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Fish and food safety: Determination of formaldehyde in 12 fish species by SPME extraction and GC–MS analysis

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Abstract

The formaldehyde (FA) content in different fish products was evaluated using a solid phase microextraction (SPME)-GC–MS method based on fiber derivatisation with pentafluorobenzyl-hydroxyl-amine hydrochloride. LOD and LOQ values of 17 and 28 μg kg^{-1}, respectively were calculated. Fish quality was assessed by the analysis of 12 species (sea-fish, freshwater-fish and crustaceans), revealing variable FA levels. Fresh, deep frozen, canned, boiled and roasted fish were analysed; cooking always produced a decrease in the analyte content. Fish belonging to the *Gadidae* family were the samples with the highest FA concentration (from 6.4 ± 1.2 mg kg^{-1} to 293 ± 26 mg kg^{-1}), in four cases out of 14 exceeding the value of 60 mg kg^{-1} proposed by the Italian Ministry of Health. Storage on ice was also investigated, showing moderate FA production also at temperature around 0 °C. FA contents lower than 22 mg kg^{-1} were finally found in all the other samples.

Keywords: Frozen fish; Food safety; Formaldehyde; SPME; PFBHA; GC–MS

1. Introduction

Recent trends in global food production, processing, distribution and preparation are creating an increasing demand for food safety research in order to ensure a safer global food supply. The World Health Organization (WHO) works closely with the Food and Agriculture Organization of the United Nations (FAO) to address food safety issues along the entire food production chain – from production to consumption – using new methods of risk analysis, taking into account the potential risks of susceptible populations as well as of combined low-level exposure to several chemicals (World Health Organization, 2002). Microbiological and chemical hazards result in the most significant sources of foodborne diseases. Chemical contamination in food can include natural toxicants, such as mycotoxins (Melchert & Pabel, 2004; Chan, MacDonald, Boughtflower, & Brereton, 2004; Tafuri, Ferracane, & Ritieni, 2004) and marine toxins (Vale, Antónia, & Sampayo, 1999); environmental contaminants, such as mercury and lead (Hui, Rudnick, & Williams, 2005; Vupputuri, Longnecker, Daniels, Guo, & Sandler, 2005; Storelli, Storelli, Giacominelli-Stuffler, & Marcotrigiano, 2005; Meador, Ernest, & Kagley, 2005; Cubadda & Raggi, 2005; Zhao et al., 2004) and naturally occurring substances. Among them, great attention has been paid toward volatile toxic aldehydes like formaldehyde, recently classified by the International Agency for Research on Cancer (IARC) in the Group 1 "as carcinogenic to humans" (International Agency for Research on Cancer, 2004).

In seafood and crustaceans formaldehyde is known to form *post-mortem* from the enzymatic reduction of trimethylamine-*N*-oxide (TMAO) to formaldehyde and dimethylamine (Sotelo, Pineiro, & Perez-Martin, 1995; Badii & Howell, 2002). This compound accumulates during frozen storage, reacts with protein and subsequently causes protein denaturation and muscle toughness (Sotelo et al., 1995).

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An *acceptable daily intake* (ADI) of 0.2 mg kg^{-1} body weight has been set by the United States Environment Protection Agency, whereas values of 60 mg kg^{-1} and 10 mg kg^{-1} for *Gadidae* and crustaceans, respectively were proposed in 1985 by the Italian Ministry of Health (MINSAN-telegram, 1985). The official method of analysis, which is based on a colorimetric procedure, presents a detection limit of 1 mg kg^{-1} (Official Methods of Analysis, 1990), but has the drawback of poor selectivity.

