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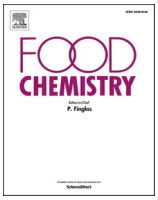

Identification and quantification of flavor compounds in smoked tuna fish based on GC-Orbitrap volatolomics approach

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ABSTRACT

Cold smoking enhances the appeal of fish products, offering consumers a smooth texture and a delicate smoky flavor. This study aims to explore variations in the volatile profile from different exposure times during cold smoking processing (light, moderate, and full-cure) in tune samples. An innovative untargeted analytical approach, headspace solid-phase microextraction combined with gas chromatography and a hybrid quadrupole-orbitrap mass analyzer, was employed to identify 86 volatiles associated with the cold smoking process. Most of these compounds, including phenols, furan derivates, aldehydes, cyclic ketones, and different aromatic species, were found to contribute to the smoke odor. The development of a QuEChERS-based extraction and clean-up method facilitated the quantification of 25 relevant smoky markers across all smoking degrees, revealing significant concentration differences after 15 h of smoking. This research sheds light on the dynamics of cold smoking impact and its on the flavor profile and safety quality of processed fish products.

1. Introduction

Food preservation methods such as smoking, drying, and salting have been employed for centuries to avoid post-harvest loss. Smoking is the most widely used way of processing fish in recent years and has become popular because of consumer demand for fish with certain taste, color, and texture attributes (Sokamte Tegang, Mbougueng, Sachindra, Douanla Nodem, & Tatsadjieu Ngoune, 2020). Frequently, product appearance is a factor in consumer decision-making when choosing between the numerous smoked fish goods available on the market (Vidal, Goicoechea, Manzanos, & Guillén, 2017). There are different types of smoking depending on the temperature at which the product is processed: cold (0–30 °C), warm (30–50 °C), and hot (50–80 °C) (Huang et al., 2019). Although color and luster are enhanced in warm and hot smoking because of Maillard reactions, high temperatures also dry the surface of the fish product, preventing smoke from being absorbed (Huang et al., 2019). In contrast, cold smoking retains the smoke flavor but preserves the moisture and texture of the untreated meat (Huang et al., 2019). The mild organoleptic characteristics of cold-smoked fish products make them especially well-suited to meet the growing demand for fish for sushi preparation in the European Union (Lacalle-Bergeron et al., 2020). Their appearance, however, might cause confusion with raw fish or products made using alternative methods that are forbidden by European law (e.g., CO or tasteless smoke) (Lacalle-Bergeron et al., 2020).

The sensory characteristics of smoked fish are mainly caused by the presence of some volatile organic compounds (VOCs), which are deposited on the flesh during the smoking process. It is known that

Abbreviations: CAR, Carboxen; **DVB**, Divinylbenzene; **EI**, Electron ionization; **FCS**, Full cure smoked fish; **FWHM**, Full width at half maximum; **HRF**, High resolution filtering score; **HRMS**, High-resolution mass spectrometry; **HS-SPME**, Headspace solid-phase microextraction; **LCS**, Light cold smoked fish; **MCS**, Moderate cold smoked fish; **PA**, Polyacrylate; **PDMS**, Polydimethylsiloxane; **RSI**, Reversed search index; **SI**, Search index; **UNT**, Untreated fish; **VOC**, Volatile organic compound; **ΔRI**, Deviation of the retention index.

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phenolic compounds contribute significantly to the smoke flavor and act as antimicrobial and antioxidant agents promoting the preservation of smoked foods (Vidal et al., 2017; Albishi, Banoub, de Camargo, & Shahidi, 2019; K. M. Yang & Chiang, 2019). Other significant classes of smoky volatiles are furans and carbonyl derivatives, particularly aldehydes and ketones (Vidal et al., 2017). The study of the changes in the highly volatile and semi-volatile fractions emitted by a biological system under specific conditions is known as volatolomics, and it has become increasingly relevant in fields connected to food (Chen et al., 2023; Hu et al., 2023). Volatolomics, as a branch of metabolomics, can follow target or untargeted approaches. While the targeted studies are focused on the determination and quantification of a list of compounds that have been pre-selected for their relevance to the research question, the untargeted studies constitute an unbiased screening that can categorize samples based on metabolite patterns that change in response to a specific factor (Lacalle-Bergeron et al., 2021).

Gas chromatography-mass spectrometry (GC–MS) has historically been the main standard method for volatolomics studies due to its high sensitivity as well as its strong identification capabilities for detecting and identifying volatile species (Diez-Simon, Mumm, & Hall, 2019). This technology is very effective for profiling applications due to its great robustness and reproducibility as well as the large amount of mass spectrum data available in commercial libraries (e.g. 394.000 mass spectra of 347 k unique compounds in NIST23) (National Institute of Standards and Technology, 2023). High-resolution mass spectrometry (HRMS) can screen a virtually unlimited number of compounds in a single analysis due to its capacity to provide accurate-mass full-spectrum data with reasonable sensitivity. Electron ionization (EI) source coupled to (quadrupole)time-of-flight ((Q)TOF) is a common technique also employed for the identification of volatiles in food products (Filatova, Bechynska, Hajslova, & Stupak, 2022; Kumar et al., 2019; Liu et al., 2018) while atmospheric pressure chemical ionization (APCI) has also been used as a source for some applications (Izquierdo-Sandoval, Fabregat-safont, Lacalle-bergeron, Sancho, & Portoles, 2022; Sales et al., 2017). In recent years, the popularity of GC-(Q)Orbitrap MS is growing thanks to its increased resolution, up to 100,000 full width at half-maximum (FWHM) at m/z 272, which leads to a higher robustness in terms of mass accuracy (Y. Yang et al., 2022; Narduzzi et al., 2023).

Among all the extraction technologies available for untargeted volatolomics, automated techniques based on direct headspace injection are the most promising since they offer the possibility of dealing with complex matrices without the use of solvents in an economical, simple, and fast application (Lacalle-Bergeron et al., 2020; Y. Yang et al., 2022). Dynamic headspace with sorbent entrapment (DHS-P&T) is a useful technique in the analysis of volatile food components with high pre-concentration power, sensitivity, and robustness that has been demonstrated in numerous applications (Frank et al., 2016; Lacalle-Bergeron et al., 2020; Thomsen et al., 2016). Headspace solid-phase microextraction (HS-SPME) has emerged as the most popular extraction technique for volatiles in the field of food analysis, including smoked fish, because of its solventless nature, requirement for short-time analysis and small sample volume, and good pre-concentration factor (Vidal et al., 2017; Saldaña et al., 2019; Feng et al., 2021; Starowicz, 2021).

