non-identified compounds had also a strong correlation with TF soy sauces (Table 2, and Fig. S4C and D as examples). However, further identification should follow to understand how these compounds appear in TF soy sauces.

Volatiles such as pyrazine derivatives, some aldehydes and quinoxaline derivatives were highly (or only) present in AH soy sauces (Table 2). Pyrazines in soy sauce can be related to the heating procedures used as they can be formed through Maillard reactions at relatively high temperatures (Lee et al., 2006). Quinoxaline derivatives were present in the AH soy sauces, as well as in the mixed and some of the poorly defined soy sauces from group II (Fig. S4E). Quinoxaline derivatives contain a bicyclic heterocycle formed from a benzene ring fused to a pyrazine ring. 5-Methylquinoxaline, for instance, has been detected in coffee and related products and it is characterized as a burnt, coffee, and corn tasting compound (www.foodb.ca). Quinoxalines have not yet been characterized before in soy sauce. The origins and formation of these compounds are diverse, however, they may be related to Maillard reactions and/or the reduction of amino acids (Mamedov, 2016). Lastly, methional had also a high PLS regression coefficient for AH/mixed soy sauces. Its intensity was observed to be higher in DAP and SSW (Fig. S4), but was however, also present in smaller amounts in some of the fermented soy sauces, including the low-salt TFs. The sulfurous compound methional (cooked potato-like odour) has been recently reported as a key aroma compound in Chinese soy sauces (Wang, Guo, Song, & Meng, 2020) and is well-known to be generated from

methionine by Strecker degradation (Pripis-Nicolau, De Revel, Bertrand, & Maujean, 2000). It has also been detected in a Korean soy sauce after long-term fermentation (Lee, Kim, & Kim, 2019). Using acid hydrolysis, this reaction may become more prominent and thus a higher abundance is found in AH soy sauces.

Overall, soy sauces from different production methods could readily be characterized using SBSE-GC-MS and volatiles annotated as being significantly present in both TF and/or AH types, as well as subgroups within. This augured well for the further determination of the possibility to link these chemical differences with sensory relevance and impact. Consequently, the following section focuses on identifying compounds responsible for the aroma characteristics.

3.4. Odour attributes linked to chemical compounds and their potential contribution to overall flavour

The typical aroma of soy sauce is associated with the large variety of volatiles found in appropriate configurations (Nunomura et al., 1976). Next to concentration, the odour threshold per compound is also important in determining sensory impact. Those compounds with a low odour threshold therefore still frequently dominate the overall odour despite perhaps only being present in trace amounts. Recently, the first flavour wheel of soy sauce has been proposed, which compiles the most important taste and aroma attributes described in the literature, together with the chemical compounds linked to each attribute (Diez-Simon, Ammerlaan, & et al., 2020). However, little is known about the differences in flavour characteristics between soy sauces produced by the contrasting TF and AH methods.

To relate the chemical similarities and differences between TF and AH soy sauces to sensory attributes and hence get a better view of their sensory relevance, gas chromatography-olfactometry-mass spectrometry (GC-O-MS) analysis was performed using four contrasting soy sauces. KNA, LDD, SJG and DAP were selected, based on their differential volatile profiles and contrasting origins. Chemical structural information obtained using GC-MS has been combined with the aroma characteristics as perceived by panellists using an Olfactory Detection Port (ODP). To trap and concentrate volatiles, SPME was chosen since it is more representative of the real-life situation of odour perception.

The list of aroma-active compounds recognized by the panellists using SPME-GC-O-MS is presented in Table 3. Most of the aroma-active compounds identified were related to sweet, floral, savoury and smoky descriptors. Benzeneacetaldehyde (floral) and guaiacol (smoky, burnt) were perceived in all samples as 'intense' odorants. These compounds had also been previously described in soy sauce as being "key odorants" with low odour thresholds (Feng, Cai, et al., 2015; Kaneko, Kumazawa, & Nishimura, 2013; Steinhaus & Schieberle, 2007; Wang et al., 2020). Phenylethyl alcohol, methional and 3-methylbutanal, characterized by having floral/sweet, cooked potato and malty notes respectively, have also been frequently reported as being key aroma compounds in soy sauce (Devanathi & Gkatzionis, 2019; Wang et al., 2020). Moreover, compounds that were perceived by panellists, but which are not yet identified, are also reported here as these represent a new set of metabolites of clear importance to sensory impact. Future attention is needed to ascertain their identities. For example, a certain popcorn aroma was perceived at 16.45 min, which was linked to the non-identified compound number 21, was only detected here in DAP soy sauce (AH) (Table 3). This aroma has not been linked to an identified compound in soy sauce yet. However, it has been characterized using GC-O-MS in TF Chinese soy sauces as being an important attribute (Wang et al., 2020). Being only detected here in DAP suggests this compound has higher concentrations in AH soy sauces. Another interesting example is compound #25, characterized by an intense liquorice/candy aroma at an R.T. of 18.90 min (Table 3). Liquorice is a unique aroma attribute that has to our best knowledge not yet been characterized in soy sauce. It was perceived in both the TF soy sauces (KNA and LDD) and again, more detailed study is needed to identify its chemical

