Multi-analyte investigation in relation to the illicit treatment of fish food with hydrogen peroxide.

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OVERWIEW

- **A multi-analyte LC-MS² method to detect polymethylamines, cadaverine and trimethylamine-N-oxide in different fishes was developed and validated**
- **We aimed to detect illicit H_2O_2 fish whitening and rejuvenating by measuring TMA/TMAO ratio.**
- **The residues of H_2O_2 in fish samples were detected by indirect SPME GC-MS determination of guaiacol formed from anisol**

INTRODUCTION

The aim of this work was the development of MS methods to recognize the illicit treatment of fish food based on the use of hydrogen peroxide (whitening and "rejuvenating").

Two analytical methods were exploited: the first one is a direct **LC-MS/MS** method for the determination of various amines and trimethylamine-oxide (TMAO) ; the second one is an indirect **SPME-GC-MS** method for the determination of residues of H_2O_2 on different fish matrices, by the hydroxylation reaction of anisole to guaiacol.

The second part of the work is the **application** of the methods to investigate about H_2O_2 fish treatment and consequent alteration of the concentration ratio of TMAO and trimethylamine (TMA) which is a known fish freshness parameter.

LC-MS/MS OF AMINES

Evaluation of amines and TMA in fish foods

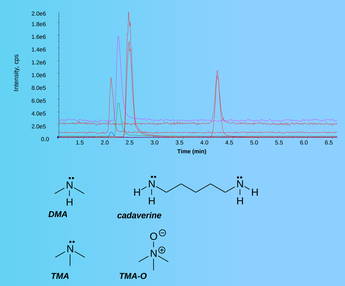
We optimized the LC-MS separation of highly hydrophilic fish nitrogen compounds (**dimethylamine, DMA; trimethylamine, TMA; trimethylamine-N-oxide, TMA-O and cadaverine**) on an ion pair RP-HPLC chromatographic system. A full validation study was then completed to make possible quantitative determination on commercial fish samples.

Sample preparation: extraction with pH 2.5 phosphate buffer (8 g fish in 40 mL, 4000 rpm centrifugation. 0.45 µm filtering.

LC: Shimadzu Nexera, column: Kinetex Evo 5 µm C18 150 x 2.1mm column, HFBA 10 mM in water – HFBA 10 mM in methanol, gradient 1:99-65:35 in 8', 0.2 mL min^{-1} Injection volume : 5 µL.

MS: Sciex 5500 Q-trap, Turbo Ion Spray ESI source, MRM acquisition (see Table 1).

Figure 1: LC-MS² chromatogram and analytes



METHOD 1

Amines LC-MS method validation:

LOQ 0.5-20 ng mL^{-1}; selectivity < 5%, accuracy 9-20%; precision (RSD% of ACC%) < 15%. Room temperature stability 24 h. Recovery > 80%. Total analysis time 15'.

Table 1: SRM parameters and quantitation limits

Analyte	Q1 (*m/z*)	Q3 (*m/z*)	CE (V)	LLOQ (ppb)
DMA	46	30	27	195
TMA	60	44	24	330
TMA-O	76	58	22	230
cadaverine	103	86	23	160

Table 2: linearity (*DMA*)

Slope	Mean	DIFF% Slope
		≤ 25.0%
12153	11.347	7.11
11300		0.41
10587		6.70

Table 3: LLOQ accuracy and precision (*TMAO*)

True Conc.	Estimated Conc.	Bias%	Mean Bias%	$RSD\%_{ACC\%}$
(ng/mL)	(ng/mL)		≤ 20.0%	≤ 15.0%
230	229	0.3	9.5	8.6
	247	7.5		
	263	14.4		
	275	19.6		
	236	2.5		
	200	12.9		

GC-MS H_2O_2-DERIVED GUAIACOL

Evaluation of H_2O_2 residues in fish foods

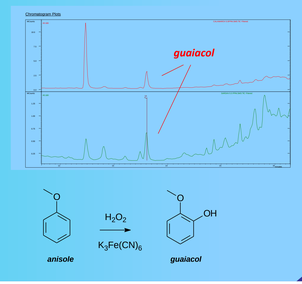
In order to identify and quantify H_2O_2 residues, we modified an old GC-ECD assay[2] based on illicit peroxide detection by oxidation of **anisole** to **guaiacol**. We developed a SPME GC-MS methodology to improve sensitivity, easiness of operation and reliability .

Sample preparation (SPME): water-suspension in phosphate buffer, pH 2.5 with anisole (2 µL) and potassium hexacyanoferrate(III) (100 µL, 0.1 M) and extraction. Fiber: Supelco Carboxen™ polydimethylsiloxane. The following parameters were optimized: reaction temperature and time, catalyst concentration, pH value, stirring, fiber exposure time.

