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Development and validation of a method for simultaneous analysis of the boar taint compounds indole, skatole and androstenone in pig fat using liquid chromatography–multiple mass spectrometry

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Abstract

Boar taint in entire male pigs remains a problem for fresh pork production. Since castration of pigs will be prohibited in the future on animal welfare reasons, attempts are made to detect boar taint pre and post mortem. Post mortem techniques focus on simultaneous quantification of the three boar taint substances by one simple and reliable method. In this study a liquid chromatographic multiple mass spectrometric (LC–MS^n) method has been developed and validated for the simultaneous determination of indole (2,3-benzopyrrole, ID), skatole (3-methylindole, SK) and androstenone (5α-androst-16-en-3-one, AEON) in pig fat samples. Sample preparation was kept as short as possible, since a single extraction method for structurally different indols and steroids was seeked after. Analytes were extracted from the fat matrix by methanol and clean-up consisted of freezing the extract in liquid nitrogen followed by a filtration step. The analytes were chromatographically separated on a Symmetry C_{18} column. Recoveries for ID, SK and AEON, as calculated from fortified fat samples using 2-methylindole (2-MID) as internal standard, were 96, 91 and 104%, respectively. However, matrix interferences were encountered determining the androstenone levels in fat. Linearity, determined in fat samples, was in the range of 50–1600 μg kg^{-1} for the indolic compounds and 125–2000 μg kg^{-1} for the steroid AEON. The correlation coefficients (R^2) of the calibration curves were 0.998 for ID, 0.997 for SK and 0.810 for AEON.

Keywords: Boar taint; LC–MS^n; Indole; Skatole; Androstenone; Fat matrix

1. Introduction

Consumer acceptance is the key to successful food products. For food products to be well received, off-odour formation during food processing for example has to be avoided. In this sense, boar taint in pork particularly is a problem when meat or meat products are heated by the consumer prior to consumption [1–3]. One of the main compounds associated with this offensive odour is the steroid androstenone (5α-androst-16-en-3-one, AEON), which is synthesised in the testes of boars, released into the blood, and because of its lipophilic properties, accumulated in fat tissue [2–7]. Boar taint has also been associated with the presence of the indolic compounds, indole (2,3-benzopyrrole, ID) and skatole (3-methylindole, SK), formed as a result of the breakdown of tryptophan by bacteria in the large intestine and marked by their high affinity for fat tissue [3–5,7–12].

Due to the lipophilic characteristics of ID, SK and AEON, a critical step in the determination of these compounds is the extraction from and clean-up of the fat matrix [6]. Contaminating matrix components of fat should be removed from the sample before analysis and several sample preparation procedures have been described for this purpose [8]. These include liquid–liquid extraction [13], steam distillation [14] and solid-phase extraction [15]. Supercritical fluid extraction and supercritical fluid chromatography have been successfully employed in the isolation of AEON from fat matrix [6]. For routine analysis these methods do not seem suitable, since they are characterised to be labour intensive and time consuming.

In recent years, many novel approaches have been developed for the determination of boar taint compounds in pig fat

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[9,10,13,16–18]. A colorimetric method as well as normal-phase and reversed phase high-performance liquid chromatography (HPLC) or gas chromatography (GC) have been reported for the analysis of SK in back fat [3,8,9]. For the measurement of AEON several detection methods have been developed, including an enzyme-linked immunosorbent assay (ELISA), GC with or without mass spectrometric (MS) detection [13], high-performance thin-layer chromatography (TLC) and HPLC [6,9]. These methods though, deal with the detection of either indolic compounds or the steroid AEON. This study, on the contrary, aims at determining both the indols, ID and SK, and the steroid AEON simultaneously in a fatty matrix.

The present paper describes the development of a methodology for rapid and effective simultaneous extraction of boar taint compounds from pig fat tissue. The applicability of a single LC–MS^n method as a specific detection method for both indolic compounds and a steroidic compound was evaluated.

2. Experimental

2.1. Reagents and chemicals

Indole (2,3-benzopyrrole, ID, purity 99.6%), skatole (3-methylindole, SK, purity 99.6%) and 2-methylindole (2-MID, purity 99.6%) were obtained from VWR International (Merck, Darmstadt, Germany). Androstenone (5α-androst-16-en-3-one, AEON, purity 99.3%) was purchased from Steraloids (London, UK). Solvents and reagents, purchased from VWR International (Merck, Darmstadt, Germany), were of analytical grade when used for extraction, and of LC-grade for LC–MS^n application.

