USE OF LIVER VOLATILE COMPOUNDS AS MARKERS OF ANIMAL EXPOSURE TO TOXIC CONTAMINANTS

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Abstract – Nowadays, the control of the contamination of the food chain by toxic xenobiotics became a major safety issue given the strong relationships pointed out between chronic exposure to contaminants and pathologies such as cancers. This issue is particularly critical for animal-derived food products. The current methods operated to ensure this control are expensive, difficult to set up and unsuitable with frequent, regular and large-scale controls required to guarantee effectively the chemical safety of food. A new approach to trace-back these toxic xenobiotics along the food chain may consist in measuring markers of animal exposure to contaminants through omics approach. Among the various classes of metabolism end-products, we focused on volatile compounds which were identified as promising biomarkers to detect pathologies [11] or exposure to contaminants [10]. The present study aims to set up a SPME-MS fingerprinting method to point out the relevance of volatile metabolites in hepatic tissues as markers of chicken exposure to three types of xenobiotics: (i) an organochlorinated insecticide _lindane (γ-HCH), (ii) an environmental micropollutants_a mix of polychlorobiphenyls (PCBs), and (iii) an antibiotic _ ampicillin (Ampisol)

Key Words – Food safety, toxic contaminants, volatile biomarkers.

I. INTRODUCTION

The chronic exposure of animals to xenobiotics via the environment or the feed constitutes a major cause of the accumulation of these toxic substances in their tissues. Consequently, the consumption of the meat-based products susceptible to contain traces of these xenobiotics can lead to their transmission to human [1]. Nowadays, the control of the transmission of the contamination to human became a major field of the food safety particularly due to the strong correlation between the development of pathologies and the chronic exposure to contaminants [2,3].

The current analytical methods used for analysis of these toxic substances are most of the time the methods used by the reference laboratories which target specifically the contaminant residues in food or tissues. However, these methods are expensive, difficult to set up, and rarely compatible with frequent, regular and large-scale of controls required to guarantee effectively the chemical quality of food to the consumer. An analytical alternative to the classical methods is based on the characterization of metabolic compounds susceptible to be biomarkers of metabolic deviations in response to a pathology [4,5], a specific nutritional condition [6,7,8], or an exposition to toxic contaminants [4,9]. Among these compounds assumed as potential markers, the volatile compounds of low molecular weights were identified as particularly promising biomarkers to detect several types of pathologies [1]. To analyze volatile compounds, headspace solid phase microextraction (HS-SPME) is a suitable method: HS-SPME is sensitive, fast, automated, without solvent, and based on a minimal preparation of samples before the analysis limiting artifacts [5]. However, it is necessary to adapt the SPME parameters according to the type of the studied matrix to guarantee the extraction efficiency. The first part of the present work is dedicated to the determination of these parameters for the extraction of volatile metabolites in liver tissues which were identified as a markers of different types of cancer [9,5,10]. This step was achieved according to three key issues:

(i) Determination of the most suitable extraction settings. The type of fiber (CAR/PDMS and DVB/CAR/PDMS), the temperature (40 °C and 60 °C) and the time (15 and 30 min) of extraction under constant agitation at 500 rpm were tested.
(ii) Minimization of analytical variability of the extraction. The time of defrosting samples from -80°C to 4°C (8, 16 and 24 h) and the number of samples by analytical series (4, 5, 7 and 10 samples) were tested.
(iii) Improvement of the sensitivity of the extraction. The sample amount put in vials (1.2g and 2g) and the salt addition (Na_2SO_4 anhydrous and saturated solution of NaCl) were tested.