Volatile components in smoked fish products serve a dual role in quality assurance, influencing sensory attributes, and contributing to food preservation. Particularly, the European Salmon Smokers Association, which advocates for good smoking practices, recommends a target total phenol content ranging from 0.4 to 2 mg / 100 g of smoked flesh (European Salmon Smokers Association, 2018). This underscores the critical necessity not only to employ screening platforms for identifying the volatile profile but also to develop validated quantitative analytical methods ensuring accurate quantification of volatile compounds in smoked fish. Although HS-SPME is gradually being used more for quantitative evaluations (Lindholm-Lehto, 2022), its applicability is primarily limited to qualitative profiling due to the meticulous experimental demands and the substantial amount of time required for quantification purposes (Nolvachai, Amaral, Herron, & Marriott, 2023). In contrast, the Quick, Easy, Economical, Effective, Robust, and Safe (QuEChERS) method offers an easy-to-use and highly versatile extraction procedure, demonstrating considerable potential for quantitative analysis across diverse food product applications (Perestrelo et al., 2019).

In this study, an analytical method was developed to explore the volatile profile of tuna fish fillets exposed to varying degrees of cold smoking. An untargeted workflow coupling HS-SPME to GC-(Q)Orbitrap MS within an automated set-up facilitates the comprehensive evaluation of the volatile fingerprint generated during the smoking process. Subsequently, the 25 most significant VOCs of interest, chosen for their contribution to the sensory attributes of smoked products, were selected for the optimization of a target strategy, employing QuEChERS for sample treatment. The final method was employed for the accurate quantification of smoky markers in 32 tuna samples subjected to different degrees of smoking exposure, including light, moderate, and full-cure smoked products.

2. Material and methods

2.1. Chemicals and reagents

Reference standards for quantification purposes were obtained from different suppliers as pure compounds (see supplementary **Table S.1**). The stable isotopic labeled internal standard (SIL-IS) Phenol-2,3,4,5,6-d_5 purity ≥99%, 98 atoms % D) was purchased from sigma-Aldrich (Barcelona, Spain). Individual stock standards solutions were prepared around 500 μg mL^{-1} in acetone and stored at −20 °C. Working solutions containing all compounds were prepared by dilution with acetone for sample fortification and acetonitrile for instrument injection. Alkane standard solution C8-C30 (sigma-Aldrich, Germany) was used for linear retention index determination

Organic solvents such as acetone (for GC residue analysis quality), n-hexane (99%, HPLC grade), and acetonitrile (LC-MS gradient) were purchased from Scharlab (Barcelona, Spain). Primary-secondary amine (PSA) was supplied by Sigma-Aldrich (Barcelona, Spain), and acetic acid (glacial, reagent grade), anhydrous magnesium sulfate ($MgSO_4$) (content ≥98%), and C18 by Scharlab (Barcelona, Spain).

2.2. Tuna samples

A total of 32 frozen tuna samples were provided by Sea Delight Europe SL: 8 untreated (UNT), 8 light cold smoked (LCS), 8 moderate cold smoked (MCS), and 8 full cure smoked (FCS). Their patented method for cold smoking treatment is characterized by keeping the temperature at 4 °C during the whole smoking process to avoid the generation of histamine in bluefish, which appears at temperatures above 4.4 °C. The samples were smoked in Vietnam using sawdust from a mixture of local hardwood and softwood, such as coconut, acacia, mango, and jackfruit, generated between 400 and 600 °C. Imported hardwoods such as hickory or cherry wood were also employed since their strong smoke aroma has been traditionally used for flavoring food. The flavor strength is directly related to the time of exposure to flavored wood smoke. Therefore, in the LCS samples, as the time of exposition is short (8 h), the smoked flavor and aroma are very light. In the MCS samples, a longer exposure time (15 h), allowed for a flavor of higher intensity. Finally, FCS presents the strongest smoked aroma and flavor due to the 48-h curation. Samples were stored in the freezer at −25 °C until the extraction.

The proper performance of the smoking process in the LCS samples was confirmed by a professional testing panel. Sensory tests of the organoleptic properties (odor/flavor) between the test subject (LCS) and the reference sample (UNT) were carried out by 12 qualified tasters (selected and trained), following the Standard UNE-EN ISO 4120:2008. Significant differences between the two samples at the level of intensity

of smoked odor and flavor were evidenced in a triangular test with 24 trials and a 100% correct response. A scale was established for the smoked flavor and aroma (from 0 to 4), obtaining 0.00 ± 0.00 in smoked odor and 0.11 ± 0.33 in smoked flavor for the UNT samples and 0.78 ± 0.67 in smoked odor and 0.67 ± 0.50 in smoked flavor for the LCS samples.

2.3. Sample treatment

2.3.1. HS-SPME qualitative screening

Tuna samples were thawed at room temperature and triturated. The operating condition for the HS-SPME was based on previous studies with some modifications (Vidal et al., 2017). Briefly, 3 g of triturated fish samples were placed in a 20-mL HS vial and sealed (before complete defrosting to avoid VOCs losses). Samples were pre-heated at 60 °C for 5 min; then SPME fiber was exposed to the headspace of the vial for 30 min. After the extraction, SPME fiber was thermally desorbed at 280 °C for 5 min on the GC injection port, maintaining the fiber for an additional post-conditioning 5 min into the injector port to avoid the carry-over. Three different coating materials were tested: Polyacrylate (PA) 85 μm, carboxen/ polydimethylsiloxane (CAR/PDMS) 95 μm, and divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/CAR/PDMS) 80 μm, 50 μm / 30 μm, all three supplied from Thermo Fisher Scientific.

2.3.2. QuEChERS extraction for quantitative analysis

The QuEChERs extraction was based on a previous methodology for the determination of GC-amenable micropollutants in fish (Dubocq, Bæringsdóttir, Wang, & Kärrman, 2022). Samples were thawed at room temperature and triturated. After that, 2 g were accurately weighed, transferred to a 50 mL falcon tube, and spiked with 50 μL of a SIL-IS solution of 10 ng·μL^{-1}. After 30 min, 10 mL of acetonitrile (1% acetic acid) was added, and the tube was vigorously shaken by vortex for 1 min. Then, 1 g sodium acetate and 4 g of $MgSO_4$ were added to the tube and subsequently shaken for 1 min. The tube was centrifuged at 3500 rpm for 5 min, and 1 mL of the upper layer of the extract was transferred to a 2-mL Eppendorf tube for the subsequent QuEChERS clean-up prior to the injection in the GC system. For the clean-up, dispersive agents PSA (50 mg), $MgSO_4$ (150 mg), and C_{18} (50 mg) were added to the acetonitrile extract, and then the mixture was vortexed for 30 s and centrifuged (3500 rpm, 2 min). Finally, the supernatant was frozen for two hours at −25 °C (freezing clean-up). Then, the final acetonitrile extract was injected into GC Q-Orbitrap.