For each component, a standard stock solution was prepared in ethanol (EtOH) at a concentration of 1000 ng μL^{-1}. Stock solutions were stored under three different temperature conditions, −20 °C, 4 °C and room temperature. Working standard solutions for spiking were prepared by appropriate dilution in EtOH of the stock solutions stored at −20 °C.

2.2. Sample preparation

Barrows (Piétrain × Hybride) were slaughtered at the age of 25 weeks, having reached a mean commercial slaughter weight of 110 kg. At the slaughter house loins with back fat were taken 24 h after slaughter. Loins were cut into slices of 2.5 cm, back fat was cut into pieces and everything was separately vacuum packed and stored at −20 °C until analysis.

A mixture of fat samples that did not exhibit measurable concentrations of the analytes of interest was used to validate the method.

2.3. Extraction and clean-up

To a 10 g of back fat sample, cut into thin flakes, 10 mL of methanol (MeOH) was added together with the internal standard (I.S.), 2-MID, added at a concentration of 200 μg kg^{-1}. After homogenisation performed with a stomacher Lab Blender 80 (Seward Medical UAC House, Great-Britain), fat matrix components were allowed to precipitate before transferring the supernatant into a new tube. Samples were cooled in liquid nitrogen and centrifuged at 6000 rpm for 5 min at 4 °C. In order to clear the upper phase, the freezing step was repeated once more. Finally, the extract was passed through 0.11 μm filter paper (Whatman, VWR International, Merck, Darmstadt, Germany). A second filtration through a 0.45 μm filter (Whatman, VWR International, Merck) was performed before dilution of 300 μL of the extract with 200 μL of 1% acetic acid (1% HAc) prior to analysis.

2.4. LC–MS^n analysis

The HPLC system consisted of a Finnigan Surveyor MS Pump Plus and a Finnigan Surveyor Autosampler Plus (Thermo Electron, San José, CA, USA). Chromatographic separation was achieved using reversed phase chromatography with gradient elution. Separation was performed using a Symmetry C_{18} column (5 μm, 150 mm × 2.1 mm, Waters, Milford, MA, USA). The mobile phase consisted of a mixture of MeOH and 1% HAc and was pumped at a flow rate of 0.3 mL min^{-1}. A linear gradient was used starting with a mixture of 50% MeOH and 50% of 1% HAc. The MeOH percentage was increased from 50 to 100% in 7 min. Between each sample the column was allowed to equilibrate at initial conditions (10 min). Analysis was carried out using a LTQ linear ion trap mass analyser (Thermo Electron) equipped with an atmospheric pressure chemical ionisation (APCI) interface. Data acquisition was carried out by Xcalibur 2.0 software (Thermo Finnigan, Austin, TX, USA).

Optimal ionisation source working parameters were: vaporiser temperature, 400 °C; sheath gas, 40 arbitrary units (a.u.); auxiliary gas, 5 a.u.; capillary temperature, 275 °C; capillary voltage, 2 V; and tube lens offset, 60 V. Data acquisition was performed in full scan mode and in product ion scan (PIS) mode, using as precursor ion the protonated molecular ion $[M+H]^+$ (Table 1). When applied, the normalised collision

Table 1
Chromatographical and mass spectrometric identification parameters, relative retention time (t_r) and precursor ion with corresponding product ions, for ID, SK, AEON and the internal standard 2-MID, as assessed from back fat fortified with a mixture of all analytes at a concentration equalling the internal action limits

Segment	Analyte	Relative retention time (t_r)	Precursor ion (m/z)	Normalised collision energy (%)	Product ions (m/z)	Product ion scan range (m/z)
1	Indole (ID)	0.66	118	55	100, 91, 86	50–125
1	Skatole (SK)	1.19	132	55	117, 100	50–140
2	Androstenone (AEON)	2.37	273	40	255, 199, 173, 159	75–300
1	2-Methylindole (2-MID)	1.00	132	55	117, 100	50–140

The internal standard was added at 200 μg kg^{-1}.

energy (N.C.E., %) was between 40 and 55%, the isolation width (IW, *m/z*) was 2.0, the activation time (AT) was 30 ms and the activation Q (AQ) was 0.25. The chromatograms were segmented into two windows, as shown in Table 1, in order to gain sensitivity.

