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Solid-phase microextraction set-up for the analysis of liver volatolome to detect livestock exposure to micropollutants☆

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ABSTRACT

Starting from a critical analysis of a first "proof of concept" study on the utility of the liver volatolome for detecting livestock exposure to environmental micropollutants (Berge et al., 2011), the primary aim of this paper is to improve extraction conditions so as to obtain more representative extracts by using an extraction temperature closer to livestock physiological conditions while minimizing analytical variability and maximizing Volatile Organic Compound (VOC) abundancies. Levers related to extraction conditions and sample preparation were assessed in the light of both abundance and coefficient of variation of 22 candidate VOC markers identified in earlier volatolomic studies. Starting with a CAR/PDMS fiber and a 30 min extraction, the reduction of SPME temperature to 40 °C resulted in a significant decrease in the area of 14 candidate VOC markers ($p < 0.05$), mainly carbonyls and alcohols but also a reduction in the coefficient of variation for 17 of them. In order to restore VOC abundances and to minimize variability, two approaches dealing with sample preparation were investigated. By increasing sample defrosting time at 4 °C from 0 to 24 h yielded higher abundances and lower variabilities for 15 and 13 compounds, respectively. Lastly, by using additives favouring the release of VOCs (1.2 g of NaCl) the sensitivity of the analysis was improved with a significant increase in VOC abundances of more than 50% for 13 out of the 22 candidate markers. The modified SPME parameters significantly enhanced the abundances while decreasing the analytical variability for most candidate VOC markers. The second step was to validate the ability of the revised SPME protocol to discriminate intentionally contaminated broiler chickens from controls, under case/control animal testing conditions. After verification of the contamination levels of the animals by national reference laboratories, data analysis by a multivariate chemometric method (Common Components and Specific Weights Analysis – ComDim) showed that the liver volatolome could reveal dietary exposure of broilers to a group of environmental pollutants (PCBs), a veterinary treatment (monensin), and a pesticide (deltamethrin), thus confirming the usefulness of this analytical set-up.

1. Introduction

The exposure of livestock to xenobiotics through the environment or feed is a major cause of their presence in derived meat products [1], where they may constitute a risk to human health [2]. Mounting evidence suggests that chronic exposure to chemical contaminants may be a cause of many health disorders [3,4]. The analytical methods implemented to test for these contaminants

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in food are most often based on those used by national reference laboratories that target specifical contaminant residues in foods or tissues. However, these strategies are too costly for the rapid batch-to-batch quality control monitoring required to ensure food safety in the production chain [5]. Promising new approaches have therefore been proposed to identify markers of exposure to micropollutants in livestock.

Among them are toxicogenomic approaches which consist in analysing the different genomic, transcriptomic, proteomic and metabolomic profiles of the tissues and fluids of livestock exposed to toxic substances in order to detect markers of contamination. Several recent studies have shown the particular utility of volatile organic compounds (VOCs) for detecting health disorders [6–8] and exposure of livestock to contaminants [9]. As attested by most of the recent work in volatolomics, solid-phase microextraction (SPME) is usually chosen for its performance and convenience [10,11]. This method, devised by Pawliszyn in 1989, is based on pre-concentrating VOCs on a fiber made from a silica film, which forms the stationary phase onto which the VOCs are adsorbed [12]. SPME is well-adapted to the qualitative identification of heterogeneous, complex mixtures and offers many advantages in terms of sensitivity, rapidity, ready automation, ease of use, and minimal sample handling before analysis thus limiting artefacts [13]. However, there is no general SPME procedure applicable to the extraction of VOCs from all biological matrices, whence the importance of adjusting extraction parameters to ensure the maximum efficiency of the SPME according to the matrix and the purpose of the analysis [14].

A first study conducted by Berge et al. [9] demonstrated the utility of the hepatic volatolome for detecting the exposure of livestock to several environmental micropollutants. In their paper, the extraction temperature of VOCs was set at 60 °C. However, since the physiological temperature of livestock animals ranges from 37 °C to 43 °C [15], an extraction temperature set as high as 60 °C may generate a representativity bias in the pattern of extracted VOCs. According to earlier works investigating food VOCs [16–18], a decrease of the extraction temperature from 60 °C to 35 or 40 °C may indeed limit the generation of thermally-induced VOCs that may be considered as analytical artefacts. However, a decrease in the peak height of less thermo-sensitive VOCs is generally to be expected when the extraction temperature is decreased from 60 to 40 °C [16], which can adversely affect the SPME extraction of these compounds and finally the sensitivity of the SPME-GC–MS method.