2.4. Instrumentation

Data were acquired using a Thermo Scientific™ Q Exactive™ GC hybrid quadrupole-Orbitrap mass spectrometer. Sample introduction was performed with a TriPlus RSH autosampler (Thermo Scientific, Bremen, Germany). This system is integrated online with a multi-purpose sampler equipped with a headspace incubation chamber and SPME sampling unit, allowing the automatization of HS-SPME and liquid injection. The chromatographic separation was based on a previous work (Lacalle-Bergeron et al., 2020) and was carried out with a Thermo Scientific™ TRACE™ 1310 GC with a split/splitless injector, working in splitless mode for both SPME desorption and liquid injection (1 μL injected) at 270 °C. The capillary column employed was a 30 m × 250 mm DB-WAXETR (0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA), with helium at 1 mL min^{-1} as a carrier gas. The oven temperature program started at 60 °C for 5 min, then increased to 260 °C at 10 °C·min^{-1} and held for 2 min (total chromatographic analysis time: 28 min). The transfer line was held at 290 °C. The electron ionization was performed at 70 eV with the source temperature set at 270 °C, with a solvent delay of 3 min in HS-SPME analysis and of 4.70 min in QuEChERS analysis. Data were acquired in full scan mode at a rate of 3.7 scan/s over a *m/z* range of 40–650 at 60,000 mass resolution (FWHM at *m/z* 272). Nitrogen gas (Praxair, Spain) was used for the C-Trap supply. The C-trap ion handling used an automatic gain control (AGC) of 1×10^6 and the injection time (IT) was set to automatic. The mass calibration procedure was performed daily (perfluorotributylamine).

2.5. Validation of the quantitative method

A quantitative method was optimized for the compounds confirmed after the non-targeted screening from LCS and MCS, and an in-house compound database was developed for those compounds (**Table S.2**).

Given the absence of reference guidelines for the determination of aromas, the validation was based on European Commission (EC) guidelines (SANTE/12682/2019). The accuracy of the method was estimated through recovery experiments, analyzing quality control (QC) samples of untreated fish considered "blank" samples ($n = 6$) spiked at three concentration levels (10, 100, and 1000 ng/g). The precision, expressed as the repeatability of the method, was determined in terms of the relative standard deviation (RSD (%)) from the recovery experiments ($n = 6$) at each fortification level. Quantification was performed using matrix-matched calibration curves obtained from a homogenized pool of untreated fish sample extracts spiked with the standards of the monitored compounds. The range of concentration studied was 1–250 ng·mL^{-1} (7 concentration points). Linearity was assumed when the regression coefficient (R^2) was >0.99 with residuals <20%, and the RSD was <30% for all the concentration points ($n = 3$). The limit of quantification (LOQ) was established as the lowest concentration level validated with satisfactory values of recovery (70–120%) and precision (RSD < 20%). The limit of detection (LOD) was estimated for all compounds by extrapolation based on the data obtained for the lowest concentration level validated and expressed as the concentration giving a signal-to-noise (S/N) of three. Specificity was evaluated considering that the response of the peak in the blank samples should be <30% of the lowest level validated.

2.6. Data processing and statistical analysis

Xcalibur 4.0 software (Thermo Scientific, Waltham, MA, USA) was employed for instrument control and data acquisition and Trace Finder™ 5.1 (Thermo Scientific, Waltham, MA, USA) was used for data processing. In the case of the screening method, the method was based on the work of (Gómez-Ramos, Ucles, Ferrer, Fernández-Alba, & Hernando, 2019) with some modifications. Deconvolution Plugin 1.5 for Trace Finder™ 5.1 performed a complete peak deconvolution, peak alignment, and peak area integration. The initial settings for deconvolution software were S/*N* >5, mass error of ±5 ppm, total ion chromatogram (TIC) intensity threshold of 100,000, and retention time (Rt) aligning window ±10 s. The compounds were characterized by their Rt and accurate *m/z* of molecular ion (if present); and they were tentatively identified by automated comparison of the deconvoluted mass spectra with the ones present in the National Institute of Standards and Technology (NIST) 2020 library (EI spectra of 306,643 compounds and retention index, RI, values of 139,382 compounds) with reversed search index (RSI), high-resolution filtering (HRF) value and a Deviation of the Retention Index (ΔRI) match higher than 700, 80 and over ±50, respectively.

Quantitative data were processed with Trace Finder™ 5.1 considering relative areas to the IS phenol-2,3,4,5,6-d5 to correct matrix effect and potential errors associated with the sample manipulation. For identification of the target compounds, it was applied a Rt tolerance of ±0.1 min from the calibration standard; the presence of, at least, two ions measured at their accurate mass (preferably including the molecular ion and with a mass accuracy of ≤5 ppm or < 1 mDa for m/z ⟨200) and a peak with a signal-to-noise ratio (S/N) higher than 3. Differences between smoking degrees were evaluated by a Mann-Whitney test using GraphPad Prism 10 (GraphPad Software, La Jolla, CA), considering a value of $p < 0.05$ statistically significant.

3. Results and discussion

3.1. HS-SPME-GC-(Q)Orbitrap MS approach for the evaluation of changes in the volatile profile derived from the smoking process.

The volatilome derived from the smoking process is composed of a diverse chemical composition derived from the processes that occur during the combustion of wood and the interaction of smoke with fish fillets (Varlet, Knockaert, Prost, & Serot, 2006). Other chemical reactions occurring during the process are the enzymatic and auto-oxidation of lipids and the interaction of their respective products with free amino acids and peptides (Huang et al., 2019). An untargeted approach has been adopted to capture information about the differences in the volatile fingerprints between the unsmoked and smoked samples, specifically light cured. The tandem HS-SPME with GC-(Q)-Orbitrap offers a powerful and sensitive technique for the high-throughput screening of the volatile fraction and collecting the necessary information to increase the identification reliability of the potential positives (Liu et al., 2023).