2.5. Quality insurance

Prior to sample analysis, standard mixtures of the analytes of interest were injected in order to check the operation conditions of the chromatographic devices. To every sample, a procedure I.S. 2-MID, was added at a concentration of 200 $\mu g\,kg^{-1}$ prior to extraction. Analytes were identified based on their retention time relative to the I.S. and on the ion ratio of their product ions, using as precursor ion the protonated molecular ion $[M+H]^+$ (Table 1). Quantification of the steroid AEON was based on ion detection in the MS^2 mode related to the product ions. The low molecular mass indolic compounds were quantified by detection of the protonated molecular ion in MS^2 mode.

European Criteria 2002/657 implements Council Directive 96/23/EC having regard to monitoring certain substances and residues thereof in live animals and animal products [19]. However, this directive does not refer to endogenous boar taint substances, accordingly it was not meant to fulfil the criteria as described in European Criteria 2002/657. Within the scope of this validation, the performance criteria for analytical residue methods as defined in the European Criteria 2002/657 were used as a guideline to systematically cover the analytical performance issues.

2.6. Data analysis

Data processing was performed using Xcalibur 2.0 software (Thermo Finnigan). All data were further analysed using Microsoft® Excel (Microsoft Corporation, USA).

3. Results and discussion

3.1. Performance of the LC–MS^n analysis

Different gradients of MeOH and 1% HAc were assayed at a constant flow rate of 0.3 mL min^{-1} in order to get all analytes separated. The chromatographic conditions as mentioned in Section 2 allow a clear separation of the three analytes and the I.S. from any endogenous components in fortified fat matrix. To prevent transfer of analytes from one sample to the next, the column was equilibrated at initial conditions in between sample runs. Relative retention times (t_r) for ID, SK, AEON and the I.S., 2-MID, are given in Table 1.

Mass spectra were acquired using direct infusion of each standard in 1% HAc applying ESI and APCI as ionisation sources in both negative and positive mode. These preliminary experiments revealed that only in positive APCI mode, effective results were obtained. Table 1 shows the precursor ion and main fragments observed for each analyte in positive APCI mode. For each analyte, identification was based on the presence of at least two product ions in the MS^2-spectra whereby detection was performed through product ion scan (PIS) (Table 1).

During the development of the method several indolic compounds, such as 2-MID and 5-methylindole (5-MID), and a steroidic compound, androstanone (5α-androstan-3-one, AAON) were investigated as internal standards. Since 5-MID coeluted with SK under the applied conditions, 2-MID was prefered. AAON was chromatographically separated well from all other analytes of interest and a specific mass spectrum was obtained when running standard solutions, but matrix interferences were encountered when analytes were determined in fat matrix. Extraction of AAON was not reproducible so that 2-MID was retained as I.S. for quantification of both indols and the steroid. Investigations on a specific I.S. for the steroidic compound AEON are ongoing. In literature several studies make use of AAON [4,5,10,13,15,18,20]. Other steroidic compounds used are 5β-androstanone [20] and 5α-androstane-3,17-dione [2,6]. Androsterone and methyl-testosterone are also considered potential alternatives.

3.2. Performance of the extraction method

Sample preparation as performed in the developed method was based on the principle as described by Hansen-Møller [15]. The simultaneous extraction of the structural different boar taint compounds was achieved with MeOH and matrix components of fat were removed from the samples by freezing in liquid nitrogen followed by filtration. This sample preparation omits time consuming solvent distribution and procedural losses due to evaporation of the extract, but still leads to a sufficient separation of all three compounds from contaminating fatty substances.

3.3. Method validation

Because no guidelines for the analysis of boar taint compounds in pig fat exist, method development was based on the European Criteria 2002/657, describing criteria for analytical residue methods for matrices of animal origin [19]. From what is reported in literature as threshold values for boar taint compounds, internal action limits were set at 100 $\mu g\,kg^{-1}$ for ID, 200 $\mu g\,kg^{-1}$ for SK and 500 $\mu g\,kg^{-1}$ for AEON in pig fat samples [7,16,21,22].

3.3.1. Specificity

The specificity of the method could be demonstrated by analysis of blank fat samples and samples fortified with each analyte separately at the concentration level of the internal action limits. Since true blank pig fat samples do not exist [15], a mixture of fat samples, originating from barrows, containing very low background concentrations of the analytes of interest was used to validate the method. For each analyte spiked, chromatograms show a significant increase of peak area and intensity at its specific retention time compared to blanks, taking a signal to noise ratio of at least 3 into account (Figs. 1 and 2). No other endogenous substances interfere at these retention times so that the developed method was found to be specific for ID, SK and AEON in the presence of endogenous matrix compounds.

