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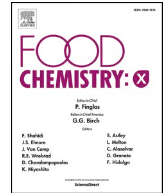

High throughput method for quantifying androstenone and skatole in adipose tissue from uncastrated male pigs by laser diode thermal desorption-tandem mass spectrometry

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ABSTRACT

The study aims at developing a rapid and robust mass spectrometric method capable of measuring the malodorous boar taint compounds androstenone and skatole in fat samples from male pig carcasses. The developed method is suited for use in commercial abattoirs as an at-line method to detect the presence of these compounds in carcasses or as a high-speed analysis in laboratories with high sample turnover. The chemical assay is based on salt-assisted liquid-liquid extraction and direct measurement with Laser Diode Thermal Desorption-Tandem Mass Spectrometry (LDTD-MS/MS). When fully automated as an at-line method, a single LDTD-MS/MS system will have a measuring capacity of >420 male pig carcasses per hour. The limit of quantification (LOQ) is 0.05 μg/g and 0.10 μg/g for skatole and androstenone, respectively, which is well below the expected sorting thresholds. The reproducibility of the method (%RSD) meets the industry requirement for an RSD of below 10%.

1. Introduction

In recent years, there has been strong and increasing opposition towards the castration of male piglets from, among others, several animal welfare NGOs. A Brussels Declaration calling for a voluntary ban on the surgical castration of pigs without sedation in Europe by 2018 has been signed by several representatives of the pig meat value chain in EU member states. However, a major challenge related to the production of meat from uncastrated male pigs is the risk of tainted meat. A variable percentage of the entire males develop boar taint, a flavour that is generally undesirable and not accepted by some consumers (Purwins & Schulze-Ehlers, 2018; Christensen, Nielsen, & Aaslyng, 2019). There is a general understanding that boar taint is predominantly caused by the compounds skatole and androstenone (Bee, Chevillon, & Bonneau, 2015; Andresen, 2006). Indole is often mentioned alongside androstenone and skatole as contributing to boar taint, even though it plays a minor role in consumer acceptance of meat from entire male pigs compared to the other components. Hence, in this work it was decided to focus our research on the two major contributors: skatole and androstenone. Assuming that the meat industry produces entire male pigs on a large scale, the impact on meat quality must be addressed, and an objective method for sorting carcasses according to their content of malodorous compounds is needed.

This has led to several attempts within the European Union to develop an analytical method for the identification and quantitation of boar-tainted meat (Buttinger, Karasek, Verlinde, & Wenzl, 2014). Methods that are potentially capable of sorting tainted carcasses at the slaughterhouses, maintaining fast line speeds and ensuring that no tainted meat reaches the consumers have been developed. In collaboration with Danish pig slaughter companies, a list of required specifications was drawn up for an analytical method capable of being implemented as an industrial at-line system:

- Reproducibility for both skatole and androstenone must not exceed an RSD of 10%.
- Limit of quantification (LOQ) must be well below the expected sorting thresholds for both skatole and androstenone.
- The chosen method must be objective and verifiable, i.e. quantitative.

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- The analytical equipment must be capable of delivering a set of results every tenth second.
- The analysis price must not exceed 1.3 EUR per carcass, excluding depreciation of the equipment.
- Daily routine maintenance and service must not exceed one hour of work for a single laboratory technician.
- The time between preventive maintenance activities requiring specialised personnel should be at least four to six months, corresponding to approx. 200,000 samples.

Furthermore, there was a request for a total analysis time (from the sample arriving at the laboratory to the analytical result being available) of less than 40 min. At Danish slaughterhouses, the sorting of carcasses according to weight, meat content, boar taint etc. takes place when the carcasses enter the cold storage rooms. Since quick chilling tunnels are used, the sorting does not take place immediately after slaughtering and classification of the carcasses. Therefore, the result of the boar taint detection is not needed immediately after sampling at the slaughter line. It is assumed that the above specifications will cover the requirements of large pig slaughter companies in general, whereas smaller companies do not need a high speed (a set of results every tenth second) corresponding to a capacity of 360 samples per hour.

Based on screening for state-of-the-art instrumentation and selection of a promising analytical technique, the hypothesis is, that it should be possible to develop a rapid and robust method to quantify skatole and androstenone simultaneously, while meeting the requirements of the slaughterhouses. The aim of this study was to develop an accurate method for simultaneous measurement of the boar taint components skatole and androstenone in small back-fat samples in accordance with industrial demands for speed of operation, thereby ensuring that it can be implemented at slaughterhouses as an at-line objective method.

