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A validated ultra-high performance liquid chromatography coupled to high resolution mass spectrometry analysis for the simultaneous quantification of the three known boar taint compounds

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ABSTRACT

Boar taint is an off-odour that can occur when meat or fat from entire male pigs is heated. Most of the currently available analytical methods are not capable of detecting the three known boar taint compounds (indole, skatole and androstenone) simultaneously, which renders their analysis often labour-intensive and time-consuming as separate analyses are required. In this study a validated U-HPLC–HR-Orbitrap-MS analysis method is described for the quantitative determination of the three boar taint compounds in fat. The sample pre-treatment involves a melting step followed by extraction with methanol and clean-up consisting of a freezing step and solid phase extraction (HLB cartridges). The analytes are then chromatographically separated and detected with an Exactive™ high-resolution mass spectrometer. Due to the absence of guidelines for the analysis of boar taint in fat, the Commission Decision 2002/657/EC [18] and ISO 17025 [19] guidelines were used as guideline for validation of the developed detection method. This resulted in limits of detection and limits of quantification between 2.5 and 7 $\mu g\,kg^{-1}$ and between 5 and 10 $\mu g\,kg^{-1}$ for the three compounds, respectively, which is far below the threshold values set at 100 $\mu g\,L^{-1}$ for indole, 200 $\mu g\,L^{-1}$ for skatole and 1000 $\mu g\,L^{-1}$ for androstenone in pig fat samples. The method obtained for the three compounds a repeatability (RSD) lower then 12.7% and a within-laboratory reproducibility (RSD) lower than 16.9%. The recovery of the three compounds ranged between 99 and 112 and an excellent linearity ($R^2 \geq 0.99$) was found. In the future, this method may be extended with other compounds that turn out to be correlated with boar taint.

1. Introduction

The castration of male animals intended for meat production has been widely practised for centuries. The main reasons were easier handling of the castrated animals and the fact that these castrated animals deposit more fat, which was at that time requested by consumers. In recent years, a trend has been observed towards consumers demanding a diet consisting of more lean meat. This trend, combined with the lower production costs of entire males, has led to cessation of castration of cattle and sheep in most countries [1]. The castration of male pigs, however, remains a common practice in most countries because 4–25% [2] of entire males produce moderate to high amounts of boar taint, an off-odour released when heating the meat of some boars.

Castration of male piglets is generally performed without anaesthesia or analgesia. This practice has led to ethical constraints [1] and several alternatives are now being explored (castration with anaesthesia, immunocastration, use of analgesia, or production of entire males in combination with management procedures) [3]. At this time European legislation does not forbid castration without anaesthesia or analgesia. Nevertheless, several countries have either already forbade these types of castration or have the intention to stop all kinds of castration completely within several years (i.e. Norway, the Netherlands, Switzerland). A European Declaration describes taking the first step to stop castration without anaesthesia or analgesia by January 2012 and in the longer term, to abandon castration by 2018. Adequate detection methods are essential to determine whether the alternatives to castration are successful in decreasing boar taint.

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The main compounds attributed to the boar taint off-odour are skatole and androstenone; indole only contributes to a lesser degree [4,5]. Because of their lipophilic characteristics, the compounds tend to accumulate in the fatty areas of the animal. The extraction and clean-up of the fat matrix is therefore considered to be the most critical and challenging step for their analytical determination [6]. Several authors have stated that the following sample preparations of this fat matrix lead to a positive influence on the sensitivity of the detection method: liquid–liquid extraction [7], saponification [8], solid phase extraction [9] and liquification of the fat [10].

In recent years, several methods have been developed to determine the boar taint compounds. However, only a limited number of authors describe analysis methods for the indolic compounds (indole, skatole) and the steroid compound (androstenone) simultaneously. Androstenone is often determined by ELISA [11] or gas chromatography coupled to electron-capture detection [8], flame ionisation detection [12] or mass spectrometry [13], while the indolic compounds skatole and indole are determined by colorimetric methods [14] or liquid chromatography coupled to fluorescence detection [7]. Analysis of all three boar taint compounds may therefore be considered to be labour-intensive and time-consuming, as separate analyses must be performed.