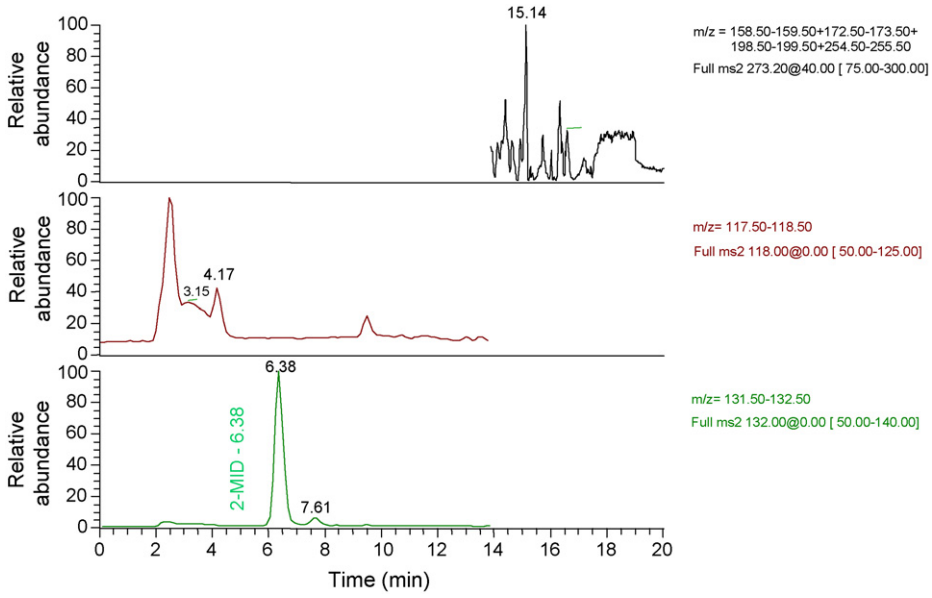

Fig. 1. Chromatograms for AEON (top), ID (middle) and both SK and 2-MID (bottom) of a blank fat sample to which only the internal standard, 2-MID is added at a concentration of 200 μg kg^{-1}.

3.3.2. Selectivity

Conform European Criteria 2002/657, analytes are identified on the basis of their relative retention time and the ion ratio of the precursor and product ions in the obtained spectrum [19]. Precursor and product ions of each analyte are given in Table 1. Since European Criteria 2002/657 was only considered as a guideline, not a rule, in the concept of this validation, identification points were not taken into account.

3.3.3. Calibration curves

The linearity of the developed method was evaluated by preparing calibration curves in matrix for the three analytes. Chromatographic peak areas were determined from the chromatograms of the precursor ion for the indolic compounds and the I.S. and from the chromatograms of the most abundant product ions for AEON. Linear plots determined with respect to 2-MID were constructed for ID ($y = 0.0048x + 0.0137$) and SK ($y = 0.0047x + 0.0251$), on the basis of eight-pount calibration curves, and for AEON ($y = 0.0002x - 0.0068$), based on seven concentration levels (Fig. 3). Linearity was achieved for both indols and for AEON within a range of 50–1600 μg kg^{-1} for ID and SK, and 125–2000 μg kg^{-1} for AEON. Correlation coefficients (R^2) obtained in this experiment were 0.998 for ID, 0.997 for SK and 0.810 for AEON.

For AEON, results were satisfactory but ask for further improvement. The lower correlation coefficient might be