3.1.1. Selection of HS-SPME fiber.

Sorbent selection is a critical aspect when implementing an SPME approach, as it significantly influences the sensitivity and selectivity of the method performance (Lancioni, Castells, Candal, & Tascon, 2022). Three different coating materials were tested: Polyacrylate (PA) (85 µm), carboxen / PDMS (95 µm), and DVB / carboxen / PDMS (80 µm, 50 µm / 30 µm). PA is a highly polar coating recognized as effective for phenol extraction, while CAR/PDMS offers sensitive and reproducible retention for volatile compounds in general (Marušić Radovčić, Vidaček, Janči, & Medić, 2016). The three-component coating (DVB/CAR/PDMS) is ideal for a wide range of polarities, and is the traditional coating used for the study of volatile compounds in smoked products (Guo, Wang, Chen, Yu, & Xu, 2021; Saldaña et al., 2019) and in fish (Lindholm-Lehto, 2022). Extraction tests were carried out on the LCS sample, as it is expected to exhibit lower concentrations of compounds derived from the smoking process due to its shorter exposure time. DVB/CAR/PDMS was the fiber that provided better performance because of the potential to extract a greater number of compounds with greater intensity and was subsequently selected for the rest of the study.

3.1.2. Volatile profile derived from cold-smoking processing.

Once the fiber was selected, it was used for the volatile evaluation of all the groups. Data acquired were processed using Trace Finder 5.1. The deconvolution process allowed the generation of clean mass spectra of coeluting peaks, yielding 400 to 900 features per sample. The peaks were thoroughly inspected in terms of peak shape, symmetry, and cleanliness. The fragmentation patterns were automatically compared with the NIST library (NIST 2020 library version) and manually reviewed. The accurate mass (± 5 ppm), fragmentation pattern, NIST library match (RSI > 700), LRI (below ±50 from NIST library), and high-resolution filtering (HRF) > 80 were the main criteria to assign a tentative identification to a feature. Following this criterion, a total of 86 compounds could be identified in the samples. **Table S2** shows the molecular formula, retention time, NIST match, HRF, LRI, and the three most abundant detected ions for each of them with a higher presence in the smoked products (LCS and MCS). As the purpose of the untargeted approach was the identification of the compounds that originated during the smoking process, only those features whose intensity we significantly higher in the LCS group, to the detriment of UNT samples, were selected. As an example of the identification workflow, Fig. 1 illustrates the tentative identification of 4-methylguaiacol in the LCS sample. Fig. 2 shows an overview of chemical families found in the MCS sample, where 37% of the compounds contained carbonyl groups (ketones, aldehydes, acids, or esters) and 26% were phenols. Furan derivatives represent 18.6% of the compounds, while 69.8% of the species had an aromatic group. Some polycyclic aromatic hydrocarbons (PAHs) were also detected (naphthalene, 1-methyl naphthalene, and 2-methyl phenanthrene) derived from the pyrolysis of the wood (Arvanitoyannis & Kotsanopoulos, 2012). Acetophenone, benzaldehyde, 5-ethyl-2 furaldehyde, phenol, and p-cresol, were presumably present in raw fish and enhanced during the wood smoke treatment.

3.1.3. Smoky odorant patterns derived from cold-smoking processing

Most of the chemical species extracted from the headspace during the screening have previously been reported as responsible for the sensory attributes of smoked fish. Phenolic compounds, which are produced mainly by thermal degradation through the oxidation and/or depolymerization of the lignin, have traditionally been identified as the main odor-active compounds associated with the pleasant smoky-aroma. Most of the detected phenols (**Table S2**) were reported on various smoked fish using different methods of smoking. (Huang et al., 2019; Lacalle-Bergeron et al., 2020; Varlet et al., 2006; Varlet, Serot, Albishi, Knockaert, & Prost, 2007). Varlet et al. found that syringol, 4-methylguaiacol, guaiacol, and cresol were contributors to the smoke and burnt odor,

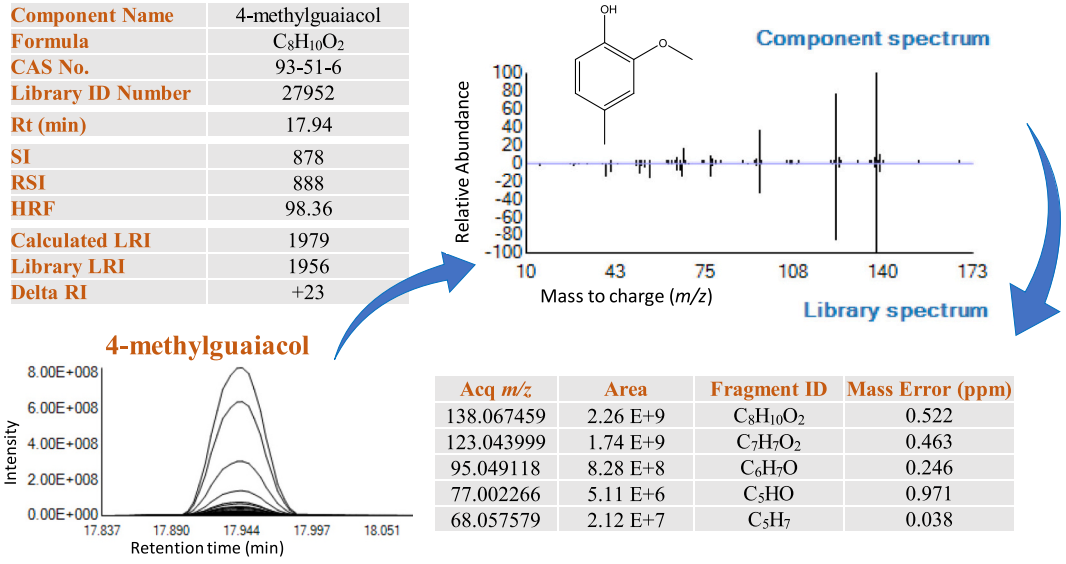

Fig. 1. Example of 4-methylguaiacol (CAS no. 93–51-6) tentative identification parameters: retention time (Rt), search index (SI), reversed SI (RSI), high-resolution filtering (HRF) value and linear retention index (LRI).