To determine FA in frozen fish and foods, different procedures have been proposed. FA is commonly determined using 2,4-dinitrophenylhydrazine as a derivatising agent or by using the Nash test followed by spectrophotometric analysis (Simeonidou, Govaris, & Vareltzis, 1998; Benjakul, Visessanguan, & Tueksuban, 2003; Bechmann, 1998; Baraniak, Nagpal, & Neidert, 1988; Renon, Biondi, & Malandra, 1991). However, as formaldehyde is a very volatile compound, solvent-free techniques like solid phase microextraction (SPME) can be easily applied for the analysis of organic compounds, thus combining sampling and preconcentration in a single step (Pawliszyn, 1997). Very recently we developed and validated an innovative method based on SPME with in situ derivatisation with pentafluorobenzyl-hydroxylamine hydrochloride (PFBHA) for the determination of formaldehyde at ultratrace levels in frozen fish samples (Bianchi, Careri, Corradini, Musci, & Mangia, 2005). Taking into account that fish and fish products fulfil an important role in human nutrition as a source of biologically-valuable proteins, fats and fat-soluble vitamins and that frozen and fresh fish are the most commercialised products, the aim of our study was to determine the formaldehyde content of different fish, crustaceans and shellfish maintained under different conditions. A SPME-GC–SIM-MS method was validated and 12 species of fresh, frozen, stored on ice, boiled, roasted and canned fish were analysed.

2. Materials and methods

2.1. Chemicals

Formaldehyde (37% v/v aqueous solution), methanol (99.8% purity), *O*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride (98+% purity) and acetone-d_6 (99.9%) were purchased from Sigma-Aldrich (Milan, Italy).

Standard and working solutions, prepared from the 1000 mg l^{-1} stock solution by dilution in distilled water, were stored at 4 °C until analysis.

2.2. SPME analysis and in situ derivatisation

All the analyses were performed following the SPME procedure developed in a previous work (Bianchi et al., 2005) and described below:

The fiber was exposed to the headspace of a 10-ml vial containing 2 ml of a 20 mg l^{-1} aqueous solution of PFBHA for a time of 10 min at the temperature of 60 °C under magnetic stirring at medium agitation speed.

The derivatized fiber was exposed to the headspace of the sample vial operating under the optimized extraction conditions, i.e. extraction temperature: 80 °C, extraction time: 30 min, matrix stirring of the sample at medium stirring speed.

SPME experiments were carried out using a 75 μm Carboxen-Polydimethylsiloxane (CAR-PDMS) fiber (Supelco, Bellefonte, PA, USA). The fiber was conditioned in the injection port of the gas chromatograph at 300 °C under helium flow for 1.5 h prior to use. Desorption was carried out in a splitless mode at a temperature of 310 °C for 3 min. A fiber blank was run between each sample to reduce memory effects.

2.3. GC–MS analysis

A HP 6890 Series Plus gas chromatograph (Agilent Technologies, Milan, Italy) equipped with a MSD 5973 mass spectrometer (Agilent Technologies) was used for GC–MS analysis. Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}; the gas chromatograph was operated in splitless mode with the PTV injector (Agilent Technologies) equipped with a PTV multi-baffled liner (i.d. 1.5 mm, Agilent Technologies).

Chromatographic separation was performed on a 30 m × 0.25 mm, d_f 0.25 μm HP-5MS capillary column (Agilent Technologies). The following GC oven temperature program was applied: 70 °C for 1 min, 3 °C min^{-1} to 100 °C, 20 °C min^{-1} to 250 °C, 250 °C hold for 2 min. Transfer line and source were maintained at the temperature of 280 °C and 230 °C respectively. The mass spectrometer was operated in time scheduled single-ion monitoring mode (SIM) by recording the current of the following ions: m/z 161, 181, 225 ions for derivatized-FA until 7 min, m/z 161, 181, 259 for derivatized-acetone-d_6 from 7 to 20 min. A dwell time of 100 ms was used for all the ions.

Initially, full scan EI data were acquired to determine appropriate masses for SIM under the following conditions: ionisation energy: 70 eV, mass range: 35–300 amu, scan time: 3 scan/s. All the analyses were performed setting the electron multiplier voltage at 1200 V. Signal acquisition and elaboration were performed using the HP Chemstation (Agilent Technologies).

2.4. Method parameters

Detection and quantitation limits, precision and trueness in terms of recovery were calculated using a blank trout sample.

