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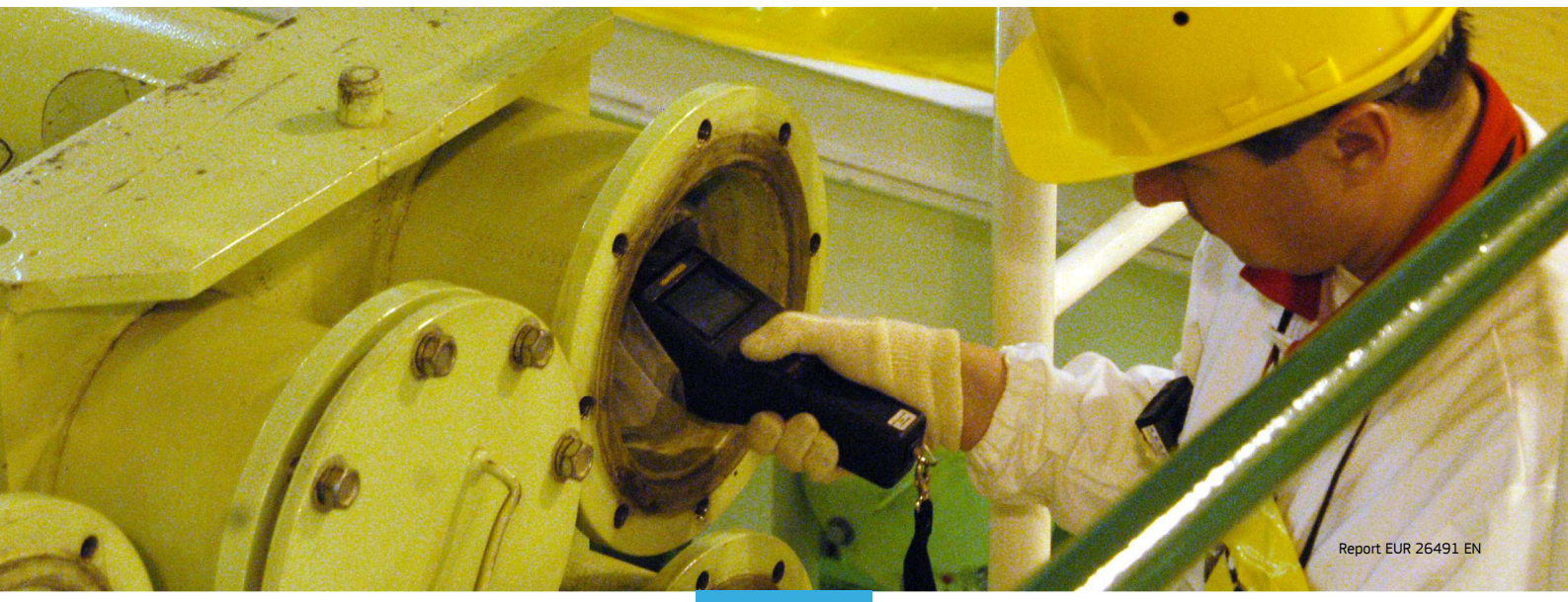

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In house validation of a reference method for the determination of boar taint compounds by LC-MSMS

G. Buttinger, L. Karasek, P. Verlinde, T. Wenzl

Executive Summary

Meat from male pigs may develop an off-flavour, commonly known as boar taint. For that reason male piglets are surgically castrated at young age to avoid the potential off-flavour formation.
Animal welfare concerns have triggered research into alternatives to surgical castration of male piglets with the long-term goal of abandoning it by 1 January 2018. (European Declaration on alternatives to surgical castration of pigs [1]).
Various analytical methods of different sophistication can be used for the detection and quantification of the two marker compounds, androstenone and skatole. Regrettably, none of the methods has been validated by collaborative study to prove its applicability in many laboratories for reference purposes.
Since the method to be validated shall serve as a standardized reference for the elaboration of rapid tests and the definition of sensory thresholds for consumer acceptance, chromatographic methods coupled to mass spectrometric detectors are proposed. It shall be stressed that it is not the intention to employ the proposed methods at the slaughter line.
In the suggested reference method, the 3 marker compounds (skatole, andostrenone and indole), for boar taint are quantified in pork fat by isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS).
The fat is separated from the ground pork fat tissue via melting and centrifugation. The fat is spiked with isotopically labelled standards and prepared for size exclusion chromatography (SEC). The SEC purified sample is evaporated nearly to dryness and, after addition of an injection standard, analysed by LC-MS/MS in selected reaction monitoring mode.
This method has been validated to obtain the method performance characteristics.
When summarising the method performance characteristics and comparing them to requirements for official food control methods in the area of food contaminants it can be concluded that the method is fit for its intended purpose.
The recovery rates are between 106 % and 115% and are therefore within the range of 60 % to 120 %.
The repeatability standard deviation ranges from 7 % to 18 % and the intermediate precision between 9 % and 18 %, all below the set limit of 20 %.
The measurement uncertainty (U), assessed with a top down approach, is below 30 % for all analytes. The method has proven to be robust and free from matrix interferences.
The method is sensitive enough to determine the off-flavour compounds at the sensory threshold values.

Introduction

Meat from male pigs may develop an off-flavour, commonly known as boar taint. For that reason male piglets are surgically castrated at young age to avoid the potential off-flavour formation.
Animal welfare concerns have triggered research into alternatives to surgical castration of male piglets with the long-term goal of abandoning it by 1 January 2018 [1].
Among the many compounds found in the offending flavour, skatole and androstenone (5α-androst-16-en-3-one) are primarily responsible for boar taint. Mass fractions between 0.50 µg and 1.00 µg for androstenone per g melted fat and between 0.20 µg and 0.25 µg for skatole per melted g fat are generally accepted as thresholds for discriminating between tainted and untainted pork samples (back fat) [2].
Various analytical methods of different sophistication can be used for the detection and quantification of androstenone and skatole. Regrettably, none of the methods has been validated by collaborative study to prove its applicability in many laboratories for reference purposes. The ALCASDE study [3] has shown that the agreement of testing results for androstenone and skatole in pig fat produced in several laboratories was not satisfactory and one of the conclusions of this project was that in a follow-on project a standardised reference method should be elaborated to rectify this shortcoming.

Since the method to be validated shall serve as a standardized reference for the elaboration of rapid tests and the definition of sensory thresholds for consumer acceptance, chromatographic methods coupled to mass spectrometric detectors are proposed. Such methods have the advantage of being highly selective and accurate, in particular when they are based on isotope dilution principles. Emphasis is given to the ease of sample handling, appropriate sample through-put, and time necessary to generate results. Other boundary conditions are a potential high level of automation and broad applicability in food control laboratories with regard to required instrumentation.
However, it shall be again stressed that it is not the intention to employ the proposed methods at the slaughter line. This shall be covered by rapid tests that are under development in dedicated projects.

Method description

The three marker compounds (skatole, andostrenone and indole), for boar taint are quantified in pork fat by isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS).
The back neck fat sample is stripped of skin, muscle and adipose tissue to only gain the hypodermis. The frozen hypodermis is then either ground with a meat grinder or with a blender after treatment with liquid nitrogen.
The fat is separated from the ground pork fat tissue sample via melting and centrifugation thereafter. The fat is spiked with isotopically labelled standards and prepared for size exclusion chromatography (SEC). Sample clean up via SEC is achieved within 45 minutes and consumes about 50 ml of organic solvent.
The SEC purified sample is evaporated nearly to dryness and after addition of an injection standard analysed by LC-MS/MS in selected reaction monitoring mode.