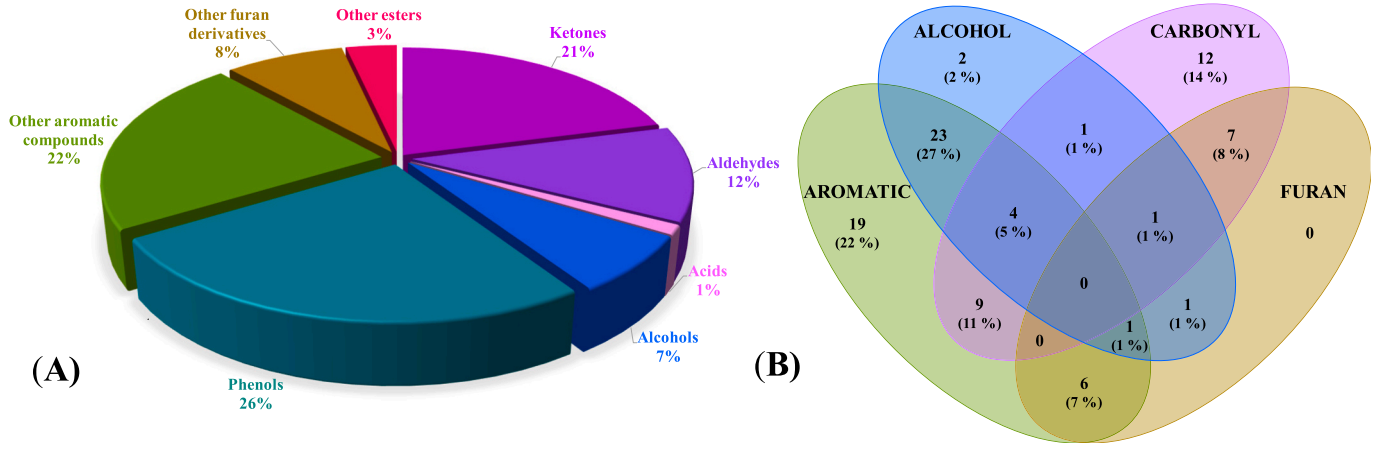

Fig. 2. (A) Pie chart listing compounds by their main functional group as identified through SPME-based untargeted metabolomics. (B) Venn Diagram showing the relationships between the functional groups contained in the selected compounds.

while isoeugenol, eugenol, 4-propylguaiacol, and 4-ethylguaiacol were responsible for the spicy notes (Varlet et al., 2006). Furanic compounds also contribute greatly to the smoke odor in smoked fish, its production is directly dependent on the pyrolysis temperature and is based on the thermal degradation of cellulose and hemicellulose (Varlet, Serot, et al., 2007). The furanic aldehydes reported in this study, such as furfural, 2-acetyl furan, and 5-methyl-2-furfural, benzofuran derivates and furfuryl alcohol are known odor-active compounds in smoked salmon (Varlet et al., 2006).

Thermally degraded wood cellulose and hemicellulose derivatives aldose and ketose interact with amino acids to produce different carbonyl acids, dicarbonyl compounds, and other aromatic chemicals via Maillard reactions and Stecker degradation (Varlet, Prost, & Serot, 2007). Cyclic ketones, aldehydes, and their derivatives contribute to the smoke flavor with sweet, spicy, and caramel-like notes (Vidal et al., 2017). There is no consensus in the literature as to whether these substances come solely from wood smoke and then are transferred to fish fillets during the smoking process or if they emerge as a result of wood smoke interacting with fish proteins. In the case of cold smoking, the first hypothesis is the most likely option since the fish fillets have no exposure to high temperatures during the process. It is worth noting the absence of aliphatic aldehydes, which are generated in enzymatic reactions of autoxidation of lipids in fish flesh, these compounds are common in the traditional smoking process where fillets are exposed to high temperatures and contribute to a characteristic boiled potato-like and rancid odor (Jónsdóttir, Ólafsdóttir, Chanie, & Haugen, 2008). None of the substances identified by Jónsdóttir et al. as useful as quality indicators for tracking the degree of spoilage in smoked products (acetic acid, 2-butanone, 3-methyl-1-butanol, 3-methyl-butanal, 3-hydroxy-butanone, and ethanol) were present at any level of cold smoking.

3.2. *Optimization of a quantitative method for the determination of relevant smoky markers in smoked fish fillets.*

From the list of compounds identified during the untargeted approach, 25 phenol, furan, ketone, and aldehyde derivates were selected for the optimization and validation of the quantitative method (**Table S.3**). **Fig. S.1** illustrates the individual levels obtained for the selected compounds during the cold smoking process, including UNT, LCS, and MCS samples. The criterion used for the selection was based on the association of these volatile species with the organoleptic characteristics (odor/flavor) of smoking foodstuffs based on previous olfactometric determinations reported in the literature (Jónsdóttir et al., 2008; Varlet et al., 2006; Varlet, Serot, et al., 2007; Vidal et al., 2017). The identity of these compounds was confirmed by the injection of analytical reference standards.

3.2.1. *QuEChERS extraction and clean-up optimization*

Developing an optimal extraction process is vital to achieve high recovery rates and precise results. To accurately quantify the compounds of interest, the simplest procedures with the fewest steps and that minimize the extraction of unwanted interfering species are preferred. In this regard, QuEChERS strategy was selected over the HS-SPME for quantitative purposes due to its robustness and versatility as well as its greater simplicity (Perestrelo et al., 2019). When implementing QuEChERS extraction method, consideration should be given to the clean-up step that is selected because it might significantly affect how sensitive and selective the method is. After extraction with acidified acetonitrile and drying with anhydrous salts, three different clean-up procedures were evaluated: i) dispersive SPE (d-SPE) sorbent which comprises a mixture of 50 mg of PSA, 50 mg of C_{18}, and 150 mg of $MgSO_4$; ii) freezing the extracts for two hours at −25 °C, and iii) the combination of both procedures. PSA is a sorbent used to remove organic acid, fatty acids, and sugars (Yin et al., 2022), C_{18} sorbent is used to retain hydrophobic interfering compounds such as lipids (El Husseini, Makkouk, Rabaa, Al Omar, & Jaber, 2018) and $MgSO_4$ is added to remove the possible remaining water. The freezing clean-up is employed to help the protein precipitation and fix lipids to the tube walls (Belarbi et al., 2021).

The selection of the most appropriate clean-up methodology was assessed in terms of extraction recovery and reproducibility. UNT samples were used as reference matrices, comparing blank extracts with extracts coming from samples spiked at 1000 ng/g, 30 min before the extraction. Recovery results ($n = 3$) are shown in **Fig. S.2** ranging from 94 to 158% for d-SPE cleanup, 62 to 90% for freezing cleanup, and 61 to 81% for the combination of both. These values improved when using IS-corrected areas in Fig. 3 reaching ranges from 73 to 111%, 68 to 96%, and 72 to 94%, respectively. According to the IS-corrected results, the bulk of compounds were effectively recovered using any of the three alternative procedures, with acceptable recoveries (70–120%). Even though the first clean-up approach had the greatest recoveries, this option was discarded due to the poor reproducibility observed for some compounds (e.g. 3-methyl-cyclopentanone (Fig. 3). Among the other two options, the combination of the d-SPE sorbents and freezing clean-up demonstrated better reproducibility (RSD from 0.03 to 6%) than freezing clean-up alone (RSD from 0.4 to 17%). For this reason, the combination of d-SPE and freezing clean-up was chosen for further method optimization.