2. Choice of instrumentation technology

Several technologies for rapid determination of the boar taint components androstenone and skatole were evaluated for their potential ability to comply with the specified requirements either through literature screening or direct contact to vendors. Haugen et al. (Haugen, Brunius, & Zamaratskaia, 2012) reviewed the literature for boar taint analysis and listed analytical methods covering sampling, extraction, derivatisation chromatographic and immunological methods. Ion mobility spectrometers used as electronic noses with various detection systems have been proposed for head space analysis of fat samples as at-line instrumentation for measuring boar taint (Van Dijk, 2001; Vestergaard, Haugen, & Byrne, 2006). However, many of these methods have either proven inadequate with respect to the demands for sensitivity and selectivity or are too slow for industrial use. Other chromatographic methods have been developed, for example LC-MS/MS (Buttinger et al., 2014), SPME-GC/MS (Verplanken et al., 2016), GC–MS/MS (Sørensen & Engelsen, 2014), dynHS-TD-GC/TOFMS (Fischer et al., 2014) or high pressure liquid chromatography (HPLC) with fluorescence detection (FD) (Hansen-Møller, 1994). Most of these methods exhibit excellent LOQ, limit of detection (LOD) and repeatability, typically with LOQ levels below 0.05 μg/g for both androstenone and skatole. When analysing hundreds of fat samples per hour, the use of chromatographic methods will result in a high turnover of expensive and vulnerable consumables, such as GC or LC columns. Furthermore, the robustness of the mass spectrometer may also be an issue, with the instrument requiring a high level of service and maintenance. Additionally, appropriate sample preparation is time-consuming and may ultimately prevent these methods from being optimised for rapid at-line methods in an industrial environment. Indeed, methods with direct injection of extracts into the mass spectrometer have also been developed. A direct injection method would result in a high-speed solution. However, due to problems with injecting large amounts of fat into the mass spectrometer, this solution could lead to increased maintenance frequency and would require extremely robust mass spectrometers. During the last few years, the introduction of the REIMS (Rapid Evaporative Ionisation Mass Spectrometry) technology has proven promising, since it has a sample-to-sample turnover time of less than ten seconds and requires no sample preparation (Hemeryck et al., 2019). However, this method does not measure skatole and androstenone directly but is semiquantitative in that it relies on chemometric analysis relating the entire mass spectrum, including the fatty acid fingerprint, to the analytes of interest (Verplanken et al., 2017). Thus, the method will be strongly dependent on the pig breed and feeding strategies used by different pig farmers and was therefore disregarded.

At the beginning of this project, DMRI (Danish Meat Research Institute) evaluated a PTR-TOF-MS (Proton Transfer Reaction-Time of Flight-Mass Spectrometer)-based method (Borggaard, Schäfer, & Kjærsgaard, 2012; Borggaard, Christensen, Hviid, & Kjaersgaard, 2012). The instrument responded rapidly to both skatole and androstenone. However, the signal-to-noise levels were such that the LOQ was prohibitively close to the expected sorting thresholds. Isobars that obscured the mass peaks for both androstenone and skatole were also present in the fat samples, and the method was therefore regarded as unsuitable. Finally, a laser diode thermal desorption ion source combined with tandem mass spectrometry (LDTD-MS/MS) was considered. The technology is based on the heating and desorption of the analytes in a dried extract which are ionised by atmospheric pressure chemical ionisation (APCI) by a corona discharge. Desorption and ionisation can be achieved in as little as 5.1 s per sample. Thermal desorption provides an attractive solution, since very little gas enters the mass spectrometer, leaving mass spectrometer contamination a negligible problem and at the same time ensuring an ultra-fast, sensitive and selective analysis. LDTD-MS/MS was therefore chosen as the most promising candidate for the development of a robust, rapid and automatable industrial assay.

3. Materials and methods

3.1. Instrumentation

Homogenisation of the adipose back-fat tissue was performed using a Kinematica Polytron PT3100D with a PT 12/2 dispersing aggregate (Kinematica, CH).

The mass spectrometer method was developed on a Sciex 6500 QTRAP MS/MS (AB Sciex, USA) connected to a laser diode thermal desorption (LDTD) model 960S with built-in atmospheric pressure chemical ionisation (APCI) (Phytronix, Quebec, Canada).

3.2. Reagents and solvents

Calibration standards were prepared from skatole (3-methyl indole, CAS No. 83-34-1, Sigma Aldrich, Germany) and androstenone (5α-Androst-16-en-3-one, CAS No. 18839-16-7, Sigma Aldrich, Germany).

Internal standards were prepared from 3-methyl-d3-indole (CAS No. 111399-60-1, CDN Isotopes, Canada) as internal standard for skatole, and androstanone (5α-androstan-17β-3-one, CAS No. 1224-95-9, 3B-Scientific, Germany) as internal standard for androstenone.

As extraction solvent, an acetonitrile solution (A) was prepared from acetonitrile (HPLC grade >99.9% purity) and spiked with 3-methyl-d3-indole and 5α-androstan-17β-3-one to 0.058 mg/L (0.25 mg/kg fat) and 0.466 mg/L (2.00 mg/kg fat), respectively, and as washing solvent, a saturated brine (>99% NaCl, Merck, USA) solution with 0.08 M sodium hydroxide (>99%, Merck, USA) was prepared, hereafter referred to as brine solution (B).

3.3. Sample pretreatment

Samples for extraction optimisation were analysed by GC/MS and HPLC according to the European Commission Joint Research Center (Buttinger et al., 2014; Hansen-Møller, 1994).