Table 2

List of compounds selected, from a PLS-DA analysis, as being characteristic of [A]: TF soy and [B]: AH soy sauces, including both identified and non-identified compounds.

A: Selected markers for traditional fermented (TF) soy sauces									
Nr.	R.T. ^a (min)	RI ^b (exp)	Name	Molecular Formula	Chemical Group	CAS	Sample Name ^c	Max. TIC value	Regression coefficient
1	9.03	813	acetic acid, butyl ester	C6H12O2	Ester	123–86-4	HBS	3,028,721	–0.00393
2	10.16	846	4-methyl-2-hexanone	C7H14O	Ketone	105–42-0	LDD	1,429,707	–0.00461
3	10.49	856	5-methyl-2-hexanone	C7H14O	Ketone	110–12-3	THD	2,147,159	–0.00434
4	13.25	937	2-methyl-2-butenic acid, ethyl ester	C7H12O2	Ester	5837–78-5	YAM	274,795	–0.00426
5	18.70	1102	benzoic acid, methyl ester	C8H8O2	Ester	93–58-3	THD	2,667,757	–0.00457
6	21.01	1176	butanedioic acid, diethyl ester	C8H14O4	Ester	123–25-1	YAM	15,190,975	–0.00404
7	21.80	1201	methylbutanedioic acid, diethyl ester	C9H16O4	Ester	4676–51-1	YAM	52,922	–0.00447
8	22.39	1221	3-pyridinecarboxylic acid, ethyl ester	C8H9NO2	Ester	614–18-6	KNA	6,499,150	–0.00442
9	24.80	1303	difurfuryl ether	C10H10O3	Furan(one)	4437–22-3	TOB	1,866,908	–0.00519
10	27.52	1401	N-acetylucine, ethyl ester	C10H19NO3	Ester	4071–36-7	YAM	465,680	–0.00539
11	28.95	1455	dimethyl phthalate	C10H10O4	Other	131–11-3	HBS	1,710,618	–0.00495
12	32.36	1591	4-hydroxy-3-methoxybenzoic acid, ethyl ester	C10H12O4	Ester	617–05-0	TAM	12,171,194	–0.00403
13	14.80	983	N.I.1				KLS	89,193	–0.00494
14	15.92	1016	N.I.2				HSL	9,380,108	–0.00490
15	16.27	1027	N.I.3				HSL	6,691,928	–0.00525
16	17.18	1055	N.I.4				HSL	81,901	–0.00514
17	19.55	1129	N.I.5				HSL	1,348,279	–0.00563
18	22.95	1240	N.I.6				SJG	685,631	–0.00554
19	23.56	1260	N.I.7				WJS	380,569	–0.00498
20	27.12	1386	N.I.8				YAM	348,751	–0.00487
21	27.75	1409	N.I.9				YAM	683,476	–0.00528
22	27.80	1411	N.I.10				LDD	3,124,467	–0.00518
23	30.06	1498	N.I.11				TAM	124,975	–0.00515
24	32.33	1590	N.I.12				LDD	587,077	–0.00486
25	34.50	1683	N.I.13				HBS	391,675	–0.00583
26	35.00	1705	N.I.14				PLS	13,428,900	–0.00534
27	36.13	1757	N.I.15				LPL	2,146,211	–0.00505
B: Selected markers for acid hydrolysed (AH) soy sauces									
Nr.	R.T. ^a (min)	RI ^b (exp)	Name	Molecular Formula	Chemical Group	CAS	Sample Name ^c	Max. TIC value	Regression coefficient
1	6.94	752	2-methyl-2-butenal	C5H8O	Aldehyde	1115–11-3	DAP	3,972,751	0.00559
2	7.88	779	3-methylthiophene	C5H6S	Sulphur compound	616–44-4	PLS	683,256	0.00552
3	9.48	826	methylpyrazine	C5H6N2	Pyrazine	109–08-0	SSW	9,494,707	0.00537
4	12.26	907	methional	C4H8OS	Sulphur compound	3268–49-3	SSW	428,404	0.00539
5	12.45	913	2,6-dimethylpyrazine	C6H8N2	Pyrazine	108–50-9	PDS	12,548,582	0.00543
6	12.59	917	ethylpyrazine	C6H8N2	Pyrazine	13925–00-3	DAP	1,455,178	0.00532
7	12.65	919	2,3-dimethylpyrazine	C6H8N2	Pyrazine	5910–89-4	SSW	1,316,767	0.00599
8	15.51	1004	trimethylpyrazine	C7H10N2	Pyrazine	14667–55-1	DAP	3,255,142	0.00490
9	18.19	1086	2-ethyl-3,5-dimethylpyrazine	C8H12N2	Pyrazine	13925–07-0	DAP	517,411	0.00533
10	21.38	1188	2,5-dimethylbenzaldehyde	C9H10O	Aldehyde	5779–94-2	DAP	10,879,260	0.00554
11	22.59	1228	quinoxaline	C8H6N2	Other	91–19-0	DAP	256,354	0.00560
12	25.21	1317	2-methylquinoxaline	C9H8N2	Other	7251–61-8	SJG	281,558	0.00552
13	26.06	1348	5-methylquinoxaline	C9H8N2	Other	13708–12-8	SSW	104,147	0.00532
14	29.07	1459	N-(4-amino-3-furazanyl)acetamide	C4H6N4O2	Other	140706–47-4	SJS	163,748	0.00570
15	12.33	910	N.I.1				SSW	2,359,853	0.00478
16	13.22	910	N.I.2				DAP	82,860	0.00518
17	13.31	936	N.I.3				PDS	58,539	0.00486
18	14.84	938	N.I.4				TOB	219,753	0.00561
19	18.63	984	N.I.5				SJS	817,662	0.00511
20	22.80	1100	N.I.6				SJG	181,093	0.00484
21	25.01	1235	N.I.7				SSW	772,663	0.00510
22	25.52	1311	N.I.8				SJG	141,437	0.00507
23	26.53	1328	N.I.9				THD	5,475,170	0.00496
24	26.63	1364	N.I.10				SJS	28,316,367	0.00502
25	29.58	1368	N.I.11				DAP	899,356	0.00560
26	35.58	1624	N.I.12				DAP	109,617	0.00538
27	40.43	1731	N.I.13				DAP	1,121,931	0.00485