Detection (y_D) and quantitation (y_Q) limits were expressed as signals based on the mean blank ($\bar{x}_b$) and the standard deviation of blank responses (s_b) as follows (1):

$$y_D = \bar{x}_b + 2ts_b \qquad y_Q = \bar{x}_b + 10s_b \tag{1}$$

where t is the constant of the t-Student distribution (one-tailed) depending on the confidence level and degrees of freedom (d_f). A 95% confidence level was chosen. The val-

ues of $\bar{x}_b$ and s_b were calculated performing ten blank measurements. The concentration values of the detection limit (LOD) and quantitation limit (LOQ) were obtained by projection of the corresponding signals y_D and y_Q through a calibration plot $y = f(x)$ onto the concentration axis.

Intra-day repeatability and between-day precision were calculated in terms of RSD% on two concentration levels, performing six replicates at each level.

Recovery (R%) was evaluated by spiking blank fresh fish with 2.5 mg kg^{-1} of formaldehyde and calculated as follows (2):

$$R\% = \frac{\overline{c_{obs}}}{c_{spike}} \times 100 \quad (2)$$

where $\overline{c_{obs}}$ is the mean concentration of the fortified sample, c_{spike} is the spiked concentration. All the measurements were replicated three times.

2.5. Fish samples

Twelve species belonging to sea fish (cod, haddock, hakes, mullets, tunas, sardines, mackerels and gilthead), freshwater fish (trouts and cat-fish), shrimps and cuttle-fish were analysed.

All the samples were purchased and analysed under different circumstances: during storage on ice, fresh, frozen, boiled, roasted and canned. For each species 3 different samples were always analysed.

As for the boiled and roasted fish, 20 g of each sample were boiled in hot water or cooked at 180 °C for 20 min. Six grams of each sample were then minced and homogenised with 6 ml of distilled water.

A calibration curve in the 1–10 mg kg^{-1} range was used for the analysis of the samples. Six concentration levels were analyzed performing three measurements at each concentration level. Acetone-d_6 was used as internal standard for formaldehyde quantitation at the final concentration of 30 μg kg^{-1}. Homoscedasticity was verified by applying the Bartlett test; lack-of-fit and Mandel's (Draper & Smith, 1981) fitting test were also performed to check the goodness of fit and linearity. The significance of the intercept (significance level 5%) was established by running a t-test.

3. Results and discussion

3.1. General

Since PFBHA proved to be a good derivatizating agent for formaldehyde determination, the aim of this study was the analysis of different *species* of fish in order to evaluate if the FA content could produce hazard effects on human health. By using a blank trout sample, LOD and LOQ values of 17 and 28 μg kg^{-1} were obtained, thus proving the capability of the SPME-GC–MS method for formaldehyde determination at trace levels.

Method precision was also evaluated both in terms of repeatability and between-day precision. CV% values lower than 3.2% and 9.7% were obtained for repeatability and between-day precision, respectively. In the last case, no significant differences were found among the mean values of the data obtained over three days ($p = 0.127$) at the 95% confidence interval.

Extraction recovery of 94.8 ± 1.7% ($n = 3$) was calculated at 2.5 mg kg^{-1} by addition of formaldehyde to blank fish samples, thus showing the good efficiency of the SPME-GC–MS method.

Another advantage of the utilised method is based on the higher selectivity if compared with the official colorimetric method. In fact, although the official procedure is simple, a drawback is that it is prone to interference when complex samples such as food are analyzed.

3.2. Fish analysis

Different fish *species* were initially analysed by using a calibration curve in the 1–10 mg kg^{-1} range. The obtained regression model: $y = 0.02817(\pm 0.00014)x$, $r^2 = 0.993$ ($n = 18$), after the mathematical verification of linearity by applying the Mandel's fitting test ($F_{calc} = 0.97$ *vs* $F_{tab(\alpha=0.01,1,17)} = 8.40$) and the lack of fit test ($F_{calc} = 0.007$ vs $F_{tab(\alpha=0.05,5,12)} = 3.10$), allowed to calculate the formaldehyde content in many fish *species*. When the concentration of the analyte was found to be greater than 10 mg kg^{-1}, an other calibration curve in the 5–500 mg kg^{-1} range was constructed by operating in the Full Scan mode. Under these conditions, the responses both of the derivatised-FA and the derivatised-IS were calculated by using the ion extract-chromatogram procedure.

On the contrary, no additional investigations were performed when the FA content was found to be lower than 1 mg kg^{-1}, since low concentration levels are not dangerous for the human health.