Prior to measurement in the LDTD-MS/MS system, back-fat adipose tissue samples were subjected to a salt-assisted liquid–liquid extraction (SALLE). Samples (0.35 g) were placed in 10 mL centrifuge tubes (polypropylene, PP), followed by addition of 1.5 mL brine solution (B) and 1.5 mL acetonitrile solution (A) containing internal standards. The content of the tubes was homogenised at 30000 rpm for 45 s. After homogenisation, the tubes were centrifuged at a relative centrifugal force (RCF) of 5000 g for three minutes at 22 °C. After centrifugation, 3 μL of the organic phase was transferred to a Lazwell™ plate, where it was left to dry for five minutes before being loaded in the LDTD-MS/MS for measurement.

Skatole is a relatively volatile compound and evaporates from the Lazwell™ plate if left to stand for a prolonged time as a dried extract.

For the trueness evaluation (defined as closeness of a measurement to the true value, ISO 5725), back-fat from 12 carcasses was analysed. Prior to analysis, the samples were trimmed by removing the skin and meat and were blended with a small household coffee grinder until homogeneous.

3.4. *Blank samples for calibration*

Homogenised back-fat adipose tissue from castrated male pig carcasses, without measurable amounts of androstenone and minimum amounts of skatole (less than the LOD), was used as a matrix for standard addition calibration curves.

3.5. *LDTD and mass spectrometer settings*

The LDTD requires the use of special microtitre plates called Lazwell™ plates (Phytronix, Canada). The tissue sample extract is deposited in a Lazwell plate well. When left to stand, the solvent evaporates at room temperature, and finally the residue is thermally desorbed by heating with a laser diode. The desorbed compounds are carried past a corona discharge needle, via a stream of clean air (carrier gas), where they are ionised by APCI and subsequently introduced directly into the MS/MS system without any chromatographic separation prior to detection in the MS/MS.

The carrier gas used in the LDTD is oil-free clean undried compressed air with a flow rate of 3 L/min. All other LDTD-APCI settings were as recommended by the manufacturer. The laser power was applied by ramping linearly from 0% to 65% of maximum output in the time interval 0.1 s to 3.0 s after laser positioning, then falling abruptly to 0%. The MS/MS system was run in positive ionisation mode with single reaction monitoring (SRM) acquisition. MS/MS signal optimisation provided the settings for the Sciex 6500 QTrap MS/MS, as shown in Table 1.

3.6. *Method performance*

The study design considered the following critical validation parameters: linearity, LOD/ LOQ, precision, trueness and recovery for validation.

The calibration curves for androstenone and skatole were made by spiking the blank fat matrix with the components corresponding to a concentration range of 0.125–6.00 μg/g and 0.03–1.00 μg/g, respectively. To each tube was added 1.5 mL of the internal standard acetonitrile solution (A) with appropriate skatole and androstenone concentrations and 1.5 mL brine solution (B).

The LOD and LOQ were estimated by ten repeated measurements on one sample of blank fat matrix. The fat was spiked with both androstenone and skatole to 0.125 μg/g. The standard deviation (SD) for the results of the repeated measurements for both components was calculated. The LOD was determined as 3 × SD, and the LOQ as 10 × SD (Wenzl, Haedrich, Schaechtele, Robouch, & Stroka, 2016).

The precision of the method originates from the repeatability (S_r) and the intermediate precision (S_R). These are calculated from the analysis of the relative standard deviation (%RSD) by ANOVA (ISO 5725-4). Here, S_r refers to the variation within experiments conducted on the same day and S_R to measurements conducted on different days.

Back-fat from seven carcasses with different levels of skatole and androstenone was selected for determining the S_r and S_R. Back-fat blocks were extracted from the carcasses and homogenised separately. From each homogenate, subsamples were subjected to duplicate analysis in the LDTD-MS/MS system over a period of five days.

Trueness was evaluated by comparing the present method with a recognised method, as certified reference material does not exist for boar taint analysis. Blended back-fat samples from 12 carcasses were analysed both by our developed LDTD-MS/MS method and at an external laboratory using GC–MS for androstenone analysis and HPLC-FD for skatole analysis. For the GC–MS analysis, the samples were extracted with an organic solvent containing a known amount of internal standard androstanone and separated using gas chromatography. For the HPLC-FD analysis, the samples were extracted with organic solvent and separated by means of liquid chromatography on a reversed column.

Recovery was evaluated by standard addition of skatole and androstenone to a fat sample matrix standard with a known concentration of androstenone (1.5 μg/g) and skatole (0.6 μg/g) in two levels: 0.75 μg/g and 1.5 μg/g androstenone and 0.3 μg/g and 0.6 μg/g skatole were added, corresponding to adding 50% and 100% to the level of components in the matrix standard. Each level was analysed in five replicates. Seven- point calibration curves in the range 0.13–6.0 μg/g for androstenone ($y = 1.979x + 0.021$, $R^2 = 0.999$) and 0.07–2.42 μg/g for skatole ($y = 1.0702x – 0.0048$, $R^2 = 0.9954$) were used for the recovery experiments.