3.2.2. *Quantitative method validation*

Table 1 compiles all information related to the validation of the method. The accuracy and precision were estimated through recovery studies in untreated fish samples which were spiked at three

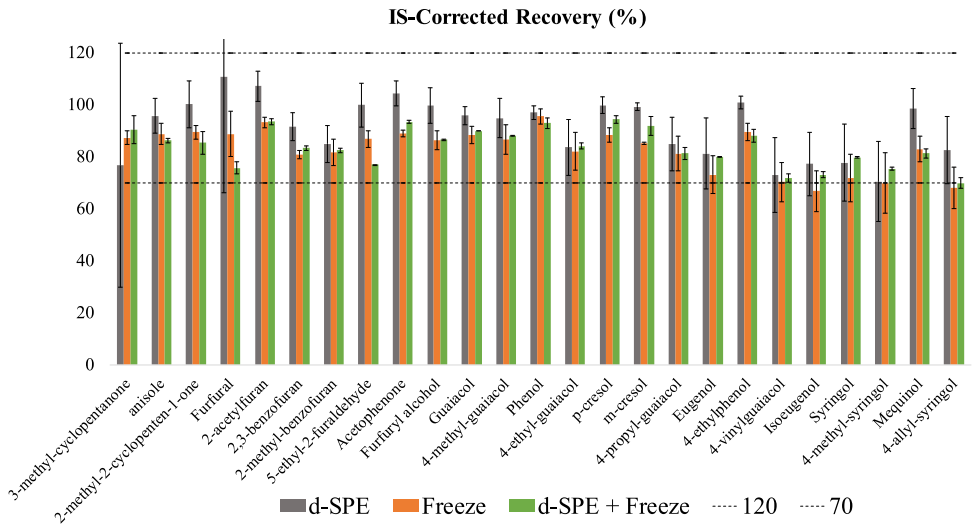

Fig. 3. Corrected recovery with the IS phenol-d5 obtained after three different QuEChERS clean-ups in terms of reproducibility ($n = 3$). Error bars show standard deviation.

Table 1
Correlation coefficients (R^2), estimated limits of detection (LOD) and quantification (LOQ), spiked mean recoveries (%) and RSDs (%, in brackets) of the compounds ($n = 6$).

Compound name	Linearity range (ng·mL^{-1})	R^2	LOD (ng/g)	LOQ (ng/g)	Recoveries %		
					10 ng/g	**100 ng/g**	**1000 ng/g**
3-methyl-cyclopentanone	1.2–300.7	0.9995	4.0	12.0	102 (5.5)	114 (12.6)	104 (4.7)
Anisole	1.3–313.2	0.9996	0.8	12.5	77 (6.4)	104 (18.5)	100 (4.7)
2-methyl-2- cyclopenten-1-one	1.1–266.3	0.9998	2.1	10.7	96 (9.0)	112 (6.9)	105 (4.6)
Furfural	0.9–226.0	0.9995	0.9	9.0	75 (11)	91 (10.5)	84 (5.0)
2-acetylfuran	1.1–268.1	0.9995	0.1	10.7	107 (3.3)	115 (9.4)	108 (4.8)
2,3-benzofuran	0.9–226.9	0.9995	1.0	9.1	81 (4.1)	104 (15.8)	99 (5.1)
2-methy-benzofuran	1.0–253.7	0.9994	0.6	10.2	90 (2.0)	104 (11.0)	99 (4.7)
5-ethyl-2-furaldehyde	1.0–259.3	0.9995	1.5	10.4	95 (1.9)	100 (4.8)	96 (5.4)
Acetophenone	1.8–438.8	0.9998	0.5	17.6	139 (3.2)	116 (2.1)	108 (4.9)
Furfuryl alcohol	1.4–347.4	0.9987	2.3	13.9	119 (14.0)	112 (17.2)	106 (5.2)
Guaiacol	1.1–283.2	0.9998	0.6	11.3	113 (7.2)	106 (5.0)	106 (4.6)
4-methylguaiacol	1.3–283.2	0.9994	0.6	13.9	95 (7.8)	101 (4.0)	106 (4.4)
Phenol	47.5–237.5	0.9959	2.6	950.0	–	–	110 (3.9)
4-ethylguaiacol	1.3–343.6	0.9999	0.4	13.7	94 (7.2)	100 (5.5)	105 (4.4)
p-cresol	1.2–293.0	0.9997	1.3	11.7	107 (6.9)	117 (5.5)	106 (4.6)
m-cresol	1.1–271.3	0.9997	1.5	10.9	119 (9.5)	105 (4.3)	105 (5.0)
4-propylguaicol	0.9–217.5	0.9996	0.3	8.7	99 (10.4)	99 (4.5)	104 (4.6)
Eugenol	1.2–310.0	0.9996	1.1	12.4	113 (7.4)	102 (4.4)	104 (4.6)
4-ethylphenol	1.1–272.6	0.9996	1.0	10.9	109 (3.3)	103 (5.4)	105 (4.9)
4-vinylguaicol	1.0–245.2	0.9996	0.7	9.8	101 (10.9)	92 (10.5)	100 (5.3)
Isoeugenol	1.4–339.3	0.9993	8.9	13.6	99 (16.0)	93 (8.5)	103 (5.3)
Syringol	1.3–315.1	0.9998	0.1	12.6	110 (18)	101 (5.6)	106 (5.9)
4-methylsyringol	0.9–231.3	0.9964	2.6	9.3	114 (10.0)	93 (6.7)	104 (5.0)
Mequinol	0.9–235.3	0.9998	0.9	9.4	120 (15.0)	107 (9.2)	105 (5.1)
4-allylsyringol	4.5–226.5	0.9996	4.5	90.6	–	100 (7.6)	104 (5.1)

concentration levels (10, 100, and 1000 ng/g). The results obtained for most of the compounds were satisfactory at all three levels, with recoveries between 70 and 120% and precision (RSD) below 20%. Apparently, acetophenone failed to comply with the requirements outlined in the guidelines at the lowest concentration level, due to the unacceptably high recovery value of close to 140%. However, because of the high observed consistency (RSD $\leq$ 3.2%) at that level, it could potentially be acceptable for acetophenone. The phenolic derivate 4-allylsyringol was not observed at the lowest level, due to the lack of sensitivity. In the case of phenol, its significant presence in untreated samples led to poor specificity results for the two first validation points, so this compound could only be validated at the highest level. Thus, LOQs for each compound were determined at 10 ng/g, apart from 4-allylsyringol and phenol, which were established at 100 and 1000 ng/g, respectively.