II. MATERIALS AND METHODS

II.1. Collect and sample preparation

Three types of xenobiotic were chosen for the exposure stage of the animals: (i) an insecticide_ lindane (γ-HCH) known for its bioaccumulation in tissues of the exposed animals, (ii) an environmental contaminant _Polychlorobiphenyls (PCBs) considering its toxicity and its accumulation in the animal products (EFSA, 2012) and (iii) an antibiotic_ ampicillin (Ampisol), the most used for preventive treatment against poultry digestive infections. Forty seven chickens (Gallus) were raised and separated in 4 groups which correspond to control animals (15 chickens) or contaminated animals (3x9 chickens) with a type of contaminant. After slaughter, livers were excised from the chicken carcasses and immersed in liquid nitrogen, wrapped in aluminum foil, vacuum packed, and stored at -80°C. Each liver tissue was ground for 3 min in liquid nitrogen into a fine homogeneous powder using a homemade stainless steel ball mill. Samples of 1.2 g of powder of liver tissue were conditioned in 20ml SPME vials, homogenized with a volume of saturated solution of NaCl. Vials were agitated for 30 seconds (Vortex T Genie 2), left for 24 hours at4°C being deposed on a metallic support at 4°C for extraction by HS-SPME.

II.2. HS-SPME-GC-MS procedure

Briefly, a multipurpose sampler (AOC-5000, Shimadzu, Kyoto, Japan) was used to carry out the following successive steps: (i) preheating of the sample in the agitator (500 rpm) for 10 min at the same temperature of the trapping step (40 or 60°C), (ii) trapping the volatile fraction by SPME fiber (CAR/PDMS or DVB/CAR/PDMS) for 15 or 30 min at 40 or 60°C, and (iii) thermal desorption of the volatile compounds at 280°C for 2 min in the GC inlet.
The desorbed VOCs were analyzed according to Berge et al.[10] with a QP2010+GC-MS system (Shimadzu) equipped with a Rxi-5SilMS column (60m × 0.32 mm I.D × 1µm film thickness, Restek, Bellefonte). Helium was used as the carrier gas at a flow rate of 1 $ml.min^{-1}$. The injections were performed in the Splitless mode (2 min). The column temperature program was as follows: 40°C for 5 min, increasing at 3°C/min to 230°C with a final isothermal period of 10 min. For the MS system, the temperatures of the transfer line, quadruple, and ionization source were 230, 200 and 150°C respectively. Positive ion electron impact mass spectra at 70eV were recorded in the scan mode in the range of m/z 33-250 amu at 10 scans s^{-1}. The chromatograms obtained in full scan by SPME-GC-MS were converted into virtual SPME-MS fingerprint according to Berge et al., 2011. Each fingerprint is a mass spectrum where the abundance of each mass fragment ranging from 33 to 250 amu corresponds to the sum of its abundance acquired at each MS scan during the GC run.

II.3. Data processing and statistical analysis

Tentative identification of targeted volatile markers in liver tissues was performed on the basis on mass spectra, by comparison with the NIST/EPA/NIH mass spectral library (NIST08), and on retention indices (RI), by comparison with published RI values and with those of our internal data bank. Data were processed using Statistica Software release 12.0 package (Statsoft, Maison-Alfort, France) and the R software version 2.1.4 (http://www.R-project.org). For the virtual SPME-MS fingerprints, the 218 mass fragments which constitute each SPME-MS fingerprint were normalized according to the Berge et al.[9]. Principal component analyses (PCA) were performed on the discriminated log ratios of mass fragment abundances selected in the SPME-MS signatures in order to visualize the structure of the data for each comparison.

III. RESULTS AND DISCUSSION

III.1. HS-SPME procedure for analysis of volatile markers in liver

In order to extract volatile metabolites in liver, it was necessary to set up optimal parameters for

the extraction which allows a large representative extract, a limited analytical variability, and a maximum of sensitivity.

III.1.1. Representativeness of the extraction

This part of the evaluation of SPME parameters concerns the selection of fiber type, the temperature and the time of extraction.

Firstly, the selection of the SPME fiber determines the range of volatile metabolites extracted via the SPME fiber coating.

Two fibers (CAR/PDMS vs DVB/CAR/PDMS) were compared to extract volatile metabolites from liver samples. The comparison of the tested fibers based on chromatographic areas and relative standards deviations (RSDs %) of identified volatile biomarkers shows that the best efficiency of the most volatile metabolites having low molecular weights was obtained with CAR/PDMS coating while DVB/CAR/PDMS fiber was rather adapted for the extraction of heavy metabolites. Thus, CAR/PDMS fiber was selected for the following optimization studies.