3.7. *Stability of volatile compounds*

During the early method development, skatole was considered too volatile to be handled in the sample preparation and laser desorption processes, and therefore derivatisation of skatole was carried out by the addition of pentafluorobenzyl bromide to the acetonitrile fraction under strong basic conditions (Blau and Halket (1993). However, due to the considerable cost of reagents, utensils and a time consumption that would fail to meet the method requirements, a great deal of effort was put into developing direct sample preparation, i.e. without derivatisation. This resulted in a method that successfully controlled the time from the extract being deposited in the Lazwell plate to measurement in the LDTD-MS-MS.

4. Results and discussion

To support the development of the new LDTD-MS/MS method, a GC/MS method using melted back-fat (Buttinger et al., 2014) and an HPLC method using fresh back-fat (Hansen-Møller, 1994) were established.

4.1. *Extraction*

During the initial optimisation of the extraction procedure, the use of lipophilic solvents such as hexane and methyl *tert*-butyl ether was not tested, since it was expected that undesirably large amounts of fats

Table 1
Settings for the Sciex 6500 QTrap MS/MS. DP: Declustering Potential (volts), CE: Collision Energy (eV), CXP: Collision Exit Cell Potential (volts) for the Sciex 6500 QTrap MS/MS with selected precursor ion and product ion masses, QL: Qualifier Ion.

MS/MS Method-ID	Q1 (Da)	Q3 (Da)	Dwell (msec)	DP	CE	CXP
Androstenone	273.1	215.1	5	80	25	13
Androstenone QL	273.2	255.1	5	80	25	13
Androstanone	275.1	217.1	5	80	25	13
Skatole	132.2	117.0	5	40	28	12
Skatole QL	132.1	90.0	5	40	40	10
Skatole-d3	135.1	117.0	5	40	28	12

would be transferred to the extracts. Hence, solvents such as methanol, cyclohexane, ethyl acetate, acetone, acetonitrile, acetone and isooctane were tested and extracts analysed by a modified method from JRC (Buttinger et al., 2014) using melted back-fat. Fresh back-fat samples containing both skatole and androstenone were analysed by HPLC-FD, and the results were converted to a melted back-fat concentration using the measured water percentage of the fresh back-fat (see results in Table 2). The results indicate that skatole was readily transferred to these extraction solvents, whereas solvents such as methanol and acetonitrile were required for the extraction of androstenone. Due to the large amount of fat impurities in the methanol extract, this solvent was found to be unsuitable for high throughput MS analyses. In addition, all the tested acetonitrile extractions had higher levels of androstenone recovery, indicating that this solvent yielded good recoveries with lower levels of impurities.

The learnings from these results were transferred to the LDTD-MS/MS, and the use of acetonitrile as an extraction solvent was further optimised by testing under acidic, basic and neutral aqueous salt conditions. Subjecting the back-fat adipose tissue to a salting-out-assisted liquid–liquid extraction procedure using acetonitrile, basic brine (0.08 M NaOH) and homogenisation of the mixture resulted in a satisfactory extraction recovery, extract purity and good MS signal response. The homogenisation proved to be a critical step in the extraction procedure. The type of homogenisation aggregate and the time of extraction were both important factors for maximising the recovery of the analytes.

4.2. Internal standards

3-Methyl-d3-indole was used as an internal standard for skatole. Four different compounds (d3-androstenone, d4-androstenone, ADD (1,4-androstadiene-3,7-dione) and androstanone) were tested as an internal standard for androstenone. The commercially available d4-androstenone (deuterated androstenone) was unsuitable due to its instability during extraction, since d4-androstenone undergoes deuterium/hydrogen interchange due to keto-enol tautomerism. The poor isotope purity and the price of the commercially available d3-androstenone also proved to be a limiting factor. Finally, androstanone was selected as an internal standard analogue due to its similar behaviour to androstenone during the analysis and due to its low price.

4.3. Optimisation of the LDTD method

The laser power profile was optimised to maximise the desorption of analytes and response, while minimising matrix desorption. Skatole desorbed easily, and only a low power laser profile was necessary to obtain a good response, whereas androstenone required a more powerful laser profile. Therefore, a compromise was made favouring both analytes to complete the desorption.

The effect of the amount of deposited sample extract in the Lazwell^{TM} plates was studied, and 3 μL was found to be optimal. Injection of larger volumes did not produce correspondingly larger responses, and peak deterioration was observed. Furthermore, the drying time of the deposited sample extracts was tested, and, in order to avoid loss of skatole after drying the extract, the samples had to be run on the LDTD-MS-MS as soon as possible after liquid was no longer visible in the wells but within ten minutes of being deposited.

Table 2
%Recovery when using different solvents compared to HPLC results.