^a Retention time (min).

^b Retention Index (experimental).

^c Sample code (see Tables 1A and 1B) which gave the maximum TIC value for each metabolite.

Table 3

Aroma-active compounds detected using SPME-GC-O-MS in four selected soy sauces. Panellists evaluation (experimental) are compared to the characteristics reported in the Food database (www.fooddb.ca). A compound was associated with each aroma quality.

No.	R.T (min)	Aroma quality (experimental)	Aroma quality (FoodDB)	Compound name	Odour threshold ^a (µg/kg)		Detected in/number of panellists				
							DAP	SJG	LDD	KNA	
1	4.46	candy, sweet		2-butenal	-	-	x	2		x	3
2	4.77	malty, savoury	malty, fatty	3-methylbutanal	1.2	low					
3	9.28	cheese, pungent, chemical		Unknown						x	3
4	9.45	fresh, floral		Unknown						x	2
5	9.85	unpleasant, sweaty, cheese	sweaty, rancid	3-methylbutanoic acid	1200	high		x	2	x	3
6	10.19	sweet, liquorice, winegum		N.L.						x	2
7	10.60	fruity, sweet		1,1-diethoxy-2-methylpropane	-	-				x	2
8	11.20	medicine, sweet		methylbutyl acetate	-	-		x	2		
9	12.15	chemical, acid		N.L.						x	3
10	12.47	cooked potato	cooked potato	Methional	1.4	low	x	2		x	1*
11	12.58	rancid, cheese		Unknown						x	3
12	12.74	sweet	cocoa, roastbeef, roastednut	2,6-dimethylpyrazine	157.6	medium	x	3			
13	12.99	popcorn, cooked rice		Unknown						x	2
14	13.03	sweaty, rancid		N.L.						x	2
15	14.19	sweet, pungent		N.L.						x	3
16	14.40	sweet	almond, burnt sugar	Benzaldehyde	751	medium			x	3	
17	14.90	mushroom, earthy		Unknown				x	2	x	3
18	14.88	rancid, sulphur	cabbage, sulphur, cooked	dimethyl trisulfide	0.016	very low	x	3			
19	15.60	sweet, floral	n.a.	2-ethyl-6-methylpyrazine	-	-	x	2			
20	15.97	mushroom, fungal		Unknown						x	2
21	16.45	popcorn, intense		Unknown			x	2			
22	17.24	floral		benzeneacetaldehyde	4	low	x	1*	x	3	x
23	17.40	caramel, sweet, candyfloss		Unknown						x	3
24	18.55	smoky, burnt	smoky, medicinal	Guaiacol	1.6	low		x	2		
25	18.90	liquorice candy		Unknown						x	3
26	19.30	caramel, honey		Maltol	35,000	high				x	3
27	19.47	floral, sweet		phenylethyl alcohol	390	medium				x	1*
28	19.60	burnt, fireplace, carbon		Unknown			x	2			
29	19.80	caramel, sweet, candyfloss		Unknown				x	2	x	3
30	21.34	sour		Unknown							x
31	21.39	savory, mushroom		Unknown						x	3
32	22.98	sweet, unpleasant	n.a.	3-phenylfuran	5.9	low					x
33	23.10	sweet		Unknown				x	3		
34	23.31	floral, caramel	floral, sweet, anise	ethyl phenylacetate	155.6	medium				x	2
35	24.15	cinnamon, spicy	spice, clove, bacon	4-ethyl guaiacol	16	medium				x	3
36	25.50	smoky, spicy		2,6-dimethoxyphenol	-	-		x	3		
37	26.19	sweet		Unknown						x	3

^aOdour threshold values were collected from the same source (Czerny et al. 2008).

*Only perceived by one panellist (less reliable).

N.L.: attribute not linked to any visible chromatographic peak.

structure. Other compounds have been characterized by having a liquorice-like odour in fermented fish and soy products, such as estragol (aniseed-like, liquorice-like) and 2-pentyl furan (beany, grassy, liquorice-like) (Czerny et al., 2008; Giri, Osako, Okamoto, & Ohshima, 2010) which could be potential candidate compounds.

In general, most odorants were detected by GC-MS in each of the four soy sauces. However, not all were also perceived by GC-O, as illustrated in Table 3. Furthermore, some compounds were detected by GC-O and not by GC-MS (labelled N.L.). This suggests that both abundance and odour threshold play a role in determining whether or not a compound is perceived in a particular soy sauce and hence, has sensory impact or not. Some compound concentrations were too low to reach human detection level in certain samples. This is visible for example when the semiquantitative differences in the GC-MS data are compared with GC-O observations across samples. For example, the attribute smoky, spicy was associated with 2,6-dimethoxyphenol (Table 3). 2,6-Dimethoxyphenol was detected by GC-MS in all 4 samples but had a four-fold higher abundance in SJG soy sauce compared to the others (Fig. S5A). However, the characteristic smoky/spicy aroma was only perceived by panellists in sample SJG. This indicates that odour threshold was only reached in the single SJG sample with the highest concentration. Another attribute, characterized by an intense cinnamon and spice-like odour, was associated with 4-ethyl guaiacol and was only

observed in LDD where it was also found to have an 18-fold higher abundance compared to the other three samples (Fig. S5B). This compound has previously been reported as a key odorant in Chinese soy sauces (Wang et al., 2020). Lastly, maltol (caramel-like) is a key odorant in soy sauce and it is characterized by having a high odour threshold value (3500 µg/kg). In our study, maltol was detected by GC-MS in all four samples, however was only perceived using GC-O in LDD (Fig. S5C). Equally important, a number of odours were conversely clearly perceived by panellists but were not visible as a peak in the GC-MS trace (annotated as N.L. in Table 3). This suggests that the causal compounds are at sub-detection limits but yet are still at levels above their odour threshold. This implies that the human receptors in our nose are more sensitive to these compounds than the Mass Spectrometer used and indicates we cannot fully rely on instrumentation to define sensory experience.