Secondly, extraction temperature will substantially affect the diffusion rates of volatile metabolites. Using an extraction time of 30 min, the effect of extraction temperature was tested at 40 and 60 °C. Raising the temperature extraction from physiological temperature at 40 to 60 °C increased the chromatographic areas of extracted metabolites. Besides, RSD values for identified metabolites at 60 °C were higher than those obtained at 40 °C. On the basis of these results, 40°C was chosen as the extraction temperature for further analyses.

Finally, the extraction time in metabolic profiling based on SPME analysis was rather minimized to avoid sample degradation during the extraction [11]. So, the influence of extraction time on the efficiency of the SPME process to extract VOCs in liver was investigated by exposing the CAR/PDMS fiber to liver sample at 40 °C for 15 and 30 min. The results demonstrated that the extraction time affected the abundances of volatile metabolites. The abundances of the heavy metabolites were higher after an extraction for 30 min than for 15 min. Moreover, RSDs of all volatile metabolites decreased after an increasing of the extraction time from 15 to 30 min. On the basis of these results, the best extraction settings were obtained with the exposition of CAR/PDMS fiber to liver sample at 40°C for 30 min.

III.1.2. Limitation of analytical variability

Outside the equilibrium of the volatile biomarkers between matrix and dynamic headspace, the quantity of volatile components is unstable which can impact the variability of HS-SPME extraction. Time of the defrosting of samples for 8, 16 and 24 h at 4°C and number of samples (4, 5, 7 and 10) by analytical series were evaluated to test their impact on the stability of the extraction of volatile metabolites. Concerning the defrosting of samples, the maximal abundances and the minimal RSDs for the targeted volatile markers were obtained after a defrosting time of samples of 24 hours à 4°C. Besides, concerning the number of samples by series, the results showed a decrease in the abundances and an increase in RSD values for all volatile metabolites after the 7th sample of the series.

III.1.3. Improvement of sensitivity

The impact of the sample amount and the salt addition on the signal intensity of the extracted volatile components were assessed.

Firstly, sample amount was fixed at 1.2g of liver powder according to Berge and al.[9]. The effect of the increase of sample amount was studied by testing a sample amount of 2 g of liver. The results demonstrated a proportional increase between the abundances of the identified volatile metabolites and the sample amount. In terms of variability, RSDs were higher with 2g of sample than those obtained with 1.2g. A best sensitivity of extraction was thus obtained with 1.2g.

Secondly, the effect of ionic strength was evaluated by testing the impact of the addition to sample of: (i) sodium sulfate anhydrous (Na_2SO_4) added in powder or (ii) a saturated solution of sodium chloride (NaCl). Signal intensities of volatile metabolites extracted by HS-SPME were higher when salts were added. This result confirm the salting-out effect of the salt on signal intensities of extracted VOCs by HS-SPME. In terms of variability, RSDs increased with the addition of salt for all identified metabolites. Particularly, RSDs obtained with the addition of Na_2SO_4 were higher than those obtained after the addition of NaCl. This behavior may be explained by the viscosity due to presence of the solid salt in contact with the

liver sample. This can influence the transfer of VOCs between matrix and headspace [12].

III.2. Application of HS-SPME procedure to study volatile metabolite markers of poultry exposure to xenobiotics

HS-SPME-GC-MS was used for the analysis of volatile fractions in liver samples from control chickens and chickens contaminated by lindane, PCBs or ampicillin.

Normed PCAs were performed on the ratios of mass fragments discriminating the control animals from those contaminated with lindane (Figure 1), PCBs (Figure 2) and ampicillin (Figure 3), respectively. The first maps of these PCAs show that that SPME-MS fingerprints of volatile fraction in liver of contaminated animals were significatively different from those found in liver of non-contaminated chickens. This result demonstrates that the contamination causes biochemical changes on the volatile profiles in the liver of the exposed animals.