	Solvents	%Recovery Skatole	
1	Acetone/Ethyl acetate/Isooctane 2:2:1	110	66
2	100% Acetone	101	61
3	100% Ethyl acetate	101	60
4	1% Formic acid in Acetonitrile	87	65
5	Acetonitrile/Acetone 3:1	100	69
6	Acetonitrile/Acetone 1:1	101	69
7	Acetonitrile/Acetone 1:3	104	71
8	100% Methanol	101	75
9	Cyclohexane/Ethyl acetate 1:1	95	58

The LDTD-Sciex software makes it possible to view the desorption peaks for androstenone and skatole resulting from the applied laser power profile. Desorption peaks for skatole and androstenone transitions are shown in Fig. 1, where it is seen that the less volatile androstenone arrives at the mass detector approximately 0.5 s later than skatole.

Although the laser power is applied for 5.1 s, the loading of the Lazwell^{TM} plate and the movement of the plate in the instrument from well to well take time, as do the plate insertion and ejection from the LDTD. Adding this to the desorption time, the LDTD-MS/MS can analyse a new sample for skatole and androstenone every 8.5 s, giving an instrument capacity of >420 analyses per hour.

4.4. Method performance

The calibration curves for skatole and androstenone seen in Fig. 2 were established using the Analyst® Software provided by Sciex using linear regression analysis with 1/x weighting (Analyst® 1.6 Software Reference Guide, 2012). This weighting of data points is appropriate when the relative measurement error can be assumed constant for all concentration ratios. The weighting ensures that data points at the high end of the curves do not dominate the least squares fits resulting in poor fits to the low-end data points. For both components and related transitions, R^2 was higher than 0.99 for qualifier and quantifier ions, and the residual analyses showed that the residuals were randomly distributed around zero (data not shown). The calculated 95% confidence intervals for the slope of the calibration curves include zero for all the quantifier ions, and they can therefore be assumed to pass through (0,0).

The MS/MS response curves for skatole and androstenone shown in Fig. 2 exhibit R^2 values of 0.9954 and 0.9992, respectively.

The LOD and LOQ for skatole were calculated to be 0.02 and 0.05 μg/g, respectively, and thus the LOQ was significantly below the threshold used at the abattoir for rejecting carcasses for fresh consumption. The LOD and LOQ for androstenone are at a similarly low level and were calculated to be 0.03 and 0.1 μg/g, respectively. Even if an extremely low sorting threshold for androstenone of 0.5 μg/g were assigned, below which the typical consumer's dislike of meat from a male pig and a castrate is not statistically different, the analytical method performance would be satisfactory (Christensen, Nielsen, & Aaslyng, 2019).

The precision of the method for related values of skatole and androstenone in seven carcasses is shown in Table 3. The reproducibility of the method (%RSD) meets the requirement for an RSD of below 10%.

Trueness verification of the methods was carried out by comparing the developed method with the results from an external laboratory. The methods used by the external laboratory were based on GC–MS and HPLC-FD analysis. Prior to analysis, 12 back-fat samples had been blended in order to obtain homogeneous samples for direct comparison of the developed method with the reference methods. For both androstenone and skatole, the samples were distributed across the calibration range. The LDTD-MS/MS results are given as double determinations, whereas the GC–MS results are given as single determinations. The results are presented in Fig. 3.

The androstenone result from one sample was excluded, since the obtained result exceeded the LDTD-MS/MS method's linear calibration range. From a paired two-tailed *t*-test, it is concluded that there is a significant difference ($P = 0.04$) between the androstenone results from the LDTD-MS/MS and the GC–MS, as would be expected since two laboratories and two different methods are compared. The androstenone results from both methods obtained on the blended back-fat homogenates are plotted against each other in Fig. 3. A linear relation is obtained with an equation for the regression curve, $y = 0.99\,x + 0.10$ and a R^2 value of 0.96, indicating a very small difference between the two