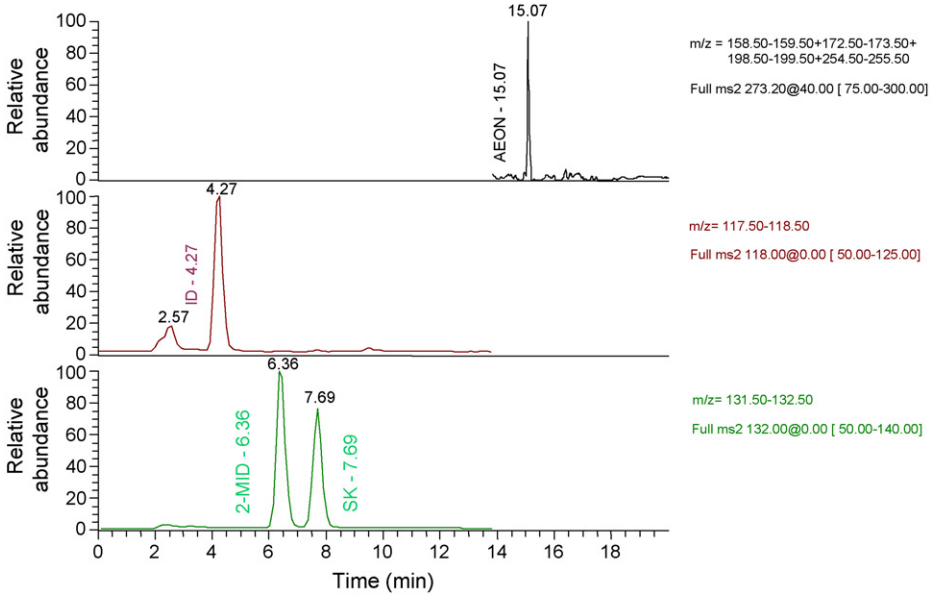

Fig. 2. Chromatograms for AEON (top), ID (middle) and both SK and 2-MID (bottom) of a blank fat sample fortified with ID, SK and AEON at their respective action limits of 100, 200 and 500 μg kg^{-1}. The I.S. 2-MID was added at a concentration of 200 μg kg^{-1}.

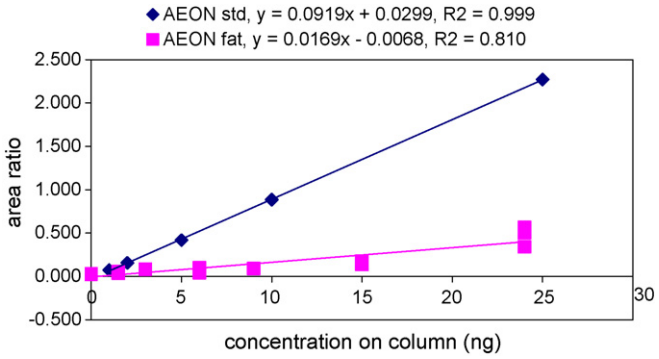

Fig. 3. Graphical representation of the calibration curves for AEON in standard solution (std) and in matrix (fat).

explained by the variation resulting from the extraction procedure that seems to hold minor difficulties in extracting the steroidic compound AEON from back fat. Precision for the determination of AEON in fat, as discussed in Section 3.3.5, is characterised by a relative standard deviation (RSD) of 33% reflecting the relatively high variability of the procedure. In literature, methods are described reporting higher correlation coefficients for extraction of AEON from fat matrix in the same concentration range but these methods only deal with AEON without taking into account indolic compounds [13]. On the contrary, the method as developed in this study allowed simultaneous extraction and detection of indols and steroids from pig fat matrix.

3.3.4. Recovery

Since no Certified Reference Material is available, the recovery is assessed by fortifying a mixture of fat of barrows, containing low background concentrations of all analytes of interest, with known amounts of these analytes. Samples of pig fat tissue were fortified at a concentration of 0.5, 1.0 and 1.5 times the concentrations set as internal action limits, 100 $\mu g\,kg^{-1}$ for ID, 200 $\mu g\,kg^{-1}$ for SK and 500 $\mu g\,kg^{-1}$ for AEON. Six replicates at each concentration level were extracted and analysed. Recovery for each analyte was calculated as the mean of all 18 samples at the level of the action limits. For samples spiked at a concentration above 10 $\mu g\,kg^{-1}$, the recovery should range from 80 to 110% [19]. The average recoveries for ID, SK and AEON were $96 \pm 17\%$, $91 \pm 13\%$ and $104 \pm 33\%$, respectively.

In comparison to recoveries for ID and SK as mentioned in other studies [3,8,9], the standard deviations as encountered for the described method are higher, though closely related to what is reported by Hansen-Møller [15]. According to this observation, it is supposed that the single step preparation procedure might explain the larger variation. Variation on recoveries reported for AEON are generally higher than those of indolic compounds [13,17]. In this study a similar observation was made. In several studies though, standard deviations on recoveries are not presented.