Layout of in house validation study

The study layout considered the following parameters for validation:

1. Linearity and range of the calibration function
2. limits of detection and quantification
3. matrix effects
4. selectivity
5. repeatability and intermediate precision
6. trueness
7. robustness

For the evaluation of the first four points a calibration was performed on eight concentration levels, each measured in triplicate. The same series of calibration standards was prepared in matrix extract instead of solvent. Additionally three calibration standards in matrix were prepared at the lower end of the calibration (between the lowest and second lowest level) for the estimation of limits of detection and quantification.
For the evaluation of points 5 and 6 three replicate samples were spiked at a low, medium and high level (including a blank sample, total of 12 samples) and measured. This experiment was performed three times by different operators.
For the evaluation of point seven a Plackett-Burman experimental design was set up including the following experimental parameters: injection volume of SEC, flow of SEC, eluent composition of SEC, column batch of SEC column, column batch of HPLC column, flow of HPLC, temperature of HPLC column, random effect.

Method performance

Linearity and range of the calibration function

Linearity was assessed using regression analysis, visual inspection of residual plots and evaluation of correlation factors. Calibrations with and without matrix were found to be linear. The range of the calibrations was set based on the limits of quantification, the sensory threshold and linearity of calibration function.
The following ranges were set:

Indol and skatole	50 ng/g – 1000 ng/g
Androsteneone	100ng/g – 5000 ng/g

Limits of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) were estimated from the calibration curve gained from matrix matched calibration standards covering the range between 10 and 50 ng/g.
Target levels for LOD and LOQ are below 10 ng/g.
The LOD is calculated as three times the standard deviation of the response divided by the slope of the calibration function and the LOQ as 10 times the standard deviation of the response divided by the slope of the calibration function.

Values calculated from the regression line are summarised in

Table 1.

Table 1 Limit of detection and limit of quantification

	LOD [ng/g]	LOQ [ng/g]
Indole	1.8	6.0
Skatole	2.8	9.3
Androstenone	14.9	49.6

Matrix effects

Matrix effects were evaluated by comparing the regression lines calculated from standards with and without matrix. No matrix effect on the calibration could be observed as both confidence intervals on slope and intercept overlap (Table 2).

Table 2 Evaluation of matrix effects

	Calibration in Solvent		Calibration in Matrix	
	Slope ± CI	Intercept ± CI	Slope ± CI	Intercept ± CI
Indole	2.362±0.215	0.031±0.011	2.212±0.190	0.003±0.010
Skatol	0.968±0.012	-0.004±0.009	0.964±0.010	-0.002±0.008
Androstenone	1.168±0.011	0.056±0.011	1.164±0.012	0.071±0.035

Selectivity

The lowest level of the matrix matched calibration solution was evaluated to check whether analyte peaks are baseline separated from other matrix constituents, and the purity of the analyte peaks checked by overlaying and plotting the recorded m/z values.

Visual inspection of a low level spiked sample (Indole, Skatol ~30 ng/g, androstenone 60 ng/g) do not show any interferences and sufficient peak resolution (Figure 1).

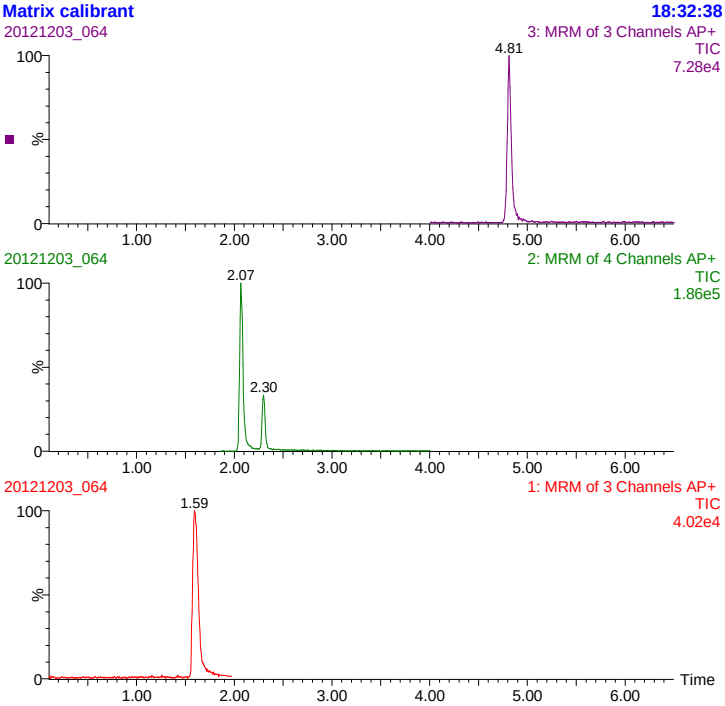


Figure 1 Low level spiked sample (Indole, Skatol ~30 ng/g, androstenone 60 ng/g)

Repeatability and intermediate precision

Intermediate precision and repeatability were evaluated at the lower and upper level of the working range as well as at a level in the centre of the working range. Therefore three replicate analyses were performed at each level on three different days by three different operators.
The results were analysed employing two way ANOVA with repetitions. This way significant differences between operators or spiking levels can be detected.
Target level of repeatability relative standard deviation was10 % and for intermediate relative standard deviation it was 15 %.
Samples were spiked at levels shown in Table 3.

Table 3 Nominal spiking levels for repeatability assessment

	Indol [ng/g]	skatol [ng/g]	androstenone [ng/g]
Low	10	10	10
Middle	500	500	2000
High	1000	1000	4000

As it turned out the low spiking level for andrestenone was below the limit of quantification and for the other two just at the limit of quantification.
The three sets of samples were measured on instrument 1 (TSQ). Later two sample sets were measured on instrument 2 (Quattro premier) to verify comparable performance (Table 4).
One of nine samples of one operator failed to inject on instrument 1, for data analysis via ANOVA its value was set to the average of the other two values of the same spiking level. This approach guaranties that the averages of the operators and spiking levels can be still compared although it leads to an underestimation of the repeatability of that spiking level of that operator.
The results for repeatability and intermediate precision based on ANOVA are summarised in Table 4.

Table 4 Repeatability and intermediate precision

	TSQ		Quattropremiere	
	r [%]	R_{IP} [%]	r [%]	R_{IP} [%]
Indole	12	14	18	18
Skatol	7	9	13	13
Androstenone	9	9	9	9

r... repeatability
R_{IP}... intermediate precision

Trueness

CRMs do not exist for boar taint analysis. Therefore, a spiking experiment is the best mean for trueness estimation. However, the standard solution used for spiking of the test samples has to be independent from the standard solution for instrument calibration. Both solutions were prepared independently starting from the neat substances. The purity of the neat substances was confirmed by GC-FID measurements of concentrated single substance standard solutions.
The recovery rates for the spiked sample were calculated for two instruments. The results are summarized in Table 5 and Table 6.

Table 5 Recovery rates achieved TSQ

TSQ	Recovery	U
Indole	112 %	19 %
Skatol	107 %	11 %
Androstenone	108 %	12 %

Table 6 Recovery rates achieved with Quattro Premier

Quattro	Recovery	U
Indole	108 %	30 %
Skatol	106 %	21 %
Androstenone	115 %	16 %

It was concluded that trueness is given if the recovery rate plus/minus its uncertainty covers 100 %. This is given for both instruments.