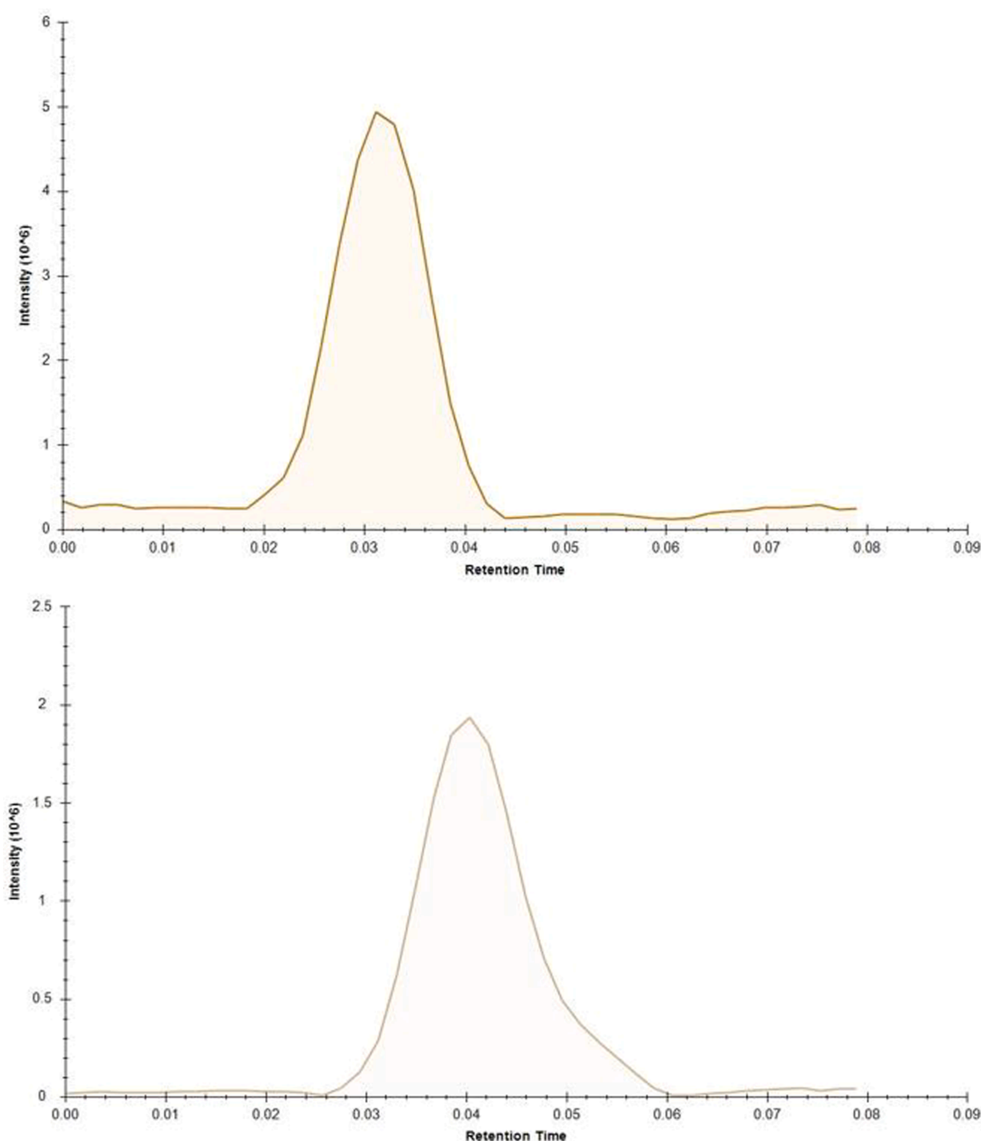

Fig. 1. Desorption profile intensities as a function of time in minutes for skatole (upper) and androstenone (lower). Skatole is desorbed 0.5 s earlier than androstenone. X- and Y-axis units are given as retention time in minutes and counts in millions, respectively.

methods and only a parallel shift of the results of 0.1 mg/kg.

A Bland-Altman statistical test was used to estimate bias and agreement of the LDTD-MS/MS and the GC–MS methods. In the Bland-Altman test, the difference of measurements is plotted versus the average values (Fig. 4). The lower limit of agreement is −0.44 mg/kg, and the upper limit of agreement is 0.21 mg/kg. The bias of the average values of the samples is −0.12 with a standard deviation of 0.17, indicating that the LDTD method measures lower values than the GC–MS method, which was also concluded from the *t*-test and the equation for the linear fit in Fig. 3.

For skatole, a paired two-tailed *t*-test for the 12 results of the blended back-fat was conducted, and it can be concluded that there is a significant difference between the results from the LDTD-MS/MS and the HPLC-FD methods ($P = 0.05$), which again is as expected. The relationship between the two methods for skatole is depicted in Fig. 5. The relationship is $y = 0.97\ x + 0.03$, $R^2 = 0.97$. As for androstenone, the linear relationship indicates a very close relationship between the skatole results from the two methods, and only a slight parallel shift in results is observed.

The Bland-Altman test for the LDTD-MS/MS method versus the HPLC-FD method gives a bias of −0.02 mg/kg with a standard deviation of 0.03 mg/kg (Fig. 6). The lower limits of agreement are −0.08 and 0.04 mg/kg. Again, it can be concluded that the LDTD-MS/MS method measured less than the HPLC-FD method.

From the above studies, it can be concluded that the developed method has good correlation with the external HPLC and GC–MS laboratory methods, which are very different in nature from the developed method based on direct desorption of dried extract.

The recovery evaluation experiments revealed 109% and 105% recoveries at 50% standard addition for androstenone and skatole, respectively, and 110% and 108% recoveries at 100% standard addition, respectively. These recoveries fell within the acceptance range (80–110%) according to the FDA's internal document "Guideline for the Validation of Chemical Methods in Food, Feeds, Cosmetics and Veterinary products" (U.S., Food, & Drug-Administration, 2019).