In conclusion, 37 aroma attributes were detected by SPME-GC-O-MS and associated with volatile compounds, of which 19 could be identified and 18 are still unknown. These volatiles also displayed a contrasting aroma pattern between the four tested soy sauces and the presence of some odorants appears to be linked to production origin. This suggests that the aroma of a soy sauce, and hereby potentially also consumer preference, can be linked to the production procedure.

4. Conclusions

This study reports a comprehensive metabolomics analysis of volatiles from soy sauces of different origins, production methods, or using different ratios of ingredients. Under the optimized experimental conditions, SBSE proved to be the extraction method which covered a large number of volatiles detected by GC-MS, in a reproducible manner. The nontargeted approach revealed that the largest difference in the volatile profiles between the twenty commercial soy sauces was related to the production procedure applied: Traditional Fermentation versus Acid Hydrolysis. The AH group was strongly associated with pyrazines, quinoxalines and sulphur compounds, whereas TF soy sauces were more affiliated with esters and ketones. The use of high temperatures during acid hydrolysis likely resulted in higher amounts of Maillard reaction products, while microbial activity in traditional fermentation gave the highest diversity in volatiles. A large number of non-identified compounds also appeared to be characteristic/unique for either of the two types of soy sauces, which delivers additional potential compounds for further identification.

Characterization of some aroma-active compounds by SPME-GC-OMS, revealed a contrasting distribution of the detected odorants across the samples related to both technical and human detection thresholds. Further investigation is needed to establish how these differences affect the overall aromas of the soy sauces.

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 animal participants performed by any of the authors.

CRediT authorship contribution statement

Carmen Diez-Simon: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Charlotte Eichelsheim:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft. **Doris M. Jacobs:** Funding acquisition, Resources, Writing - review & editing. **Roland Mumm:** Conceptualization, Methodology, Supervision, Writing - review & editing. **Robert D. Hall:** Conceptualization, Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors acknowledge funding from the Netherlands Organisation for Scientific Research (NWO Proj. No. 731.015.207), DSM Food Specialities and Unilever Research in support of this work. The authors also thank Jasper Engel for supplying the R script of the heatmap.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2021.110348>.

References

Chen, Z. Y., Feng, Y. Z., Cui, C., Zhao, H. F., & Zhao, M. M. (2015). Effects of koji-making with mixed strains on physicochemical and sensory properties of Chinese-type soy sauce. *Journal of the Science of Food and Agriculture*, 95(10), 2145–2154.

