# DEVELOPMENT AND VALIDATION OF A UHPLC-HR-ORBITRAP-MS METHOD FOR THE SIMULTANEOUS DETERMINATION OF BOAR TAINT IN PORCINE MEAT AND MEAT PRODUCTS

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**Abstract – Boar taint is an off-odour that entails negative consumer reactions. In this study, two extraction and UHPLC-HRMS analysis methods, valuable for evaluation of consumer acceptance towards boar tainted meat, were developed for quantification of indole, skatole, and androstenone in different meat products. Sample pretreatment consisted of extraction with methanol and a homogenizing step (cooked ham, minced meat, tenderloin, bacon, cutlets, blade loin and uncooked ham) or a melting step (salami sausage and liver paste). Both methods were validated according to CD 2002/657/EC and ISO17025 guidelines, and good performance characteristics were obtained. Both methods showed good linearity ($R^2 \geq 0.99$) and no lack of fit was observed. Also good recoveries (89 % - 110 %), repeatabilities (RSD ≤ 14.9 %) and within-laboratory reproducibilities (RSD ≤ 17.2 %) were obtained. Analysis of cooked ham and salami sausage samples proved the applicability of both methods for routine analysis.**

**Key Words – androstenone, consumer acceptance, indole, skatole**

## I. INTRODUCTION

The surgical castration of male piglets has been widely practiced for centuries. The main reason for this practice is the prevention of boar taint (BT), i.e. an off-odour that can be released by heating the meat or fat of non-castrated boars [1]. The main compounds contributing to this taint are 5α-androst-16-ene-3-one (AEON)[2], skatole or 3-methylindole (SK) [3], and to a lesser extent indole (IND) [4]. Since research showed that surgical castration causes pain, even in very young animals, societal pressure against the surgical castration of pigs has risen [5]. For this reason, in 2010 the European declaration on alternatives to surgical castration of pigs was signed, in which participating member states engage to no longer perform surgical castration of pigs without anaesthesia and/or analgesia, and in the long run to ban surgical castration of pigs by January 2018 [6]. A valuable alternative is the production of entire males, which is associated with a lower feed conversion ratio, faster growth and more lean meat production [7]. However, the main problem remains the possible presence of BT. In light of the impending ban on surgical castration, it is important to valorize meat from entire males and to assess its impact on consumer acceptance. Since general agreement on acceptable levels of BT compounds is lacking [8], determination of odour thresholds in meat instead of neck fat could increase the understanding of consumer acceptance of meat from entire males. Accordingly, the determination of thresholds necessitates analytical methods for the quantification of the BT compounds in different meat matrices. For this reason, the aim of this study was to develop accurate, robust and fast extraction and UHPLC-HRMS analysis methods for the simultaneous quantification of AEON, SK, and IND in different meat products. The methods were validated according to the guidelines of CD 2002/657/EC and ISO17025 [9, 10].

## II. MATERIALS AND METHODS

*Reagents and Chemicals*

The reference standards IND (2,3-benzopyrrole), SK (3-methylindole), and AEON (5α-androst-16-ene-3-one) and the internal standards 2-methylindole (2-MID) and androstadienedione (1,4-androstadiene-3,17-dione, ADD) were obtained from Sigma Aldrich (St. Louis, MO, USA). Reagents were of analytical grade when used for extraction purposes and of MS-grade for

UHPLC-MS applications. They were obtained from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific (Lechestershire, VS), respectively. Solid phase (SPE) columns were purchased from Waters Corporation (Milford, US).

*Sample extraction and clean-up*

Two different protocols for extraction and clean-up of the samples were optimized. The meat products were subdivided into two categories in accordance with their fat percentage.

Cooked ham, minced meat, tenderloin, bacon, cutlets, uncooked ham and blade loin were subjected to the protocol including direct extraction with methanol. Two grams of blended meat were fortified with a mixture of internal standards (500 µg $kg^{-1}$ 2-MID and 1000 µg $kg^{-1}$ ADD). Methanol (5 ml) was added and each sample was vortexed for 30 seconds. Further homogenization was carried out using an Ultra-Turrax (IKA® T18 Digital) and the samples were centrifuged at 12,300 x g for 10 min. Next, the supernatant was transferred into 15 ml tubes, which were frozen (-20 °C) for 60 min to clarify the supernatant. Afterwards, the 15 ml tubes were centrifuged at 12,300 x g for 5 min and 2 ml of the extract was diluted with 38 ml water prior to solid phase extraction. The cartridge was conditioned with 2 ml of 100 % methanol and equilibrated with 2 ml of 5 % methanol. 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, 100 µl was diluted with 100 µl of 0.05 % formic acid prior to HPLC analysis.