4.5. Stress test of the method and instrumentation

When implemented at a large Danish slaughterhouse as an at-line measuring system, the LDTD-MS/MS will be required to analyse between 700,000 and 1,400,000 male pig carcasses per year. Thus, it is of utmost importance that the sample analysis does not contaminate the

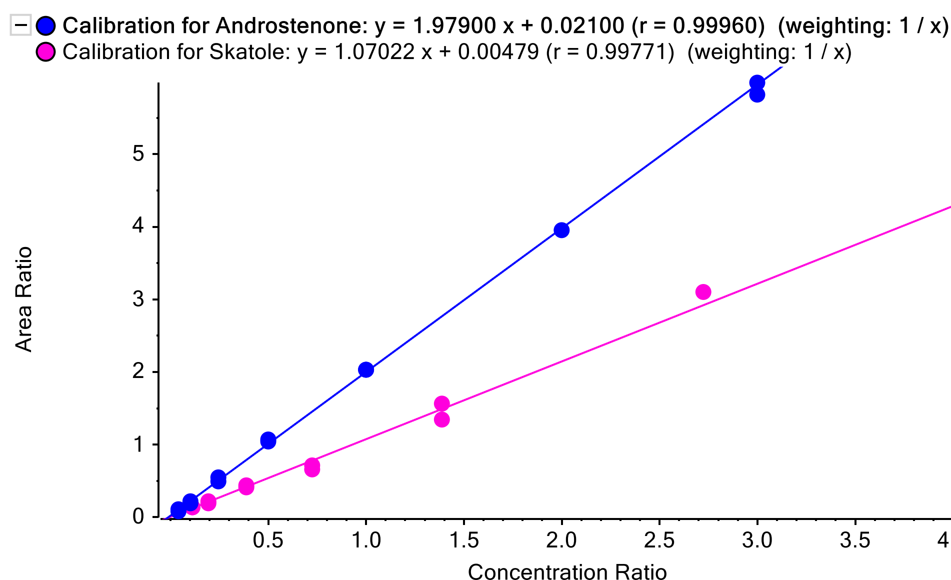

Fig. 2. Linear regression curves for skatole (magenta) and androstenone (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
The precision of the method for the quantification of skatole and androstenone. C = concentration, SR = intermediate precision, Sr = repeatability, %RSD = the relative standard deviation.

	Skatole					Androstenone				
Sample	C (µg/g)	S_R (%RSD)	S_R (µg/g)	S_r (%RSD)	S_r (µg/g)	C (µg/g)	S_R (%RSD)	S_R (µg/g)	S_r (%RSD)	S_r (µg/g)
1	0.1	6	0.003	5	0.003	2.4	3	0.076	3	0.066
2	0.1	9	0.008	7	0.006	0.3	8	0.021	4	0.009
3	0.2	6	0.012	3	0.007	3.9	3	0.121	3	0.103
4	0.2	6	0.009	6	0.009	1.5	4	0.059	4	0.059
5	0.4	7	0.028	5	0.023	9.0	4	0.349	4	0.349
6	0.7	7	0.044	4	0.029	5.7	3	0.187	2	0.122
7	0.9	5	0.048	5	0.048	1.6	3	0.042	3	0.042

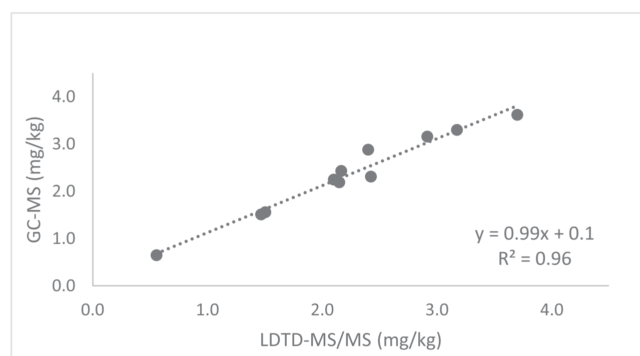

Fig. 3. Trueness evaluation for androstenone. The linear regression between the results obtained by LDTD-MS/MS and GC–MS for androstenone from 11 blended back-fat samples.

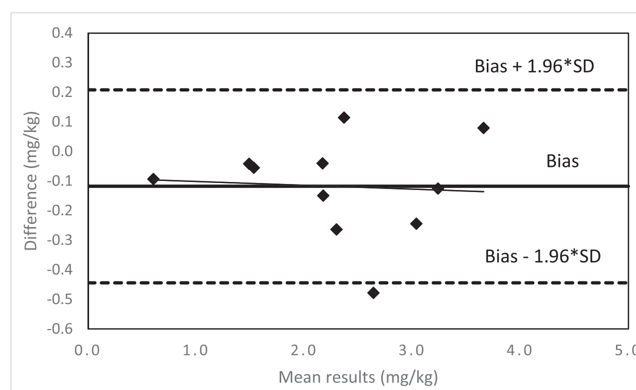

Fig. 4. Bland-Altman plot representing bias/agreement of the two androstenone methods. Solid line represents mean (bias) and dashed line represent the lower and upper limit of agreement.

MS/MS system and result in accuracy deterioration and the need for frequent service maintenance.