The recovery rates for the internal standards based on injection standard were calculated to evaluate overall loss of analytes during the procedure. A recovery of >70 % was set as target.
The results are summarized in Table 7

Table 7 Recovery rates for the internal standards based on the injection standard (results of one day)

Quattro	Recovery	RSD
D_7-Indole	95 %	11 %
D_3-Skatol	91 %	6.6 %
D_4-Androstenone	94 %	5.4 %

The relative standard deviation of the peak areas of internal standards from samples are comparable to those obtained for calibration solutions.

Robustness

Robustness testing was executed applying a Plackett-Burman experimental design.
The experimental parameters were:

Injection volume: 750 ± 30 µl
Flow of GPC eluent ± 2%,
GPC-eluent composition: ethyl acetate content ±2 %
Batch to batch variability of GPC column
LC-column
LC-flow ± 2%
Column temperature ± 1.0 °C
Random effect

The studied effects were:

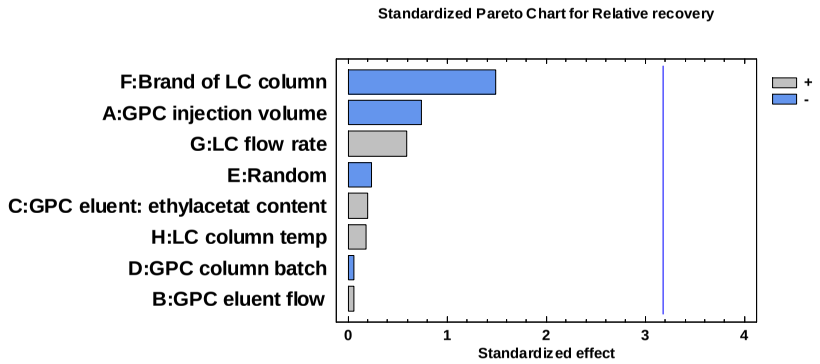
- Recovery of spiked substances relative to injection standard (target above 70 %)
- Peak resolution between indole and skatole (target above 1.5)

The recoveries for all samples for indol and skatol were within ± U of the values found for the trueness assessment. For androstenone all were within ± 2 *U of the values found for the trueness assessment.
Recoveries of internal standard based on injection standard were for all samples above 70 %.
Recoveries of internal standard based on injection standard were for all samples on average higher than found for the samples used for the trueness assessment.

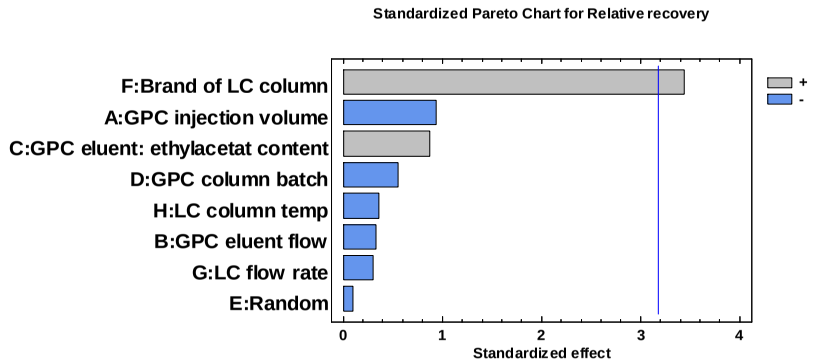
The peak resolution for Indol/skatol was above 5 for all samples.

On the next page a statistical evaluation of the study can be seen. It can be seen that none of the studied parameters have significant impact on the method performance except for the type of LC column for skatol. The significance value is just above the threshold for this parameter. Therefore it cannot be excluded that it is a statistical artefact. On the other hand a change in type of HPLC column is normally accompanied with a re-optimisation of the HPLC parameters.
The overall conclusion is that the method has proven to be robust.

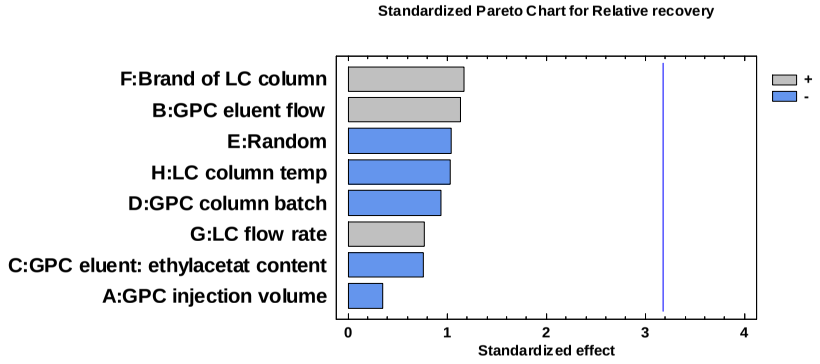
Indol



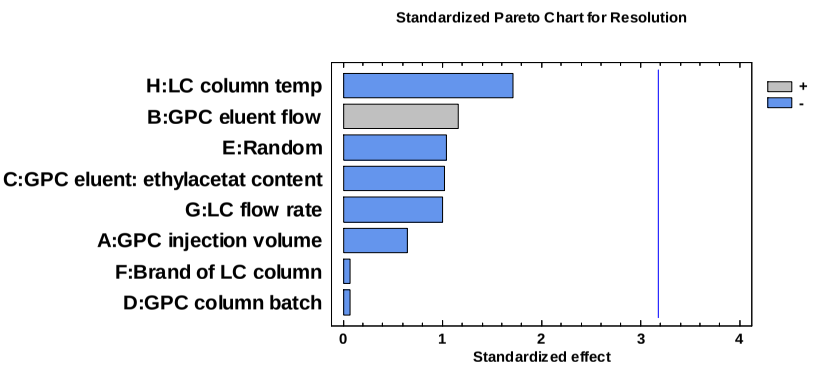
Skatole



Androstenone



Resolution Indole/Skatole



Measurement uncertainty

Uncertainty was estimated via a bottom-up approach covering the uncertainties of calibrants, intermediate precision, and uncertainty of recovery. The targeted combined standard uncertainty was 20 % or below.

Contributions from calibration (purity of calibrant, gravimetric preparation, linear regression) are negligible compared to contributions from repeatability and intermediate precision.
Contributions from recovery are included in the contribution from repeatability and intermediate precision.
Uncertainty is calculated according to

$$U = 2 * \sqrt{\frac{s_r^2}{n} + \frac{s_{IP}^2}{d}}$$

Where: U is the expanded uncertainty
s_r is the repeatability
n is the number of replicates
s_{IP} is the intermediate precision
d is the number of days/operators

the measurement uncertainties are summarised in Table 8.

Table 8 Measurement uncertainties achieved on the Quattro Premier

Quattro	U
Indole	27 %
Skatol	20 %
Androstenone	14 %

Conclusions

When summarising the method performance characteristics and comparing them to requirements for official food control methods in the area of food contaminants it can be concluded that the method is fit for its intended purpose.
The recovery rates are between 106 % and 115% and are therefore within the range of 60 % to 120 %.
The repeatability standard deviation ranges from 7 % to 18 % and the intermediate precision between 9 % and 18 %, all below the set limit of 20 %.
The measurement uncertainty (U) is below 30 % for all analytes. The method has proven to be robust and free from matrix interferences.
The method is sensitive enough to observe the sensory threshold values.

References

1 http://ec.europa.eu/food/animal/welfare/farm/initiatives_en.htm
2 Bonneau, M; Meat Science 49 (Supp1) 257
3 http://ec.europa.eu/food/animal/welfare/farm/alcasde_study_04122009_en.pdf

ANNEX 1

SOP

Determination of boar taint compounds indole, skatole and androstenone in pork neck hypodermis.