Because of the higher fat content of salami sausage and liver paste, these products were subjected to an extraction protocol including a melting step. Five grams of salami sausage or 8 grams of liver paste were weighed. Both samples were fortified with a mixture of internal standards (500 µg $kg^{-1}$ 2-MID and 1000 µg $kg^{-1}$ ADD) and each sample was vortexed thoroughly. Next, the samples were melted in the microwave oven (salami sausage: 3 min at 200 Watt, liver paste: 3 min at 100 Watt), and the melted fraction of liver paste was centrifuged at 17,000 x g for 1 min at room temperature to separate the fat fraction from the supernatant. From both salami sausage and liver paste samples, an aliquot of 150 µl of melted fat was taken and mixed with 750 µl methanol. The eppendorfs were transferred into an ultrasonic bath (Elma® Transsonic Digital) for 10 min at 32 °C. Afterwards the eppendorfs were frozen (-20 °C) for 15 min and centrifuged at 17,000 x g for 5 min at 4 °C. Of the extract, 500 µl was diluted with 9500 µl water prior to solid phase extraction as previously described.

*Instrumentation*

Analysis of the BT compounds was carried out on an Accela UHPLC pumping system coupled to an Exactive™ benchtop mass spectrometer (Thermo Fisher Scientific, San José, USA) fitted with an atmospheric-pressure chemical ionization (APCI) source, as described by Bekaert et al [11].

*Method Validation*

Both optimized methods were validated according to the criteria of the European Commission, by evaluating the specificity, selectivity, linearity, trueness, precision, and limits of detection and quantification. To this end, a complete validation was carried out for cooked ham and salami sausage.

*Analysis of cooked ham and salami sausage samples*

In a pilot study, BT positive carcasses were selected at the slaughter line by means of the soldering iron method as optimized by Bekaert et al. [12]. To confirm the presence of IND, SK, and/or AEON, a neck fat sample from each carcass was analyzed according to Bekaert et al. [11]. After slaughter, commercial meat companies, located in Belgium, produced cooked ham and salami sausage from the selected carcasses. Subsequently, a sample was taken from each meat product and analyzed in duplicate using the newly developed methods.

## III. RESULTS AND DISCUSSION

*Method validation*

To evaluate specificity and selectivity, both blank and fortified samples were analyzed. In the blank samples, low concentrations of IND and AEON were found. A possible explanation could be the endogenous presence of the boar taint compounds in pigs. When fortifying the blank samples, a significant increase in peak area intensity of the

chromatographic peaks at their specific retention times could be observed, taking into account a signal-to-noise ratio of at least 3, also no other interfering substances could be found. The Linearity of both methods was assessed through 14-point based matrix-matched calibration curves. The obtained regression model showed good linearity ($R^2 \geq 0.99$) and no lack of fit was observed. Trueness was assessed as recovery by fortifying blank meat matrices, at three spike levels. The recoveries calculated for both extraction methods meet the permitted levels (-20 % to + 10 %) (Table 1 & 2). To evaluate precision (Table 1 & 2) of the methods, the repeatability and within-laboratory reproducibility were determined. The RSD values calculated for the repeatability were below 15 %, indicating good repeatability according to the criteria of the European Commission. Moreover, for the within-laboratory reproducibility, RSD values were in accordance with the performance limits as calculated by the Horwitz equation, indicating good precision of both methods.