- Czerny, M., Christbauer, M., Christbauer, M., Fischer, A., Granvogel, M., Hammer, M., ... Schieberle, P. (2008). Re-investigation on odour thresholds of key food aroma compounds and development of an aroma language based on odour qualities of defined aqueous odorant solutions. *European Food Research and Technology*, 228(2), 265–273. <https://doi.org/10.1007/s00217-008-0931-x>.
- David, F., Ochiai, N., & Sandra, P. (2019). Two decades of stir bar sorptive extraction: A retrospective and future outlook. *TrAC - Trends in Analytical Chemistry*, 112, 102–111. <https://doi.org/10.1016/j.trac.2018.12.006>.
- Devanathi, P. V. P., & Gkatzionis, K. (January 2019). Soy sauce fermentation: Microorganisms, aroma formation, and process modification. *Food Research International*, 120, 364–374.
- Diez-Simon, C., Eichelsheim, C., Mumm, R., & Hall, R. D. (2020). Chemical and sensory characteristics of soy sauce: A review. *Journal of Agricultural and Food Chemistry*, 68(42).
- Diez-Simon, C., Ammerlaan, B., van den Berg, M., van Duynhoven, J., Jacobs, D., Mumm, R., & Hall, R. D. (2020). Comparison of volatile trapping techniques for the comprehensive analysis of food flavourings by Gas Chromatography-Mass Spectrometry. *Journal of Chromatography A*, 1624, 461191. <https://doi.org/10.1016/j.chroma.2020.461191>.
- Fan, W., & Qian, M. C. (2006). Characterization of aroma compounds of Chinese “Wuliangye” and “Jiannanchun” liquors by aroma extract dilution analysis. *Journal of Agricultural and Food Chemistry*, 54(7), 2695–2704. <https://doi.org/10.1021/jf052635t>.
- Fan, W., Shen, H., & Xu, Y. (2011). Quantification of volatile compounds in Chinese soy sauce aroma type liquor by stir bar sorptive extraction and gas chromatography-mass spectrometry. *Journal of the Science of Food and Agriculture*, 91(7), 1187–1198. <https://doi.org/10.1002/jsfa.4294>.
- FAOSTAT (2017). Retrieved February 19, 2020, from <http://www.fao.org/faostat/en/#data/TP>.
- Feng, Y., Cai, Y., Su, G., Zhao, H., Wang, C., & Zhao, M. (2014). Evaluation of aroma differences between high-salt liquid-state fermentation and low-salt solid-state fermentation soy sauces from China. *Food Chemistry*, 145, 126–134. <http://www.sciencedirect.com/science/article/pii/S0308814613010017>.
- Feng, Y., Cai, Y., Sun-Waterhouse, D., Cui, C., Su, G., Lin, L., & Zhao, M. (2015). Approaches of aroma extraction dilution analysis (AEDA) for headspace solid phase microextraction and gas chromatography-olfactometry (HS-SPME-GC-O): Altering sample amount, diluting the sample or adjusting split ratio? *Food Chemistry*, 187, 44–52. <https://doi.org/10.1016/j.foodchem.2015.03.138>.
- Feng, Y., Su, G., Sun-Waterhouse, D., Cai, Y., Zhao, H., Cui, C., & Zhao, M. (2017). Optimization of headspace solid-phase micro-extraction (HS-SPME) for analyzing soy sauce aroma compounds via coupling with direct GC-olfactometry (D-GC-O) and gas chromatography-mass spectrometry (GC-MS). *Food Analytical Methods*, 10(3), 713–726.