Contents Page

1 Scope

The three marker boar taint compounds (skatole, andostrenone and indole), are quantified in pork fat either by gas-chromatography with mass-spectrometry detection (GC-MS) or liquid chromatography with tandem mass spectrometry (LC-MS/MS).
The fat is removed from the pork tissue via melting. The fat, spiked with isotopically labelled boar taint compounds, is purified by means of size exclusion chromatography (SEC).
The SEC purified sample is analysed either by GC-MS in selected ion monitoring mode or LC-MS/MS in selected reaction monitoring mode.
Sensorial threshold levels of consumers were reported in literature for indole and skatole to 200 µg/kg to 250 µg/kg and for androstenone to 500 µg/kg to 1000 µg/kg respectively. Thus, the working ranges of the LC method and GC method was set to 50 – 1000 µg/kg fat for indole and skatole and to 100 – 5000 µg/kg fat for androstenone.

2 Principle

The fat is separated from the pork fat tissue sample via melting. The fat is separated via centrifugation from the remaining tissue material. Boar taint compounds in an aliquot of the fat , spiked with isotope labelled boar taint compounds, are purified by means of size exclusion chromatography (SEC), using a mixture of cyclohexane and ethyl acetate as eluent.
For GC or UHPLC analysis 100 µL of nonane or 1-octanol, respectively are added as a keeper to the collected SEC fraction. The SEC fraction is then evaporated to about 100 µL. Finally the sample is reconstituted using an injection standard prior to measurement by GC-MS or LC-MS/MS.
For GC-MS analysis:
The injection is performed with a splitless injection port. The chromatographic separation is obtained on a capillary column with 5%-phenyl-methylpolysiloxane as stationary phase. The analytes are ionised by electron ionization (EI) at 70 eV. The target ions are recorded in Single Ion Monitoring (SIM) mode, and quantified by an isotope dilution method.
For UHPLC-MS/MS analysis:
The chromatographic separation is obtained on a sub-2-µm reversed phase C18 column. The analytes are ionised by atmospheric pressure chemical ionisation (APCI). The target boar taint compounds are detected in selected reaction monitoring (SRM) mode and quantified by an isotope dilution method.
For HPLC-MS/MS analysis see Annex 1.

3 Definitions

Laboratory sample: sample as prepared for sending to the laboratory and intended for inspection or testing (i.e. the sample or subsample(s) received by the laboratory).
Test sample: sample prepared from the laboratory sample and from which test portions will be taken.
Test portion: the quantity of material drawn from the test sample and on which the test or observation is actually carried out (i.e. for this study the test portion is of 4 g).
Final extract: solution containing the analytes; obtained after the last evaporation step and reconstitution of the extract.

Resolution (R_s): Ability of a column to separate chromatographic peaks; $R_s \equiv (t_{R2} - t_{R1})/[(w_{b1} + w_{b2})/2]$, where t_{R2} and t_{R1} are the retention times of the two peaks and w_b is the baseline width of the peaks. It is usually expressed in terms of the separation of two peaks (A value of 1.5 is considered sufficient for baseline resolution for two peaks of equal height.)
Base peak (BP): The peak in a mass spectrum corresponding to the separated ion beam which has the greatest intensity.
Injection standard: Compound added before the GC-MS or LC-MS/MS analysis to check the recovery of the labelled standards.
Labelled standard: deuterated or ^{13}C-labelled analogue of native boar taint compounds. The labelled standards are used to correct the losses of native boar taint compounds during analysis. They are added to the test portion prior to the sample preparation.
Quantifier ion (Q_1): ion monitored in the mass spectra to quantify the boar taint compounds (normally it coincides with the base peak).
Qualifier ion (Q_2): ion monitored in the mass spectra for identification purpose.
Procedural blank: a blank sample made up of all reagents foreseen for the preparation of a test portion and processed in all respects as a test portion. This kind of blank, tests the purity of the reagents but also other possible sources of contamination, like the glassware and the analytical instrument (for this study 4 g of vegetable oil (e.g. palm oil), 2 g of sodium sulphate (4.13)).

4 Reagents

4.1 General

Use only reagents of recognized analytical quality/standard, unless otherwise specified. Commercially available solutions with equivalent properties to the reagents listed may be used.
For storing of substances and commercially available solutions, supplier indications are followed. For opened commercial solutions or for in-house prepared solutions, the indications given in this procedure are such to minimise the evaporation of the solvent and to protect the analytes (boar taint compounds) from degradation.
Standard solutions are prepared gravimetrically. For the preparation of solutions of native or labelled boar taint compounds, a micro-balance (6.4) and an analytical balance (6.5) are used. All quantities are expressed as mass fractions (weight/weight). If necessary, the quantities expressed as mass concentration (weight/volume) could be obtained applying the density equation (Equation 1).

$$\rho = \frac{m}{V}$$

Equation 1

with ρ the density [g/mL]
with *m* the mass of substance [g]
with *V* the volume of the solution [mL]

Densities of toluene at 20°C is 0.8669 g/ml. Comprehensive information on the density of solvents at various temperatures is given in the Handbook of Chemistry and Physics. All solutions and substances are used at room temperature.
WARNING 1 — Indole is considered a potential carcinogen and just like its derivatives it is irritating to eyes, respiratory system and skin.

People using these instructions should be familiar with normal laboratory practise. It is the responsibility of the user of these instructions to apply safety and health practices which are in agreement with the local requirements.

4.2 Helium purified compressed gas

(purity equivalent to 99.995% or better)

4.3 Nitrogen purified compressed gas

(purity equivalent to 99.995% or better)

4.4 Water

Type 1 (ASTM D1193)

4.5 n-Hexane

4.6 Methanol

HPLC grade or better

4.7 Cyclohexane

HPLC grade or better

4.8 1-Octanol

HPLC grade or better

4.9 Toluene

GC grade or better

4.10 Ethyl acetate

HPLC grade or better

4.11 Formic acid

Purity 98 % or better

4.12 Nonane

Purity 99 % or better

4.13 Sodium sulphate, anhydrous

p. A.

4.14 SEC eluent

Mix 1 part per volume of cyclohexane (4.7) with 1 part per volume of ethyl acetate (4.10).

4.15 LC eluent A: 0.1 % (v:v) aqueous formic acid

Mix 1 part per volume of formic acid (4.11) with 1000 parts per volume of water (4.4).

4.16 LC eluent B: 0.1 % (v:v) methanolic formic acid

Mix 1 part per volume of formic acid (4.11) with 1000 parts per volume of methanol (4.6).

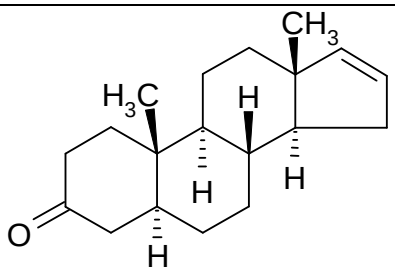
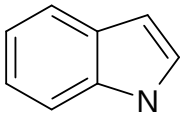
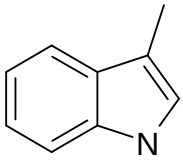
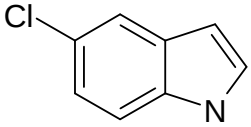
4.17 Reference material for quality control

In descending order of preference, a certified reference material, or during a proficiency test round tested, or a self-prepared test material may be applied for this purpose.
This material will be analysed with every sample batch and used to control the method performances along time.