Hansen-Møller [15] was the first to describe a simple method for the simultaneous determination of the three compounds with HPLC coupled to fluorescence detection. The obtained limits of detection (LODs) for the indolic compounds and androstenone were <3 $\mu g\,kg^{-1}$ and 20 $\mu g\,kg^{-1}$, respectively. Androstenone was detected using derivation with dansylhydrazine. However, the use of a derivation step may lead to possible false-positive results and is labour-intensive [16]. In addition, the determination of the limits of detection and quantification are determined in standard solutions instead of in a matrix. The possibility therefore exists that the LODs and LOQs would be higher in a matrix. More recently, a similar method was developed which relys on HPLC coupled to mass spectrometry [17]. It has a limit of quantification for the indolic compounds of 50 $\mu g\,kg^{-1}$ and for androstenone of 125 $\mu g\,kg^{-1}$. For routine purposes, however, this method was not robust enough (mainly for androstenone). The extraction procedure in particular needed improvement. Fischer et al. [18] developed a method with limits of quantification of 0.5 and 1 $\mu g\,kg^{-1}$ for skatole and indole, respectively, and 60 $\mu g\,kg^{-1}$ for androstenone using HS-SPME-GC–MS. Prior to the extraction, the fat was melted by a microwave step, after which methanol was added. This was followed by a freezing and evaporation step. Afterwards HS-SPME was used for extraction, while separation and detection occurred by GC–MS.

The objective of this study was to develop a quantitative, accurate, robust and fast U-HPLC–MS-based method that is capable of quantifying the three known boar taint compounds simultaneously in fat. The method was validated according to the guidelines of 2002/657/EC [19] and/or ISO 17025 [20] which for makes the method suitable for use when comparing boar taint alternatives.

2. Materials and methods

2.1. Reagents and chemicals

The reference standards indole (2,3-benzopyrrole), skatole (3-methylindole) and androstenone (5α-androst-16-ene-3-one) and the internal standards 2-methylindole (2MID) and androstadienedione (1,4-androstadiene-3,17-dione, ADD) were obtained from Sigma–Aldrich (St. Louis, MO, USA). For each compound a stock solution was prepared in methanol at a concentration of 1 $mg\,mL^{-1}$. Working solutions were made for each compound in methanol at a range of 5–100 $ng\,\mu L^{-1}$. Solutions were stored in dark glass bottles at −20 °C.

Reagents were of analytical grade when used for extraction purposes and of MS-grade for U-HPLC–MS applications. They were obtained from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific (Leichestershire, VS), respectively.

Table 1
Linear gradient for the separation of the boar taint compounds on a U-HPLC system.

Time	% 0.05 formic acid	% methanol
0.00	50	50
0.01	53	47
2.57	50	50
3.00	5	95
6.00	0	100
8.00	0	100
8.01	50	50
10.00	50	50

2.2. Samples

Barrows are normally slaughtered at a mean commercial slaughter weight of 110 kg. For the control samples, neck fat of barrows was collected 24 h after slaughter. Skin and muscle were separated from the fat before packaging and the samples were stored at −20 °C until analysis.

2.3. Extraction and clean-up

Two grams of fat were sliced into pieces and spiked with a mixture of internal standards (500 $\mu g\,L^{-1}$ 2-MID and 1000 $\mu g\,L^{-1}$ ADD). The fat was melted in a microwave oven for 3 min at 220 Watt and allowed to rest for another 3 min. This was repeated until at least 300 μL of liquid fat was obtained. An aliquot of 150 μL was taken and mixed with 750 μL of methanol by vortexing. The eppendorfs were put into a hot water bath (60 °C) for 60 min to enhance the liquid–liquid extraction. Next, the samples were frozen (−20 °C) for 60 min to clarify the supernatant. Afterwards the eppendorfs were centrifuged at 14,000 rpm for 5 min and 500 μL of the extract was diluted with 9500 μL water prior to solid phase extraction. Solid phase extraction (Oasis HLB 3 cm^3 (60 mg), (Waters)) was performed for further clean-up. The cartridge was conditioned and equilibrated with 2 mL of 100% and 5% methanol, respectively. After loading the sample, the cartridge was washed with 2 mL of 20% methanol and eluted with 1 mL of 100% methanol. Of the obtained extract, 500 μL was diluted with 500 μL of 0.05% formic acid, and 10 μL was injected directly onto the column.

2.4. Instrumentation

The U-HPLC system consisted of a Thermo Fisher Scientific (San José, CA, USA) Accela U-HPLC pumping system coupled to an Accela Autosampler and Degasser. Chromatographic separation was achieved using reversed phase chromatography with gradient elution. Separation of the compounds was carried out on a Hypersil Gold column (1.9 μm, 50 mm × 2.1 mm ID) (Thermo Fisher Scientific). The mobile phase consisted of a mixture of methanol and 0.05% formic acid, pumped at a flow rate of 0.3 $mL\,min^{-1}$. Optimized separation of the compounds was obtained using a linear gradient (Table 1).

Mass spectrometric analysis was carried out using an Exactive™ benchtop mass spectrometer (Thermo Fisher Scientific) fitted with an atmospheric-pressure chemical ionisation source (APCI) operated in the positive ion mode. The optimal ionisation source working parameters are given in Table 2. A scan range of m/z 100–500 was chosen and the resolution was set at 50,000 full width