Two levers dealing with sample defrosting time and salt addition may compensate for these expected lower abundances while minimizing the extraction variability. In Berge et al. [9], liver samples stored at −80 °C were placed at 4 °C on the autosampler for immediate extraction. Several papers suggest that thawing time may be a key issue to consider prior to SPME analysis [19,20]. Extraction of the volatolome by SPME cannot be considered satisfactory unless the partitioning of the analytes between sample matrix and fiber film reaches a steady state, and the overall mass transfer to the fiber is typically limited by mass transfer rates from the sample to the headspace [21]. Since extraction temperature is to be set close to physiological temperature at 40 °C, a longer time may be needed to reach equilibrium state of VOCs, which could reduce the analytical variability of the SPME procedure as implemented by Berge et al. [9]. As well, the sensitivity for the detection of certain trace constituents of the volatolome might be improved by increasing the abundances of liver VOCs through the addition of salting out agents to the sample to be extracted by SPME [22,23]. Several studies have shown that adding salt, in particular NaCl [24] and Na_2SO_4 [25], to the sample may be useful. Other studies demonstrated a linear relationship between salt concentration and increase in analyte abundances, up to NaCl saturation [22]. Thus, maintaining a constant ionic strength in the samples by adding a saturated salt solution is crucial to achieving higher and reproducible analyte extractions.

The present work is devoted to improving the SPME protocol proposed by Berge et al. [9] so as to obtain more representative extracts by using an extraction temperature closer to the livestock physiological conditions (40 °C) while minimizing analytical variability and maximizing VOC abundances. The first part of the paper aims to set up a revised SPME protocol by adjusting extraction conditions and sample preparation. The suitability of the SPME protocol will be assessed using a chicken liver sample in terms of both abundances and the coefficients of variation of 22 VOC candidate markers identified in earlier volatolomic studies. In the second part, the applicability of the SPME-GC–MS method will be evaluated on a set of liver samples from control chickens, and chickens intentionally fed with diets contaminated by micropollutants potentially found in the meat chain, to reveal markers of livestock exposure to different types of contaminants. The micropollutants under study were: (i) a pesticide, deltamethrin; (ii) a group of environmental contaminants, polychlorobiphenyls (PCBs); and (iii) a coccidiostatic agent, monensin. A multivariate chemometric method named Common Components and Specific Weights Analysis (ComDim) [26] was applied to compare volatolomic signatures in livers of chickens exposed and unexposed to deltamethrin, PCBs, and monensin in order to detect differences that could be associated with the type of contaminant exposure.

2. Experimental section

2.1. Liver samples

Animal experimentation was conducted from 5/11/2013 to 28/01/2014 in PEAT at INRA of Nouzilly (France; Accreditation C37-175-1, delivered on 28/08/2012). Experimentation was evaluated by ethics committee n°19 of Val de Loire and authorized under reference 01012.01 according to rural articles (R. 214-78 and R. 214-126). In this animal test, chickens have been exposed or not to dietary micropollutants. Forty-four male chickens (*Gallus gallus*) (JA 657, Boyé Accouvage, La Boissière-en-Gâtine, France) were raised together until 28 days of age. They were then distributed into four groups, one control group (n = 17 chickens) and three other groups each exposed to a diet contaminated with one type of micropollutant (n = 9 chickens). The chickens were fed in individual cages for 8 weeks (28–84 days of age) with control or contaminated feed. The feed given to the chickens was a granulated mixture of commercial feed with 5% sunflower oil with or without added micropollutant. The amount of feed distributed each day was the same for all chickens and followed the breeder recommendations. Three contaminants were chosen for their different behaviour in the environment and the body: (i) deltamethrin, known for its persistence and bioaccumulation in animal tissues [27]; (ii) a mixture of PCBs (Arochlor 1260), known for their toxicity and their potential for bioaccumulation in animal products [28]; and (iii) sodium monensin, a coccidiostat authorized as a feed additive by European Commission Regulations. Table 1 presents the concentrations of contaminants in the feed chosen to be at the maximum levels fixed by the

regulations in poultry feed and sufficient to induce a response in the metabolism of exposed livestock. At age 84 days, all the chickens were slaughtered, after fasting for 12 h, by electronarcosis followed by bleeding according to European recommendation standard conditions. Two livers from the control group were dedicated to setting up the SPME parameters for the analysis of liver volatolome, while the rest of the livers were used to validate the SPME extraction protocol for the detection in liver volatolome of candidate markers of livestock exposure to micropollutants. After slaughter, the liver