- Feng, Y., Su, G., Zhao, H., Cai, Y., Cui, C., Sun-Waterhouse, D., & Zhao, M. (2015). Characterisation of aroma profiles of commercial soy sauce by odour activity value and omission test. *Food Chemistry*, 167, 220–228. <http://www.sciencedirect.com/science/article/pii/S0308814614009431>.
- Giri, A., Osako, K., Okamoto, A., & Ohshima, T. (2010). Olfactometric characterization of aroma active compounds in fermented fish paste in comparison with fish sauce, fermented soy paste and sauce products. *Food Research International*, 43(4), 1027–1040. <http://www.sciencedirect.com/science/article/pii/S096399691000030X>.
- Harada, R., Yuzuki, M., Ito, K., Shiga, K., Bamba, T., & Fukusaki, E. (2018). Microbe participation in aroma production during soy sauce fermentation. *Journal of Bioscience and Bioengineering*, 125(6), 688–694. <https://doi.org/10.1016/j.jbiosc.2017.12.004>.
- Hosking, R. (1996). Soy Sauce. In N. Ishige (Ed.), *A dictionary of Japanese food: Ingredients & culture* (1st ed., pp. 204–206). Tokyo: Tuttle Publishing.
- Kaneko, S., Kumazawa, K., & Nishimura, O. (2013). Studies on the Key Aroma Compounds in Raw (Unheated) and Heated Japanese Soy Sauce. *Journal of Agricultural and Food Chemistry*, 61(14), 3396–3402. <https://doi.org/10.1021/jf400353h>.
- Lee, B. Q., & Khor, S. M. (2015). 3-chloropropane-1,2-diol (3-MCPD) in soy sauce: A review on the formation, reduction, and detection of this potential carcinogen. *Comprehensive Reviews in Food Science and Food Safety*, 14(1), 48–66. <https://doi.org/10.1111/1541-4337.12120>.
- Lee, K. E., Lee, S. M., Choi, Y. H., Hurh, B. S., & Kim, Y.-S. (2013). Comparative volatile profiles in soy sauce according to inoculated microorganisms. *Bioscience, Biotechnology, and Biochemistry*, 77(11), 2192–2200. <https://doi.org/10.1271/bbb.130362>.
- Lee, S. M., Seo, B. C., & Kim, Y. S. (2006). Volatile compounds in fermented and acid-hydrolyzed soy sauces. *Journal of Food Science*, 71(3), C146–C156. <https://doi.org/10.1111/j.1365-2621.2006.tb15610.x>.
- Lee, S. M., Kim, S. B., & Kim, Y. S. (2019). Determination of key volatile compounds related to long-term fermentation of Soy Sauce. *Journal of Food Science*, 84(10), 2758–2776. <https://doi.org/10.1111/1750-3841.14771>.
- Lommen, A. (2009). MetAlign: Interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. *Analytical Chemistry*, 81(8), 3079–3086. <http://proxy.libraries.smu.edu/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=a9h&AN=39362778&site=ehost-live&scope=site>.
- Luh, B. S. (1995). Industrial production of soy sauce. *Journal of Industrial Microbiology*, 14, 467–471.
- Mamedov, V. A. (2016). Quinoxaline—As a parent heterocycle. In Quinoxalines: Synthesis, reactions, mechanisms and structure (pp. 1–437).