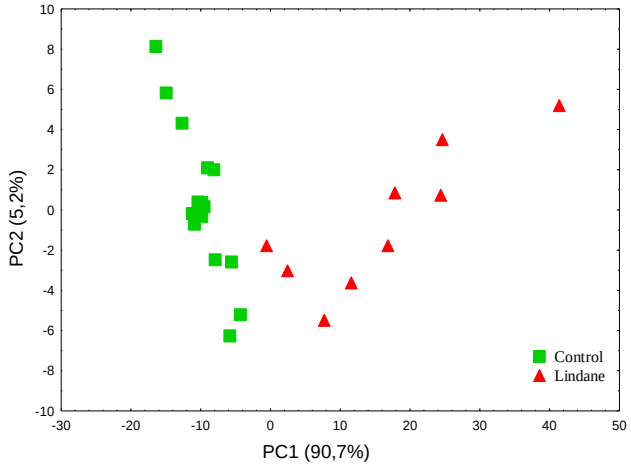

Fig 1. Normed PCA plotted on the discriminative mass fragment ratios selected in SPME-MS fingerprints of the volatile fraction in liver of control chickens and "lindane" contaminated chickens.

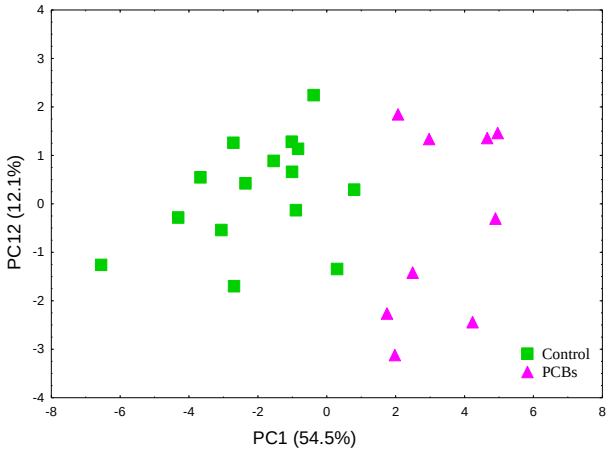

Fig 2. Normed PCA plotted on the discriminative mass fragment ratios selected in SPME-MS fingerprints of the volatile fraction in liver of control chickens and "PCBs" contaminated chickens.

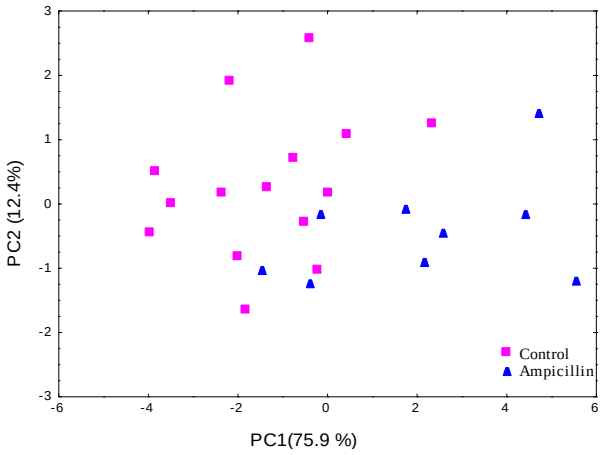

Fig. 3 Normed PCA plotted on the discriminative mass fragment ratios selected in SPME-MS fingerprints of the volatile fraction in liver of control chickens and "ampicillin" contaminated chickens.

IV. CONCLUSION

HS-SPME-GC-MS procedure has been developed to obtain the best representativeness, the minimal analytical variability and the maximal sensitivity for the extraction of volatile biomarkers in liver tissues. The use of this HS-SPME-GC-MS procedure enabled to reveal differences between the volatile fingerprints in animal liver of control group and contaminated groups.

Further investigations are in progress to identify the volatile biomarkers which allow non-

contaminated and contaminated animals to be discriminated.

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