To evaluate the linearity, a homogenized pool of untreated fish sample extracts was spiked in duplicate at seven different concentration points. The concentration range for 4-allyl-syringol was 5–250 ng·mL^{-1}, 50–250 ng·mL^{-1} for phenol, and 1–250 ng·mL^{-1} for the remaining compounds. The calibration curves showed in all cases correlation coefficients (R^2) higher than 0.99, and residuals lower than 20%. LODs were estimated by extrapolation from the chromatogram at LOQ concentration and were in the range of 0.1–4.5 ng/g for all the compounds. As an example of the determination of LOQ, **Fig. S3** shows the extracted ion chromatograms (XIC) for the quantification and confirmation ion at the lowest concentration level for 5-ethyl-furaldehyde, 2-acetylfuran, 4-methylguaiacol, and 4-propylguaiacol.

3.3. Quantitation of the selected smoky marker compounds

The optimized and validated quantitative method was applied to 8 samples of each group (UNT, LCS, MCS, and FCS). Blank samples ($n = 3$) spiked at each concentration level validated (10, 100, and 1000 ng/g) were included in the analysis batch as quality control (QC) samples. As certain species exceeded the highest point of the calibration curve in MCS and FCS samples, these extracts were 10-fold diluted with blank extract and then re-injected. Consequently, a new QC at 10,000 ng/g and then diluted 10 times with blank extract was also added to the analysis. The analytical results are summarized in Table 2**,** including the concentration range observed across the 8 samples of each smoking treatment (UNT, LCS, MCS, and FCS) and the *p*-value of the non-parametric Matt-Whitney *U* test comparing the average value between consecutive treatments (for individual concentration see **Table S4**). It was considered a detected compound when the compounds were clearly present in the samples (S/N > 3) but lower than the LOQ of the method. Undetected and detected values were imputed from the statistics by assigning them a value corresponding to half the LOQ. In Fig. 4, the comparison of the accumulation of volatiles between the different treatments can be seen more clearly, that is, the exposure time to the cold smoking treatment.

Generally, the results obtained were consistent with the untargeted analysis **(Fig. S1),** although with some noticeable differences. For example, some species including furfural, guaiacol, 4-methylguaiacol, m-cresol, 4-ethylphenol, 4-vinylguaiacol, and mequinol were detected in untreated fish samples. A plausible explanation for not having detected these compounds in the untargeted approach could be the application of minimum intensity thresholds. Supporting this hypothesis, 3-methyl-cyclopentanone, guaiacol, 4-methylguaiacol, 4-propylguaicol, eugenol, 4-ethylphenol, 4-vinylguaiacol, isoeugenol, syringol and 4-methylsyringol present a considerable concentration in MCS samples, while the response of these samples in the untargeted approach was negligible. At the same time, it seems that SPME fiber has a higher affinity for 5-ethyl-2-furaldehyde than the quantitative extraction. In the FCS group, some species presented a wide range of concentrations within samples. As an example, in the case of isoeugenol, the highest concentration found was 14 times the lowest. Given that the smoked tuna samples were ground and homogenized before extraction, and that the precision was validated in the optimization of the method, this variation must have originated during the smoking process itself. Large variations have been previously reported in smoked fish, regardless of the extraction technique used (Varlet et al., 2006. Vidal et al., 2017). In some FCS samples furfuryl alcohol and phenol exceeded the calibration ranges even with diluted samples, and therefore for those samples, only an estimation of concentration was possible.

Comparing the average concentrations resulting from the different treatments, it can be seen that, except for furfural, there are no significant differences between the untreated fish and samples subject to a minimum of 8 h of cold smoking treatment. At the same time, the accumulation of the studied VOCs is notable after a 15-h of exposure, with the exception of mequinol, anisole, and 4-allylsyringol exhibiting concentrations that remain stable throughout the entire smoking process. The concentrations of the remaining compounds continue to increase significantly for the remainder of the smoking process, only benzofuran derivates (2,3-benzofuran 2-methy-benzofuran) remains stable in the FCS group. It is important to note that odor thresholds can vary greatly within the same compound family and can be influenced by the presence of other odor-active compounds (Varlet et al., 2006). For example, the odor threshold for isoeugenol in water is reported as 0.71 ng/g (Kreissl, Mall, Steinhaus, & Steinhaus, 2022) which is below its LOQ for the current method. While these values cannot be directly extrapolated to the concentrations reported in the present study, it is reasonable to assume that the differences in smoke flavor and odor between the UNT and LCS samples, as detected by the sensory panel, cannot be measured in terms of concentration differences due to this limitation in the current quantitative method.

Apart from its sensory attributes, many of the volatile compounds derived from the smoking process are valuable for their potential antimicrobial and antioxidant activity, which has a direct effect on the product shelf-life (Vidal et al., 2017). In this context, the European Guide to Good Practice for Smoked and/or Salted and/or Marinated draws particular attention to total phenol content, recommending values exceeding 4 $mg{\cdot}kg^{-1}$ during the smoking process (European Salmon Smokers Association, 2018). To calculate this parameter, the concentrations of all phenolic species were added together within each

Table 2
Range of concentrations (ng/g) found in smoked fish samples. ND: not determined, and d: detected.

Compound name	UNT (ng/g)	LCS (ng/g)	MCS (ng/g)	FCS (ng/g)	Mann-Whitney (P-value)		
					UNT - LCS	LCS - MCS	MCS - FCCS
3-methyl-cyclopentanone	ND	ND	38–61	91–156	>0.9999	0.0002	0.0002
Anisole	ND	ND - d	d	d	>0.9999	>0.9999	>0.9999
2-methyl-2-cyclopenten-1-one	ND	d - 11	582–1013	167–389	>0.9999	0.0002	0.0002
Furfural	d	d - 43	494–1032	6964 - 15,524	0.0256	0.0002	0.0002
2-acetylfuran	ND	d	565–976	1700 - 3394	>0.9999	0.0002	0.0002
2,3-benzofuran	ND	d - 9.7	31–111	24–117	>0.9999	0.0002	0.3658
2-methy-benzofuran	ND	d	d - 36	d - 50	>0.9999	0.007	0.179
5-ethyl-2-furaldehyde	ND	ND	d - 11	19–48	>0.9999	>0.9999	0.0002
Acetophenone	d	d	94–132	139–302	>0.9999	0.0002	0.0002
Furfuryl alcohol	ND	d - 46	1686 - 4238	9371–21,585 [a]	0.0796	0.0002	0.0002
Guaiacol	d	d	1265 - 3176	3457 - 13,706	>0.9999	0.0002	0.0002
4-methylguaiacol	d	d	725–1904	1497 - 8224	>0.9999	0.0002	0.0047
Phenol	d	d	1371 - 3560	3885–13,595 [a]	>0.9999	0.0002	0.0002
4-ethylguaiacol	ND	ND	182–425	291–2207	>0.9999	0.0002	0.0045
p-cresol	d	d	206–482	424–2071	>0.9999	0.0002	0.0006
m-cresol	d	d	412–998	823–4084	>0.9999	0.0002	0.003
4-propylguaicol	ND	ND	28–65	29–319	>0.9999	0.0002	0.0463
Eugenol	ND	ND	50–117	70–629	>0.9999	0.0002	0.0135
4-ethylphenol	d	d	55–125	79–576	>0.9999	0.0002	0.0207
4-vinylguaicol	ND - d	ND	29–96	54–538	>0.9999	0.0002	0.003
Isoeugenol	ND	ND	187–471	196–2826	>0.9999	0.0002	0.0207
Syringol	ND	ND	302–1109	589–4708	>0.9999	0.0002	0.0047
4-methylsyringol	ND	ND	210–388	204–3620	>0.9999	0.0002	0.0499
Mequinol	d	d	d	d	>0.9999	>0.9999	>0.9999
4-allylsyringol	ND	ND	d	d - 532	>0.9999	>0.9999	0.256