GC: Varian Saturn 3900, equipped with a 1177 injector. Column: Phenomenex Zebron ZB-624 30 m, i.d. 0.25 mm column. T 40 - 240 C. Injector temperature 240 C / split. Helium gas 1.2 mL min^{-1}

MS: Varian Saturn 2100 T ion trap analyzer. EI ionization. Full MS acquisition (40-500 *m/z*)

Figure 2: GC-MS chromatogram evidencing guaiacol formation (real samples: top *squid*, bottom *atlantic bonito*)



METHOD 2

Guaiacol GC-MS method validation:

LOQ 0.5-20 ng mL^{-1}; selectivity < 27%, accuracy 7-27%; precision (RSD% of ACC%) < 6%. Room temperature stability 24 h. Total analysis time 16'.

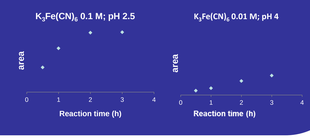
Table 4: SPME parameters optimization

Analyte	pH	reaction T (°C)	$K_3Fe(CN)_6$ $mol.L^{-1}$	reaction time	exposure time	stirring
guaiacol	2.5	60	0.1	1 h	20'	no

Table 5: linearity (*guaiacol*)

Slope	Mean	DIFF% Slope
		≤ 25.0%
4548402	5308622	14.3
5118199		3.60
6259267		17.9

Figure 3: correlation signal/reaction time

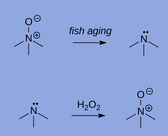


DISCUSSION

We use the method to determinate the concentration of the main amines in order to evaluate fish quality[1] (the quantity of amines contributes to the TVB-N value (Total Volatile Basic Nitrogen). Cadaverine derives mainly from lysine decomposition, methylamines from TMAO reduction.

Hydrogen peroxide is reported to be used for (illicit) fish food washing in order to whiten it or to eliminate mucus. It can quantitatively transform TMA in TMAO (chemical synthesis preparation).

The simultaneous determination of TMA and TMAO by LC-MS/MS evidences that the reaction can occur in fish food also.



Scheme 1: TMA/TMAO transformation

CONCLUSIONS

Different MS approaches were developed to characterize the presence of hydrogen peroxide residues and the effects on fish foods. Both H_2O_2 traces and TMA to TMAO conversion were evidenced in different fish food matrices. The methods show to be reliable and sensitive enough to detect illicit treatment of these foods.

Squid color appearance is a significant organoleptic parameter

APPLICATION TO FISH SAMPLES

Evaluation of H_2O_2 illicit treatment of fish food

The developed methods were applied both to two mollusks (squid) and to two oily fishes (atlantic bonito) as model matrices.

We performed all of the measurements on fresh samples (collected by fishing and brought to the laboratory in ice in < 6 hours) and on the same samples stored for 48 h and more (data not shown) at 4 C, or at room temperature.

The formation of guaiacol, then measured by SPME GC-MS, showed to be linear in the range 0-10 mg L^{-1}, in all cases of low-temperature stored samples. A great interference from the decomposing matrix was evidenced in the case of squids stored at room temperature, the only case in which the method is not applicable.

Similar results were obtained evaluating TMA/TMAO ratio evolution in the presence or in the absence of hydrogen peroxide. We can confirm that this treatment is able to reduce the TVB-N (Total Volatile Basic Nitrogen) by reverting the equilibrium between the amine and its N-oxide.

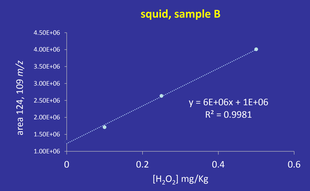
Figure 4: fish matrices studied

The developed methods are suitable to be used in **forensic analysis** to evidence illicit H_2O_2 fish food treatment.

RESULTS

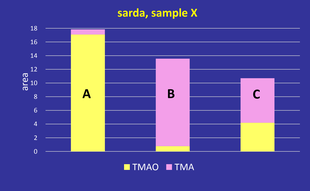
Hydrogen peroxide residues evaluation:

Figure 5: standard addition curve on a real sample. Actual concentration 0.22 mg/Kg



TMA/TMAO ratio evaluation:

Figure 6: TMA/TMAO evaluation in real samples (atlantic bonito). A: fresh sample; B: 48h-aged untreated; C: H_2O_2-treated 48h-aged sample.



References: 1) S.W.C. Chung, B.TP. Chan, trimethylamine-N-oxide, dimethylamine, trimethylamine, formaldehyde in main traded fish species in Hong Kong. *Food Add. Contamin. Part B.* **2**, 44-51 (2009)
2) A. Tanaka, M. Ijima, Y. Kikuchi. Determination of Hydrogen Peroxide in Fish Products and Noodles (Japanese) by Gas-Liquid Chromatography with Electron-Capture Detection. *J. Agric. Food Chem.*, **38**, 2154-2159 (1990)