4.18 Native reference standards

Commercially available neat material or solutions of boar taint compounds.
The list of native substances analysed with this method is reported in Table 9.
Commercially available, preferably certified, standard solutions are preferred due to the higher level of safety in handling.

Table 9 Names and structure of boar taint compounds and injection standard

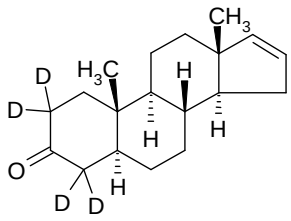
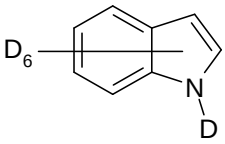
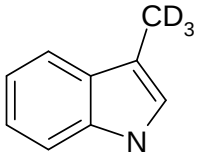
Name	CAS #	structure
Androstenone (5α-Androst-16-en-3-one)	18339-16-7	
Indole (1H-Benzo[b]pyrrole)	120-72-9	
Skatole (3-Methylindole)	83-34-1	
5-Chloroindole (injection standard)	17422-32-1	

4.19 Labelled reference standards

In form of neat material or commercially available solutions.
The labelled standards, applied for the quantification of the target boar taint compounds included in the scope of this instructions are listed in Table 10.

Remark
The labelled standard D4-androstenone has 4 deuterium atoms in alpha positions to the carbonyl group in ring A. It is well known that deuterium can be lost from such a position via the keto/enol tautomerism (it is indeed the way of production). Therefore a back exchange in methanolic solutions is possible. It is recommended to keep methanolic solutions containing D4-androstenone at -20 °C, although no change in isotope ratio could be detected in a methanolic solution after 1 year at 4 °C.

Table 10 List of labelled boar taint compounds

Labelled standard	structure
D4-Androstenone	
D7-Indole	
D3-skatole	

4.20 5-Chloroindole

Neat material or commercially available solutions.
5-Chloroindole (Table 9) is used as injection standard and added to the sample extract prior to GC-MS or LC-MS/MS analysis.

5 Standard preparation

5.1 Injection standard stock solution

Prepare gravimetrically a 20 mL solution of 5-chloroindole (4.20) in toluene (4.9) with a concentration of approximately 5 mg/mL (i.e. 100 mg neat substance). The conversion of units to mg/mL is done via the density equation. For toluene a density value of 0.8669 g/mL is applied at 20 ºC.

5.2 Methanolic injection standard stock solution

Prepare gravimetrically a 20 mL solution of 5-chloroindole in methanol (4.6) with a concentration of approximately 50 µg/mL based on the injection standard stock solution (5.1) (i.e. 200 µl of 5.1). The conversion of units to µg/mL is done via the density equation. For methanol a density value of 0.7918 g/mL is applied at 20 ºC.

5.3 Methanolic injection standard solution

Prepare gravimetrically a solution of 5-chloroindole in methanol (4.6) with a concentration of approximately 250 ng/mL based on the methanolic injection standard stock solution (5.2). The conversion of units to µg/mL is done via the density equation. For methanol a density value of 0.7918 g/mL is applied at 20 ºC.

5.4 Toluene injection standard stock solution

Prepare gravimetrically a 20 mL solution of 5-chloroindole in toluene (4.9) with a concentration of approximately 50 µg/mL based on the injection standard stock solution (5.1) (i.e. 200 µl of 5.1). The conversion of units to µg/mL is done via the density equation. For toluene a density value of 0.8669 g/mL is applied at 20 ºC.

5.5 Toluene injection standard solution

Prepare gravimetrically a solution of 5-chloroindole in toluene (4.9) with a concentration of approximately 250 ng/mL based on the toluene injection standard stock solution (5.4). The conversion of units to ng/mL is done via the density equation. For toluene a density value of 0.8669 g/mL is applied at 20 ºC.

5.6 Native reference standards single-substance stock solutions

Prepare gravimetrically from the native reference standards (4.18) skatole and indole, individual solutions in toluene (4.9) with a concentration of approximately 5 mg/mL. Prepare gravimetrically from the androstenone native reference standard a solution in toluene (4.9) with a concentration of approximately 1.5 mg/mL.
Single-substance stock solutions prepared by weighing a limited amount of native reference standard (below 20 mg) should be prepared by substitution weighing. The amount of reference standard should be determined in a glass weighing cylinder (6.1) using the microbalance (6.4). For amounts above 50 mg an analytical balance can be used. After determination of the amount of standard present in the weighing cylinder, cylinder plus standard are transferred into an amber volumetric flask (6.2) and its weight is determined using an analytical balance (6.5). Subsequently, the appropriate amount of toluene (4.9) is added and weighted with an analytical balance (6.5). For toluene a density value of 0.8669 g/mL is applied at 20 ºC.
To dissolve the substances, each solution shall be sonicated for a couple of minutes. Once the solutions are homogeneous, they are transferred into amber glass vials (6.6) and stored in the dark and at a temperature below 10 ºC.

5.7 Methanolic indole and skatole stock solution (25 µg/mL)

Prepare, from the single-substance stock solutions of indole and skatole (5.6), gravimetrically a solution in methanol (4.6) with a concentration of approximately 20 µg/mL. For this purpose, both the single standard stock solutions (5.6) and methanol (4.6) are weighed with an analytical balance (6.5). The conversion of units to µg/mL is

done via the density equation. For methanol a density value of 0.7918 g/mL is applied at 20 ºC.
The solution is homogenised by shaking and subsequently transferred into an amber glass vial (6.6) and stored in the dark and at a temperature below 10 ºC.

5.8 Methanolic indole and skatole stock solution (500 ng/mL)

Prepare, from the mixed methanolic indole and skatole stock solution (5.7), gravimetrically a solution in methanol (4.6) with a concentration of approximately 500 ng/mL. For this purpose, both the mixed methanolic stock solution (5.7) and methanol (4.6) are weighed with an analytical balance (6.5). For methanol a density value of 0.7918 g/mL is applied at 20 ºC.
The solution is homogenised by shaking and subsequently transferred into an amber glass vial (6.6) and stored in the dark and at a temperature below 10 ºC.

5.9 Methanolic androstenone solution (25 µg/mL)

Prepare, from the single-substance stock solution of androstenone (5.6), gravimetrically a solution in methanol (4.6) with a concentration of approximately 25 µg/mL. For this purpose, both the single standard stock solution (5.6) and methanol (4.6) are weighed with an analytical balance (6.5). For methanol a density value of 0.7918 g/mL is applied at 20 ºC.
The solution is homogenised by shaking and subsequently transferred into an amber glass vial (6.6) and stored in the dark and at a temperature below 10 ºC.

5.10 Labelled reference standards single-substance stock solutions

Prepare gravimetrically from the labelled reference standards (4.19) skatole-D_3, indole-D_7 and androstenone-D_4, individual solutions in toluene (4.9) with a concentration of approximately 100 µg/mL.
Single-substance stock solutions prepared by weighing a limited amount of labelled reference standard (below 20 mg) should be prepared by substitution weighing. For amounts above 50 mg an analytical balance can be used. The amount of labelled standard should be determined in a glass weighing cylinder (6.1) using the microbalance (6.4). After determination of the amount of standard present in the weighing cylinder, cylinder plus standard are transferred into an amber volumetric flask (6.2) and its weight is determined using an analytical balance (6.5). Subsequently, the appropriate amount of toluene (4.9) is added and weighted with an analytical balance (6.5). The conversion of units to µg/mL is done via the density equation. For toluene a density value of 0.8669 g/mL is applied at 20 ºC.
To dissolve the substances, each solution shall be sonicated for a couple of minutes. Once the solutions are homogeneous, they are transferred into amber glass vials (6.6) and stored in the dark and at a temperature below 10 ºC.