[a] Upper limit of the range if out of the limits of quantification, therefore it is an estimation.

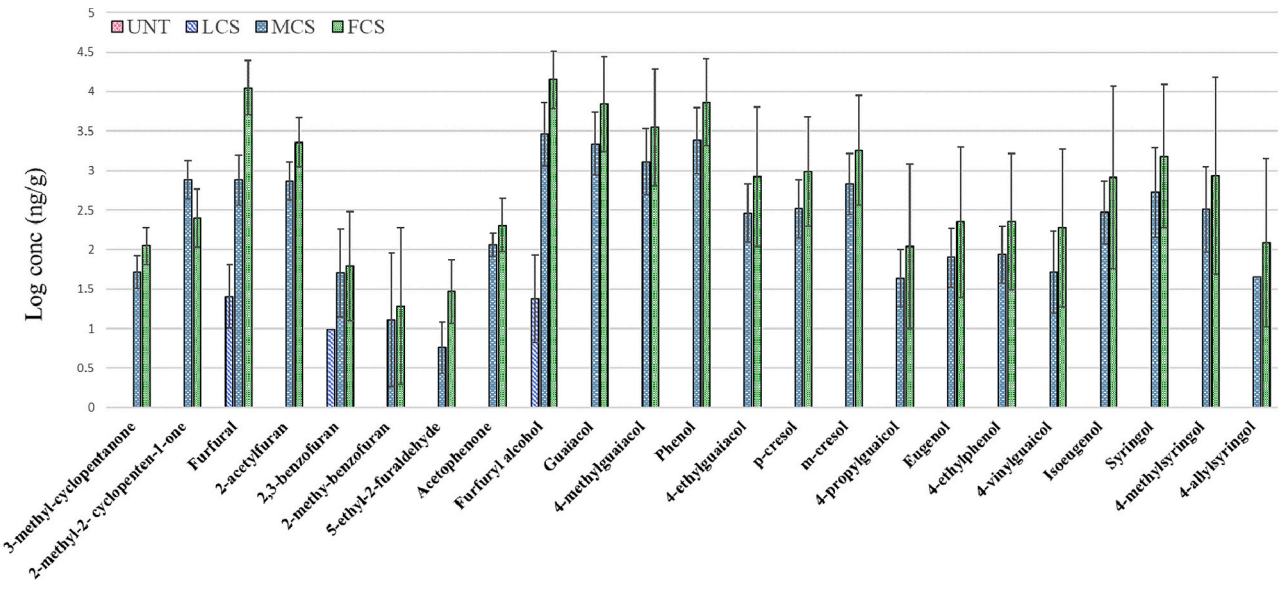

Fig. 4. Logarithm of the concentration of the 25 volatile compounds quantified in the different smoking treatment, untreated (UNT), light cold smoking (LCS), moderate CS (MCS) samples and full cure CS ($n = 8$). Error bars show full range.

smoking degree (LCS, MCS, and FCS). MCS and FCS samples yielded values of 9 ± 3 and 29 ± 15 mg·kg^{-1}, respectively. Unfortunately, the developed quantitative method was not sensitive enough to provide concentrations of compounds in LCS group. These findings evidence that an exposure time of at least 15 h in the cold smoking technique is enough to reach acceptable yields of phenol content in tuna fillets and consequently, ensuring the organoleptic and safety quality of the product.

4. Conclusions

In this study, the potential of HRMS for the monitoring and quantification of volatile substances in cold-smoked fish at different degrees of exposure has been demonstrated. HS-SPME coupled to GC-(Q)Orbitrap and the subsequent data processing and identification workflow, has allowed a fast and robust determination of the profile of volatile compounds derived from the cold smoking process, by comparing the volatile fraction of raw fish and long-exposure smoked fish, it was possible to determine up to 86 volatile compounds with a high level of identification confidence. Among the reported compounds are mainly different phenolic, furan, aldehydes, ketones, and aromatic derivates, although some acids and esters are also found. Volatile compounds present in smoked products in this study are known to come mainly from the thermal degradation of wood, while other odd-flavors derived from lipid oxidation in fish filets were not detected.

In the second stage of the study, the 25 most relevant odorous compounds are selected among the species identified through the untargeted approach, and a target analytical method is developed for their quantification within the samples of the different degrees of cold smoking. The developed analytical strategy includes a QuEChERS-derived sample treatment for the extraction of the selected volatile species and the removal of interferences, whose most successful application, in terms of reproducibility and robustness, includes the combination of dispersive sorbents and a final freezing clean-up step. The results derived from the application of the quantitative method showed trends for the accumulation of volatile compounds in the fish fillets as the smoking exposition time increases, as had been observed in the undirected approach. From the results obtained, it was possible to calculate the phenolic content of the samples, showing that at moderate levels of exposure in the cold smoking process, the levels are acceptable according to European guidelines. But it also evidences the need to develop methods that achieve greater sensitivity to evaluate this parameter in samples with a short exposure in the smoking process.

CRediT authorship contribution statement

Samia Mokh: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Leticia Lacalle-Bergeron:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **David Izquierdo-Sandoval:** Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Validation, Visualization, Writing – original draft, Writing – review & editing. **M. Carmen Corell:** Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. **Joaquim Beltran:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Writing – original draft. **Juan Vicente Sancho:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology. **Tania Portolés:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.139312.

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