Table 1 Summary of the method validation performance criteria as determined for cooked ham

| Analyte | Nominal Concentration (µg kg$^{-1}$) | Recovery Mean ± SD n = 18 | Precision | |
|---|---|---|---|---|
| | | | Repeatability RSD % n = 18 | Within-Laboratory Reproducibility RSD % n = 24 |
| **IND** | 50 | 109 ± 7 | 6.6 | 6.0 |
| | 100 | 106 ± 7 | 6.3 | 7.2 |
| | 200 | 107 ± 6 | 3.8 | 5.8 |
| **SK** | 100 | 97 ± 10 | 10.7 | 9.9 |
| | 250 | 101 ± 15 | 14.3 | 12.6 |
| | 500 | 98 ± 7 | 7.5 | 6.9 |
| **AEON** | 250 | 89 ± 13 | 14.9 | 17.2 |
| | 500 | 95 ± 10 | 11.0 | 15.3 |
| | 1000 | 110 ± 10 | 9.6 | 9.3 |

Table 2 Summary of the method validation performance criteria as determined for salami sausage

| Analyte | Nominal Concentration (µg kg$^{-1}$) | Recovery Mean ± SD n = 18 | Precision | |
|---|---|---|---|---|
| | | | Repeatability RSD % n = 18 | Within-Laboratory Reproducibility RSD % n = 24 |
| **IND** | 50 | 100 ± 6 | 5.9 | 6.5 |
| | 100 | 98 ± 6 | 6.2 | 5.6 |
| | 200 | 101 ± 3 | 3.4 | 3.2 |
| **SK** | 100 | 100 ± 3 | 2.9 | 2.6 |
| | 250 | 94 ± 10 | 10.1 | 8.9 |
| | 500 | 99 ± 3 | 3.4 | 3.3 |
| **AEON** | 250 | 104 ± 6 | 6.4 | 6.7 |
| | 500 | 103 ± 6 | 5.9 | 5.6 |
| | 1000 | 109 ± 11 | 10.5 | 10.2 |

*Analysis of cooked ham and salami sausage samples*

To illustrate the applicability of the newly developed methods, cooked ham and salami sausage samples produced with meat from BT positive carcasses were analyzed. In the cooked ham samples all BT compounds could be found (Figure 1A). However, levels obtained for the meat matrix were low in comparison to analysis of a neck fat sample from the same carcass. A possible explanation for this finding is the distribution and storage of the BT compounds in pork tissue. IND, SK, and AEON possess high partition coefficients (LogP IND: 2.14; LogP SK: 2.60; LogP AEON: 4.9), which translates into a strong lipophilic character. For this reason, the BT compounds are mainly present in adipose tissue and to a lesser extent in muscle tissue [13, 14]. In the salami sausage samples, all BT compounds were detected with signal-to-noise ratios > 10 and good peak shape was observed (Figure 1B). These findings show the applicability of the newly developed methods on cooked ham and salami sausage samples produced from boar-tainted meat.

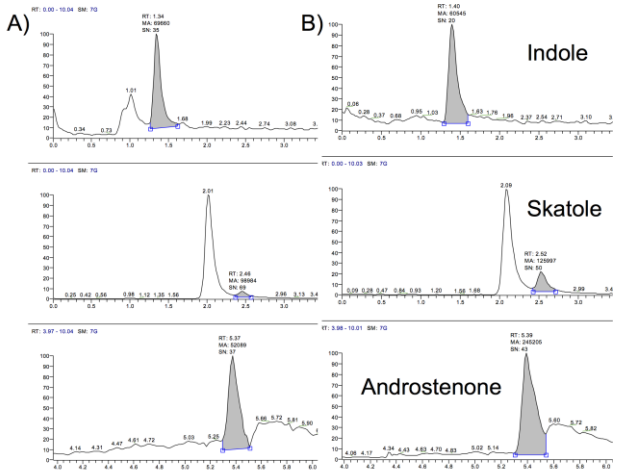


Figure 1 Chromatogram of a cooked ham (A) and salami sausage (B) sample produced from a carcass affected with boar taint

## IV. CONCLUSION

For the first time, robust, specific and selective extraction and UHPLC-HRMS analysis methods for the simultaneous quantification of IND, SK, and AEON in a wide variety of meat products were developed and validated according to the

criteria of the European Commission (CD 2002/657/EC). Two extraction protocols were optimized for the detection of the boar taint compounds in cooked ham, minced meat, tenderloin, bacon, cutlets, uncooked ham and blade loin on the one hand and salami sausage and liver paste on the other. Both methods showed a large linear range and good accuracy and precision. Additionally, analysis of cooked ham and salami sausage samples from boar taint positive carcasses proved the applicability of both methods for routine analysis and their suitability to study consumer acceptance towards boar tainted meat.

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