5.11 Labelled standards process solution

Prepare, with the individual solutions of skatole-D_3, indole-D_7 and androstenone-D_4 (5.10) a solution in toluene (4.9) with a concentration of 8 µg/mL androstenone-D_4, 4 µg/mL skatole-D_3, and 2 µg/mL indole-D_7. For toluene a density value of 0.8669 g/mL is applied at 20 ºC. For this purpose, both the labelled single standard stock solutions (5.10) and toluene (4.9) are weighed with an analytical balance (6.5).

This solution will be used for spiking the test portion (7.2). The solution is homogenised by shaking and subsequently transferred into an amber glass vial (6.6) and stored in the dark and at a temperature below 10 ºC.

5.12 Methanolic labelled standards calibration solution

Prepare, with the individual solutions of skatole-D_3, indole-D_7 and androstenone-D_4 (5.10) and of Cl-Indole (5.2) a solution in methanol (4.6) with a concentration of approximately 4 µg/mL androstenone-D_4, 2 µg/mL skatole-D_3, and 1 µg/mL indole-D_7 and 2 µg/mL Cl-Indole . For this purpose, both the labelled single standard stock solution (5.10), (5.2) and methanol (4.6) are weighed with an analytical balance (6.5). For methanol a density value of 0.7918 g/mL is applied at 20 ºC.
This solution will be used for preparation of the methanolic calibration stock solutions (5.13). The solution is homogenised by shaking and subsequently transferred into an amber glass vial (6.6) and stored in the dark and at a temperature below 10 ºC.

5.13 Methanolic calibration stock solutions

Prepare gravimetrically the calibrants presented in Table 11 in methanol (4.6) using the labelled standards calibration solution (5.12), the methanolic indole and skatole solutions (5.7 or 5.8), the methanolic androstenone solutions (5.9).

Table 11. Nominal target concentrations in methanolic calibration stock solutions.

Calibrant stock	indole D_7	skatole D_3	androstenone D_4	5-Cl-indole	indole	skatole	androstenone
	Nominal concentration in ng/mL						
CAL 1	100	200	400	200	0	0	0
CAL 2					50	50	100
CAL 3					100	100	250
CAL 4					150	150	500
CAL 5					200	200	1 500
CAL 6					400	400	2 500
CAL 7					600	600	3 500
CAL 8					900	900	4 500

Table 12. Volumetric preparation scheme to prepare 10 mL of the methanolic calibrant stock solutions

	Indole D_7 skatole D_3 androstenone D_4 5-Cl-indole	Indole skatole	androstenone	methanol
CAL 1	1 000 µL (5.12)	-	-	9 000 µL
CAL 2		1 000 µL (5.8)	50 µL (5.9)	7950 µL
CAL 3		2 000 µL (5.8)	100 µL (5.9)	6 900 µL
CAL 4		60 µL (5.7)	200 µL (5.9)	8 740 µL
CAL 5		80 µL (5.7)	600 µL (5.9)	8 320 µL
CAL 6		160 µL (5.7)	1 000 µL (5.9)	7 840 µL
CAL 7		240 µL (5.7)	1 400 µL (5.9)	7 360 µL
CAL 8		360 µL (5.7)	1 800 µL (5.9)	6 840 µL

5.14 Toluene indole and skatole stock solution (25 µg/mL)

Prepare, from the single-substance stock solutions of indole and skatole (5.6), gravimetrically a solution in toluene (4.9) with a concentration of approximately 20 µg/mL. For this purpose, both the single standard stock solutions (5.6) and toluene (4.9) are weighed with an analytical balance (6.5). The conversion of units to µg/mL is done via the density equation. For toluene a density value of 0.8669 g/mL is applied at 20 ºC.
The solution is homogenised by shaking and subsequently transferred into an amber glass vial (6.6) and stored in the dark and at a temperature below 10 ºC.

5.15 Toluene indole and skatole stock solution (500 ng/mL)

Prepare, from the mixed tolene indole and skatole stock solution (5.14), gravimetrically a solution toluene (4.9) with a concentration of approximately 500 ng/mL. For this purpose, both the mixed toluene stock solution (5.14) and toluene (4.9) are weighed with an analytical balance (6.5). For toluene a density value of 0.8669 g/mL is applied at 20 ºC.
The solution is homogenised by shaking and subsequently transferred into an amber glass vial (6.6) and stored in the dark and at a temperature below 10 ºC.

5.16 Toluene androstenone solution (25 µg/mL)

Prepare, from the single-substance stock solution of androstenone (5.6), gravimetrically a solution in toluene (4.9) with a concentration of approximately 25 µg/mL. For this purpose, both the single standard stock solution (5.6) and toluene (4.9) are weighed with an analytical balance (6.5). For toluene a density value of 0.8669 g/mL is applied at 20 ºC.

The solution is homogenised by shaking and subsequently transferred into an amber glass vial (6.6) and stored in the dark and at a temperature below 10 ºC.

5.17 Toluene labelled standards calibration solution

Prepare, with the individual solutions of skatole-D_3, indole-D_7 and androstenone-D_4 (5.10) and of Cl-Indole (5.4) a solution in toluene (4.9) with a concentration of approximately 4 µg/mL androstenone-D_4, 2 µg/mL skatole-D_3, and 1 µg/mL indole-D_7 and 2 µg/mL Cl-Indole . For this purpose, both the labelled single standard stock solution (5.10), (5.4) and toluene (4.9) are weighed with an analytical balance (6.5). For toluene a density value of 0.8669 g/mL is applied at 20 ºC.
This solution will be used for preparation of the toluene calibration stock solutions (5.18). The solution is homogenised by shaking and subsequently transferred into an amber glass vial (6.6) and stored in the dark and at a temperature below 10 ºC.

5.18 Toluene calibration stock solutions

Prepare gravimetrically the calibrants presented in Table 14 in toluene (4.9) using the labelled standards calibration solution (5.17), the toluene indole and skatole solutions (5.14or 5.15), the toluene androstenone solutions (5.16).

Table 13. Nominal target concentrations in toluene calibration stock solutions.

Calibrant stock	indole D_7	skatole D_3	androstenone D_4	5-Cl-indole	indole	skatole	androstenone
	Nominal concentration in ng/mL						
CAL 1	100	200	400	200	0	0	0
CAL 2					50	50	100
CAL 3					100	100	250
CAL 4					150	150	500
CAL 5					200	200	1 500
CAL 6					400	400	2 500
CAL 7					600	600	3 500
CAL 8					900	900	4 500

Table 14. Volumetric preparation scheme to prepare 10 mL of the toluene calibrant stock solutions

	Indole D_7 skatole D_3 androstenone D_4 5-Cl-indole	Indole skatole	androstenone	toluene
CAL 1	1 000 µL (5.17)	-	-	9 000 µL
CAL 2		1 000 µL (5.15)	50 µL (5.16)	7950 µL
CAL 3		2 000 µL (5.15)	100 µL (5.16)	6 900 µL
CAL 4		60 µL (5.14)	200 µL (5.16)	8 740 µL
CAL 5		80 µL (5.14)	600 µL (5.16)	8 320 µL
CAL 6		160 µL (5.14)	1 000 µL (5.16)	7 840 µL
CAL 7		240 µL (5.14)	1 400 µL (5.16)	7 360 µL
CAL 8		360 µL (5.14)	1 800 µL (5.16)	6 840 µL

6 Apparatus

WARNING 3 — All glassware must be meticulously cleaned (except disposable glassware). The glassware is first thoroughly washed with laboratory detergent and hot water. All glassware is rinsed before use with n-hexane (4.5).