- Nogueira, J. M. F. (2015). Stir-bar sorptive extraction: 15 years making sample preparation more environment-friendly. *TiAC Trends in Analytical Chemistry*, 71, 214–223. <http://linkinghub.elsevier.com/retrieve/pii/S016599361500165X>.
- Nunomura, N., Sasaki, M., Asao, Y., & Yokotsuka, T. (1976). Identification of volatile components in Shoyu (Soy Sauce) by gas chromatography-mass spectrometry. *Agricultural and Biological Chemistry*, 40(3), 485–490. <https://doi.org/10.1080/00021369.1976.10862082>.
- Ohyama, T. (2013). Traditional and modern Japanese soy foods : Manufacturing, nutrition and cuisine of a variety of soy foods for health and joy of taste. *Food science and technology TA - TT*. Hauppauge, New York SE: Nova Science Publishers Inc.
- Pripis-Nicolau, L., De Revel, G., Bertrand, A., & Maujean, A. (2000). Formation of flavor components by the reaction of amino acid and carbonyl compounds in mild conditions. *Journal of Agricultural and Food Chemistry*, 48(9), 3761–3766.
- Shurtleff, W., & Aoyagi, A. (2012). *History of soy sauce (160 CE to 2012)* (1st ed.). Lafayette: Soyinfo Center.
- Steinhaus, P., & Schieberle, P. (2007). Characterization of the key aroma compounds in soy sauce using approaches of molecular sensory science. *Journal of Agricultural and Food Chemistry*, 55(15), 6262–6269. <https://doi.org/10.1021/jf0709092>.
- Sumner, L. W., Amberg, A., Barrett, D., Beale, M. H., Beger, R., Daykin, C. A., ... Viant, M. R. (2007). Proposed minimum reporting standards for chemical analysis: Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics*, 3(3), 211–221. <https://doi.org/10.1007/s11306-007-0082-2>.
- Sun, S. Y., Jiang, W. G., & Zhao, Y. P. (2010). Profile of volatile compounds in 12 Chinese Soy Sauces produced by a high-salt-diluted state fermentation. *Journal of the Institute of Brewing*, 116(3), 316–328. <https://doi.org/10.1002/j.2050-0416.2010.tb00437.x>.
- Tikunov, Y. M., Laptinok, S., Hall, R. D., Bovy, A., & de Vos, R. C. H. (2012). MSClust: A tool for unsupervised mass spectra extraction of chromatography-mass spectrometry ion-wise aligned data. *Metabolomics*, 8(4), 714–718. <https://doi.org/10.1007/s11306-011-0368-2>.
- U.S. Environmental Protection Agency. CompTox Chemicals Dashboard. (n.d.). Retrieved from <https://comptox.epa.gov/dashboard/>.
- Wanakhachornkrai, P., & Lertsiri, S. (2003). Comparison of determination method for volatile compounds in Thai soy sauce. *Food Chemistry*, 83, 619–629. <http://www.sciencedirect.com/science/article/pii/S0308814603002565>.
- Wang, X., Guo, M., Song, H., & Meng, Q. (April 2020). Characterization of key aroma compounds in traditional Chinese soy sauce through the molecular sensory science technique. *Lwt*, 128, 109413. <https://doi.org/10.1016/j.lwt.2020.109413>.
- Zheng, J., Wu, C. D., Huang, J., Zhou, R. Q., & Liao, X. P. (2013). Analysis of volatile compounds in Chinese soy sauces moromi cultured by different fermentation processes. *Food Science and Biotechnology*, 22(3), 605–612.