A simulated stress test was carried out over a period of five working days. During the test, 10,000 LazWell™ wells were analysed to evaluate the extent to which the LDTD-MS/MS instrument was affected in terms of response and instrument contamination.

Before the test, the MS/MS system had been running for approximately one and a half years without more than just routine cleaning. During this period, approximately 15,000 analyses were carried out in the laboratory with LDTD, and the MS/MS system had also been used as detection equipment in other laboratory projects where UPLC equipment was used instead of LDTD. After the stress test with an additional 10,000 analyses, a maintenance overhaul of both the MS/MS system and the LDTD was carried out by the supplier's service technicians. Upon inspection of the MS/MS system, the supplier's service technicians assessed that the system showed a low level of contamination after 25,000 analyses with LDTD.

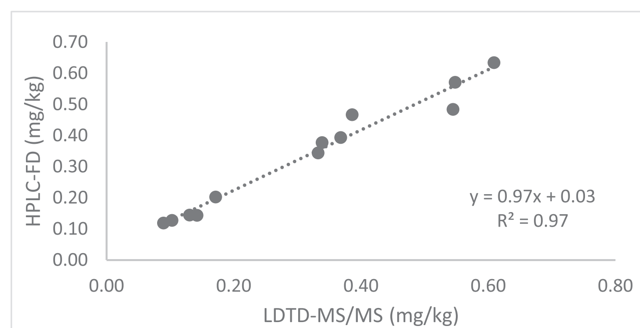

Fig. 5. Trueness evaluation for skatole. The linear regression between the results obtained by LDTD-MS/MS and HPLC-FD for androstenone from 12 blended back-fat samples.

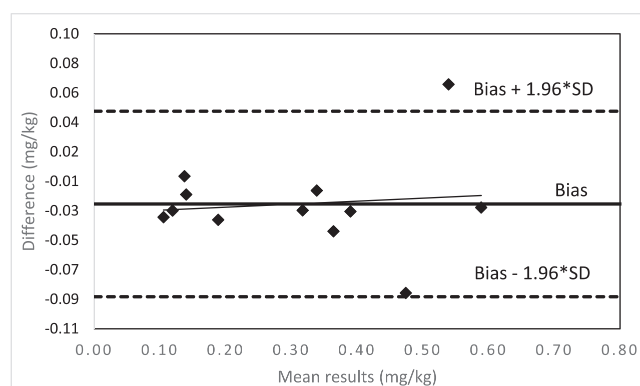

Fig. 6. Bland-Altman plot representing bias/agreement of the two skatole methods. Solid line represents mean (bias) and dashed line represent the lower and upper limit of agreement.

The results from the stress test showed that the reproducibility and sensitivity of the method were highly acceptable. The RSD over the five days was below 5% for androstenone and below 9% for skatole, regardless of the sample concentration. The calibration curves for both androstenone and skatole were made daily and revealed that the response factors were stable throughout the test, and the correlation coefficient (R^2) of all standard curves was above 0.99, which is satisfactory.

4.6. Quality assurance of the method validation and suitability as an at-line testing system

The overall conclusion from the validation of the critical parameters is that the method proved to be robust and sufficiently sensitive to observe the sensory threshold values for androstenone and skatole.

The method has been accredited by DANAK (Danish Accreditation Fund) in accordance with ISO 17025:2017.

Several requirements for an at-line analytical testing system were specified early in the project, and the developed method proved to meet these requirements in terms of analysis speed, price and method performance. Even though sample preparation must take place, the request for a total analysis time (from the start of the sample preparation to the availability of the analytical results) of less than 40 min has been met. Despite the delay due to the sample preparation, the method is capable of delivering a set of analytical results to the abattoir database within nine seconds. Correct batch-wise sample handling ensures that the developed method is applicable as an at-line boar taint detection system (Borggaard, Birkler, Meinert, & Støier, 2017).

Although we have not discussed the analytical sample price in this article, it is estimated that the total sample price is less than 1 EUR, including utensils, reagents and solvents but excluding financial depreciation of the equipment. Routine maintenance and preventive maintenance are expected to be low due to the analytical principle of LDTD.

5. Conclusion

In the present study, a method for simultaneously analysing skatole and androstenone in adipose back-fat tissue, where the adipose tissue is subjected to a salt-assisted liquid-liquid extraction followed by LDTD-MS/MS analysis, was developed. The use of LDTD as mass spectrometer front end allows for an objective method for quantifying boar taint with high accuracy. The method is robust and meets the production speed requirements of a slaughter line. The method has been validated and accredited by DANAK. In conclusion, the results obtained in this study show that the developed high throughput method for analysis of skatole and androstenone meets the requirements of a large production site, with an analysis price of less than 1 EUR. Although adipose tissue is extracted and analysed in the MS/MS system, the use of LDTD as MS/MS front end ensures that only small amounts of molecules enter the mass spectrometer. Furthermore, the use of tandem mass spectrometry provides objective, selective and quantitative analytical results.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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