6.1 Glass weighing cylinders,

approximately 1 mL volume

6.2 Amber glass volumetric flasks,

of various volumes (5 mL to 100 mL)

6.3 Reaction glass tubes (16 x 100 mm, Pyrex)

with PTFE layered screw caps

6.4 Micro-balance,

with a readability of 0.000 001 g

6.5 Analytical balance,

with a readability of at least 0.000 1 g

6.6 Amber crimp cap glass vials

6.7 Laboratory balance,

with a readability of 0.01 g

6.8 (Ceramic) knife or scalpel,

6.9 Heating device,

with the capability to heat the reaction tubes (6.3) to 90 °C ± 3 °C for at least 60 min

6.10 Centrifuge

6.11 Glass Pasteur capillary pipettes,

230 mm length

6.12 PTFE® membrane filter,

Ø 13 mm and 0.45 µm pore size

6.13 Size Exclusion Chromatography (SEC) apparatus,

comprising the following

6.13.1 Liquid pump,

suitable for a flow rate of 1.0 mL/min

6.13.2 Sample carousel,

for vials of 2 mL capacity (optional)

6.13.3 sample collection vials,

20 mL capacity

6.13.4 Injection system,

capable of injecting 750 µL.

6.13.5 SEC column,

with the following characteristics: Bio-beads S-X3® in 10 x 450 mm glass column, preconditioned in cyclohexane: ethly acetate = 1:1
Alternative columns may be applied provided that equality of performance is demonstrated.
The SEC column shall be kept following the supplier specifications.

6.13.6 Solvent collection bottles

6.13.7 Instrument control and data processing system,

e.g. computer based

6.13.8 Autosampler vials for SEC apparatus

6.14 Sample concentration apparatus:

sample concentrator, comprising a block heater capable of evaporating small volume samples at controlled temperature and under gas stream (e.g. Techne® sample concentrator) (4.3)

6.15 Microliter syringe(s) or calibrated microliter pipette(s),

with 25 µL to 500 µL capacity

6.16 Gas-chromatography – mass spectrometry (GC-MS) apparatus, (e.g. Agilent 6890 GC with Waters Quattro Micro GC),

comprising the following

6.16.1 Injection system,

split/splitless injector, suitable for temperatures up to 400 ºC.

6.16.2 GC oven,

suitable for temperatures up to 350 ºC and capable of temperature programming

6.16.3 Sample tray

6.16.4 Amber sample vials for the sample tray (6.16.3),

with a capacity of about 1,5 ml

6.16.5 GC capillary column,

DB-5MS (5%-Phenyl-methylpolysiloxane), 30 m, 0.25 mm i.d., 0.25 µm film thickness. A capillary column with these characteristics shall ensure an acceptable resolution.

6.16.6 An interface with the mass spectrometer,

with a temperature control device, suitable for temperatures up to 350ºC (see 9.1.4)

6.16.7 Mass spectrometer

with the following characteristics:
- Electron Ionisation source with inert inner surface
- Ionisation energy of 70 eV
- Mass resolution: at least 1 amu
- Temperature control devices for the ion source (up to 300 °C), the quadrupole (up to 200 °C), the GC-MS interface (up to a 350 °C)
- Tuning stability at least of 48 h (allowing for the analysis of a sequence of samples or standards)
- Response linearity range of at least two orders of magnitude

6.16.8 Computer based instrument control system,

capable of programming different acquisition modes in different time intervals.

6.16.9 Data processing system,

computer based

6.17 Liquid-chromatography – mass spectrometry (LC-MS/MS) apparatus, (e.g. Waters Acquity UHPLC with Quattro Premier TQD),

comprising the following

6.17.1 Liquid pump,

suitable for a flow rate of 0.3 mL/min and a back pressure of up to 1000 bar

6.17.2 Sample carousel,

for vials of 2 mL capacity

6.17.3 Injection system,

capable of injecting 10 µL.

6.17.4 C18 column,

Macherey-Nagel, Nucleodur C18 Pyramid 1.8 µm 100 x 2.0 mm or equivalent
The column shall be kept following the supplier specifications. To extend column life it shall be protected with a 0.2 µm inline filter and if possible with a guard column.

6.17.5 Solvent collection bottles

6.17.6 Instrument control and data processing system,

e.g. computer based

6.17.7 Autosampler vials for sample carousel

6.17.8 Triple quadrupole mass spectrometer

with the following characteristics:
- atmospheric pressure chemical ionisation (APCI)
- Mass resolution: at least 1 amu

7 Procedure

For each batch of samples, a procedural blank shall be run in parallel, to assess interferences deriving from the applied reagents and apparatus. A reference material (quality control sample) shall be also included in the batch, for checking the method performances along time.

7.1 Sample treatment

As a general precaution, all of the sample material received by the laboratory shall be used for obtaining a representative and homogeneous laboratory sample without introducing secondary contamination. This is achieved by blending the frozen sample or mincing.

Remark

If the sample received contains not only subcutaneous fat tissue (hypodermis) but also skin, muscle or visceral fat (adipose tissue) these tissues have to be removed before homogenization.

Only the hypodermis is used for analysis!

7.2 Test portion preparation and fat extraction by melting

To obtain the test portion weigh, 2 g of sodium sulphate (4.13) into a reaction tube (6.3), thereafter weigh 4 g ± 0.1 g of the homogenised test sample into the reaction tube with an analytical balance (6.5).
Close the reaction tube and heat it for 1 h to 90 °C.
Centrifuge the tube at 40°C for 5 min at 3220 g.
Transfer 1 g ± 0.01 g (W_{sample}) of the fat into a glass vial using a glass Pasteur pipette (6.11) and an analytical balance (6.5)
Add 100 µL of labelled process solution (5.11) for the quantification of the analytes by isotope dilution, add and dilute with 5 mL SEC eluent (4.14).

7.3 SEC cleanup

Around 1.5 ml of test sample, prepared as described in 7.2, are filtered using a 0.5 µm syringe filter (6.12) into the SEC autosampler vial (6.13.8).
The SEC takes place under the following conditions:
Injection volume: 750 µL (if necessary adaptable to a maximum of 200 mg fat loaded on column)
Flow rate: 1 mL/min
Collected fraction: 25.0 min to 37.0 min (approximately 12 mL)
Eluent: 1:1 (v:v) cyclohexane: ethyl acetate (4.14)

7.4 Preparation of the sample for the LC-MS/MS analysis

At the end of the SEC cleanup, 100 µL 1-octanol (4.8) are added to the collected fraction and the fraction is thereafter evaporated under a gentle stream of nitrogen down to 100 µL at 40 ºC in the sample evaporation apparatus (6.14).
Care has to be taken not to evaporate to dryness!
Subsequently, the sample is reconstituted in 300 µL of methanolic injection standard solution (5.3) and thoroughly vortexed. The reconstituted sample is transferred into a 2 mL amber vial (6.17.7) for analysis.

7.5 Preparation of the sample for the GC-MS analysis

At the end of the SEC cleanup, 100 µL of nonane (**Error! Reference source not found.**) are added to the collected fraction and the fraction is thereafter evaporated under a gentle stream of nitrogen down to 100 µL at 40 ºC, in the sample evaporation apparatus (6.14).
Care has to be taken not to evaporate to dryness!
Subsequently, the sample is reconstituted in 300 µL of toluene injection standard solution (5.5) and thoroughly vortexed. The reconstituted sample is transferred into the autosampler vial (6.16.4) for analysis.