Different categories of fish were submitted to analysis. The FA content of some samples of cod, hakes, mullets and haddock is reported in Table 1. As shown in the Table, fresh, home-frozen and deep-frozen fish were analysed showing different FA-levels. As general results it has to be mentioned that all the fresh samples revealed low FA concentration levels, also in the case of fish belonging to the *Gadidae* family. As for the mullets, the fresh samples always revealed a FA content lower than 1 mg kg^{-1}. Data obtained also evidenced that the analysed hake samples as well as two cod exceeded the 60 mg kg^{-1} limit stated by the Italian Ministry of Health.

The different FA levels observed among the *species* under investigation as well as between fresh and frozen samples could be explained taking into account both that TMAO levels differ from *species* to *species* (with higher amounts especially in fish belonging to the *Gadidae* family) and that TMAO reduction to FA and DMA is typically obtained in frozen fish by means of enzimatic reactions (Gill, Keith, & Smith Lall, 1979). A different behaviour is usually observed in fresh fish, where the bacterial activity is the main responsible for the TMAO reduction to

Table 1
FA content in some sea fish

Species	Samples	FA (mg kg^{-1})[a]
Haddock	1	1.47 ± 0.27[b]
	2	4.87 ± 0.50[b]
	3	4.17 ± 0.50[b]
Cod	1	106 ± 15[b]
	2	10.38 ± 0.82[c]
	3	222 ± 27[b]
	4	6.4 ± 1.2[c]
	5	8.5 ± 1.3[c]
	6	21.8 ± 2.8[c]
Hake	1	293 ± 26[b]
	2	232 ± 19[b]
Mullet	1	3.38 ± 0.71[d]
	2	1.38 ± 0.30[d]
	3	5.16 ± 0.49[d]

[a] $n = 3$.
[b] Deep-frozen fishes.
[c] Fresh fish.
[d] After 3 months of home-frozen.

trimethylamine (Sotelo et al., 1995). The observed FA variability could be also ascribed to differences in fish dimension, feeding quality and fishing zone; in fact, all these parameters are able to strongly influence the TMAO levels of fish.

Since fish products are generally eaten after cooking, some of the samples were analysed just after boiling and roasting in order to evaluate the possible decrease in the formaldehyde content. As reported in Table 2, a decrease in the FA concentration was generally observed, even if for some cod and hakes FA values higher than 60 mg kg^{-1} were obtained. The decreasing behaviour was not surprising, in fact, all the samples were cooked in open pots, thus allowing the evaporation of the analyte during the cooking process.

On the basis of these findings and taking into account the possibility of a daily consumption of fish (200 g/day) characterised by the highest levels of formaldehyde, hazard effects should not be neglected, being that the ADI value is 0.2 mg kg^{-1} body weight. This hypothesis is however rather pessimistic since people usually do not eat only frozen cod or hake samples which are the products with the higher FA levels.

Table 2
FA content in some boiled and roasted sea fish

Species	Samples	FA (mg kg^{-1})[a] roasted fish	FA (mg kg^{-1})[a] boiled fish
Haddock	1	<1	<1
	2	1.95 ± 0.45	2.70 ± 0.21
Cod	1	86 ± 5	91 ± 7
	3	181 ± 26	216 ± 11
	5	6.38 ± 0.65[b]	6.62 ± 0.34[b]
	6	10 ± 1[b]	8.3 ± 1.4[b]
Hake	1	212 ± 29	218 ± 22
	2	195 ± 17	188 ± 19

[a] $n = 3$.
[b] Fresh fish.

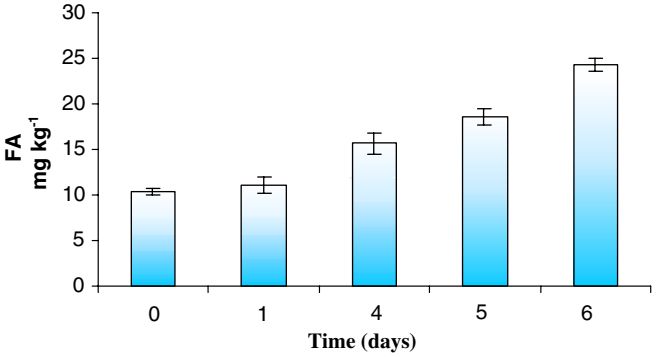

Fig. 1. FA production during storage on ice.