3.3.5. Precision

According to the Horwitz equation, the mean relative standard deviation (RSD) for repeated analysis of fortified blank back fat for a mass fraction of 100 $\mu g\,kg^{-1}$ should not exceed 23%, for a mass fraction of 200 $\mu g\,kg^{-1}$ and 500 $\mu g\,kg^{-1}$, the level of respectively 20% and 18%, should not be exceeded [19]. For each boar taint compound, the RSD at each concentration level and the overall RSD are summarised in Table 2 . Indolic compounds are characterised by RSDs lower than what is described by the Horwitz equation for the respective mass fractions. By contrast, for AEON, the RSD is not within the permitted range following European Criteria 2002/657. This indicates the need to improve the sample preparation procedure, in order to validate the method according to European Criteria 2002/657 [19].

Table 2
Overview of the validation parameters, recovery (%) and the corresponding relative standard deviation (RSD, %) at 0.5, 1.0 and 1.5 times the concentrations as set as internal action limits for ID, SK and AEON

Analyte	Spiked concentration ($\mu g\,kg^{-1}$)	Recovery (%)	RSD (%)
Indole (ID)	50	99	19
	100	90	12
	150	101	17
			Overall RSD (%) = 17
Skatole (SK)	100	94	17
	200	86	9.0
	300	93	14
			Overall RSD (%) = 15
Androstenone (AEON)	250	121	30
	500	101	26
	750	89	31
			Overall RSD (%) = 31

3.3.6. Limit of quantification

Different procedures to determine the limit of quantification (LOQ) are reported in literature [19]. In this study the LOQs were determined in matrix and were defined as the lowest concentration of the calibration curve which could be differentiated from zero, relating to peaks with a signal to noise ratio of at least 3. Due to the identical treatment of fortified and biological samples, a possible reagent blank is automatically compensated for. The LOQ for both ID and SK was set at 50 $\mu g\,kg^{-1}$, for AEON it was set at 125 $\mu g\,kg^{-1}$.

Lower LOQ values have been reported in literature, even for simultaneous analysis of all three analytes in fat matrix [15]. These LOQs were determined in standard solutions though, so that the extraction procedure was not considered. Since it is more accurate to include the extraction procedure when assessing LOQ values, in this study LOQ values were based on experiments in matrix.

3.3.7. Matrix effect

The effect of matrix on the analysis of boar taint compounds was evaluated by comparison of calibration curves

in standard solution and in matrix. For ID in standard solution ($y = 0.3804x - 0.0491$; $R^2 = 0.999$) and in fat ($y = 0.4015x + 0.0137$; $R^2 = 0.998$) curves parallel each other. A similar result was obtained for SK in standard solution ($y = 0.4224x + 0.0084$; $R^2 = 0.999$) and in fat ($y = 0.3931x + 0.0251$; $R^2 = 0.997$). From these results, no matrix effect was encountered for the indolic compounds. For AEON, by contrast, matrix has got an effect on the analysis (Fig. 3). Whenever analysing a batch of samples, spikes at three concentration levels are analysed and fitted into the calibration curve to account for this effect.

3.3.8. Stability of the compounds

The stability of standard stock solutions was evaluated at different storage temperatures, −20 °C, 4 °C and room temperature. Successive injections ($n = 3$) over 10 weeks revealed that RSDs of repetitive injections generally were the lowest when storage occurred at −20 °C, ranging from 3.70 to 7.63% for ID, from 0.75 to 7.67% for SK, from 0.67 to 6.16% for AEON and from 0.11 to 5.29% for 2-MID and that, regarding higher absolute peak areas, the sensitivity was highest at this storage temperature. Experiments regarding the stability of standard stock solutions in time are ongoing.

4. Conclusion

Many laboratories are searching for rapid, easy to perform assays to accurately determine tainted pigs in order to discard carcasses at the slaughter line [3,23]. Till now, these assays do not exhibit the same sensitivity, specificity and accuracy as analytical LC–MS^n methods. Therefore, analytical methods still are of value for screening of pigs for boar taint. Moreover, to gain insight in the contribution of certain compounds to boar taint, or their correlations with one another, accurate analytical determination of concentrations remains indispensable.

This method was designed for large-scale laboratory research on the investigation of different strategies in order to reduce boar taint.

The results obtained in this study showed that the simultaneous determination of ID, SK and AEON in back fat matrix was enabled combining minimised sample handling with a high specific mass spectrometric detection technique. However, as simultaneous analysis of structurally different indolic compounds and a steroid was assessed, future research will specifically address improvement of the extraction procedure.

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