Further experiments were also performed in order to follow the production of FA in two cod samples during storage on ice. As shown in Fig. 1, data obtained revealed an increase of the FA content until 134% after 6 days, thus confirming the production of this compound also at temperatures around 0 °C.

Since many other fish products for which no FA limit values have been set, are commonly eaten, different *species* like mackerels, trouts, sardines, and tunas were also analysed (Table 3). All the sardine, mackerel, trout and gilthead samples reported in the Table were analysed also fresh, just before freezing, revealing FA levels lower than 1 mg kg^{-1}. Generally, in the frozen samples FA values were lower than 25 mg kg^{-1}, with the exception of two cat-fish samples. Roasting was also applied both to the mackerel samples 1 and 3 and to the sardine samples 2 and 3 allowing the observation of decrease in the formaldehyde content from 10.3 ± 0.8 mg kg^{-1} to 7.1 ± 1.1 mg kg^{-1} and from <1 mg kg^{-1} to 5.53 ± 0.25 mg kg^{-1} for mackerels and sardines, respectively.

Table 3
FA content of different fish

Species	Samples	FA (mg kg^{-1})[a]
Mackerel	1	24.9 ± 5.4[b]
	2	<1[b]
	2	2.6 ± 0.4[c]
	3	10.14 ± 0.29[c]
Sardine	1	<1[b]
	2	1.34 ± 0.27[b]
	3	5.93 ± 0.5[b]
Gilthead	1	1.31 ± 0.18[b]
	2	1.85 ± 0.35[b]
Trout	1	2.74 ± 0.32[b]
	2	3.51 ± 0.25[b]
Cat-fish	1	29.5 ± 6.4[d]
	1	53.1 ± 9.2[c]

[a] $n = 3$.
[b] After 2 months of home-frozen.
[c] After 4 months of home-frozen.
[d] After 3 months of home-frozen.

Again, the differences in the FA levels among fish belonging to the *Gadidae* family and other sea-fish or fresh-water fish could be explained on the basis of the different TMAO levels usually found in these products. In fact, pelagic fish like sardines, tunas and mackerels are recognised to be characterised by lower TMAO content with respect to cod and hakes.

Since a FA limit value of 10 mg kg^{-1} was set for crustaceans, the SPME-GC–MS method was applied also for the analysis of shrimps and cuttle-fish. Both the products, belonging to different producers were acquired already frozen in big trades. All the two shrimp samples showed a FA content ranging from 6.04 ± 0.25 mg kg^{-1} to 8.68 ± 0.53 mg kg^{-1}, whereas values from 2.91 ± 0.26 mg kg^{-1} to 3.27 ± 0.20 mg kg^{-1} were found for the two cuttle-fish. By boiling the samples an higher FA decrease was observed for the shrimps (2.31 ± 0.29 mg kg^{-1} and 3.81 ± 0.71 mg kg^{-1}) with respect to the cuttle-fish (2.06 ± 0.35 mg kg^{-1} and 2.78 ± 0.47 mg kg^{-1}). These results could be explained taking into account the higher consistency of the meat and its ability to retain the analyte.

Finally, some samples of canned tuna fish were analysed showing FA values lower than 1 mg kg^{-1}. These findings could be related both to a low analyte content in the fresh fish and to the thermic treatment of the canned products before their sale. Under these circumstances, the volatile formaldehyde could be released from the matrix.

4. Conclusions

A SPME-GC–MS method based on in-situ fiber derivatisation with PFBHA was used to determine the formaldehyde content in different fish products. Higher FA levels were founds in *species* belonging to the *Gadidae* family, whereas fresh-water fish as well as crustaceans were generally characterised by lower values.

The effect of cooking was also investigated, thus showing a reduction of the formaldehyde content in the analysed samples.

As general results, data obtained showed that no adverse effects on human health related to the fish consumption have to be supposed. In fact, the formaldehyde *ADI* value is sufficiently high to guarantee consumer safety.

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