8 Sample analysis by LC-MS/MS

Chromatographic conditions have been optimized for the current configuration of the Acqcuity UHPLC chromatograph (6.17) and the characteristics of the current column (6.17.4, column void volume of 187 µL)

8.1 Instrumental conditions

8.1.1 UHPLC conditions:

Injection volume: 5 µL
Column temperature: 28.4 °C
Flow rate: 0.3 mL/min
Gradient:

Time	%LC eluent A (4.15)	Curve
0	39	6
3.58	3.6	6
4.80	3.6	6
4.81	39	6
6.50	39	6

Above conditions shall result in a representative chromatogram as shown in Figure 2. Indicative retention times are presented in Table 15.

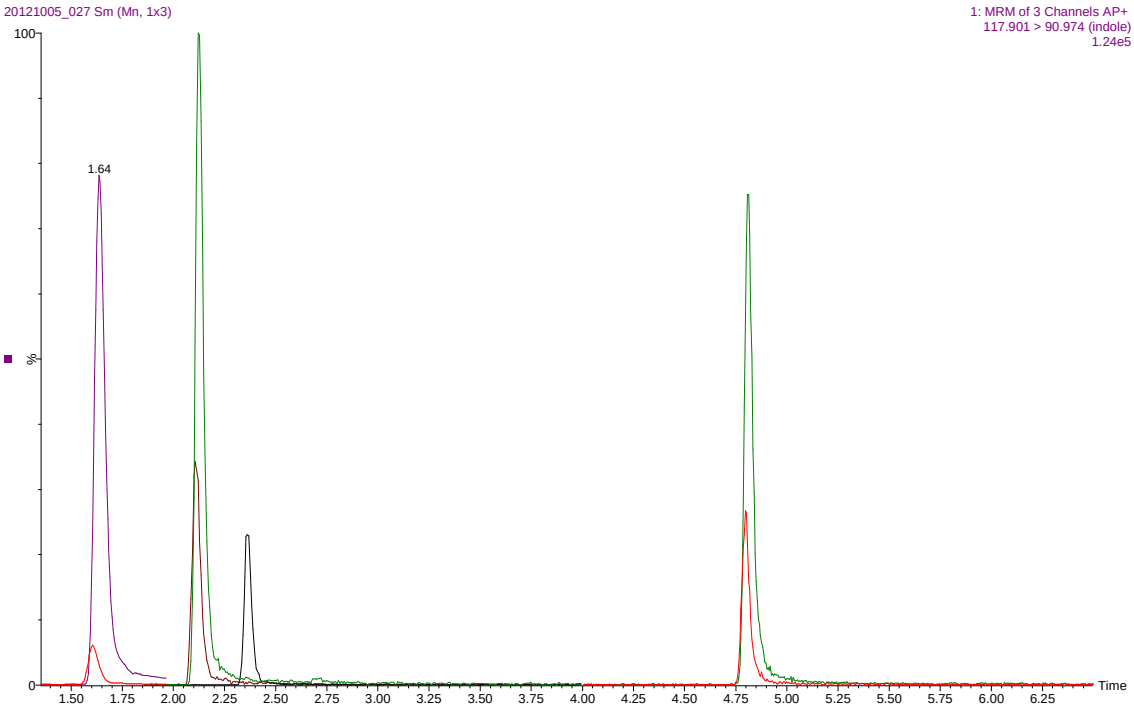


Figure 2. Chromatogram of calibration standard CAL7 in 3:1 (v:v) methanol : 1-octanol (5µL injection volume)

Table 15. Indicative analyte retention times.

Compound	Retention time (min)
Indole	1.64
Indole D_7	1.60
Skatole	2.12
Skatole D_3	2.12
5-Chloroindole	2.36
Androstenone	4.80
Androstenone D_4	4.80

8.1.2 Mass spectrometer acquisition parameters and peak identification

Analytes are ionised by positive mode atmospheric pressure chemical ionisation (APCI+) and specific fragment ions are detected after collision induced dissociation (CID) by selected reaction monitoring (SRM). Ionisation parameters are shown in Table 16. Detected SRM transitions and corresponding parameters are shown in Table 17. All measurements are performed with a span of 0.5 m/z, an Inter Channel Delay time of 5 ms and an Inter Scan Time of 5 ms. Dwell times shall be optimised to reach at least 12 points across the peak.

With reference to Commission Decision (EC) 2002/657 - Table 4 of the Annex, a tolerance of 10% to 50% in the value of the ratio is accepted, depending on the amount of the diagnostic ion in relation with the target ion (for this method qualifier ion transition Q_1 and quantifier ion transition Q_2 respectively).

A substance eluting from the chromatographic column is identified as one of the target analytes only:

- if the retention time of the unknown substance coincides with the retention time of the native compound ± 2 SD as found in the calibration solutions.
- if both quantifier and qualifier ion transitions are detected

- if for indole and skatole the Q_1/Q_2 peak area ratio values are within ± 2 SD as found in the calibration solutions. (during method development 7.9 ± 2.3 and 5.1 ± 1.4 respectively)
- if for Cl-indole the peak area in the sample is within ± 2 SD as found in the calibration solutions.

Table 16. Ionisation parameters for Waters Quattro Premier

Mode	Positive APCI
Corona discharge	7.5 µA
Cone Voltage	Depending on analyte (see below)
Extractor	5 V
RF Lens	0 V
Source Temperature	120°C
Desolvation Temperature	600°C
Cone gas flow	500 L/h
Desolvation gas flow	100 L/h
Collision gas flow	0.35 mL/min
Collision energy	Depending on analyte (see below)
Multiplier Voltage	650 V

Table 17 SRM detection parameters for Waters Quattro Premier

Time	SRM Transition	Dwell Time (s)	Cone Voltage	Collision Energy (eV)	Compound
0.00-1.98	117.90 > 65.01	0.100	40.00	29.00	Indole‡
	117.90 > 90.97	0.100	40.00	20.00	Indole†
	123.97 > 95.88	0.100	40.00	20.00	Indole D_7†
1.90-4.00	131.98 > 89.80	0.100	34.00	31.00	Skatole‡
	131.98 > 116.90	0.100	34.00	23.00	Skatole†
	134.98 > 116.90	0.100	34.00	23.00	Skatole D_3†
	151.95 > 116.95	0.100	30.00	18.00	5-Chloroindole†
4.00- 6.50	273.23 > 158.37	0.100	30.00	17.00	Androstenone‡
	273.23 > 255.12	0.100	30.00	14.00	Androstenone†
	277.23 > 259.12	0.100	30.00	14.00	Androstenone D_4†

†: quantifier SRM transition
‡: qualifier SRM transition

8.2 Sample analysis

Before starting the sequence, two solvent blanks (methanol, 4.6) and the calibration standard solution CAL2 (5.13) have to be injected to verify the instrumental performances for this specific method. At the end of the sequence analysis of these solutions is repeated.
The chromatogram of the second solvent blank shall be checked for peaks which could indicate the need of cleaning the system.
The chromatogram of the CAL2 standard solution shall be examined to check the instrument's sensitivity and column performance, i.e. retention times, peak tailing, resolution between compounds.
In each sequence of analysis, after having checked the system, the calibrants, the procedural blank, the QC sample and the sample extracts shall be injected.
At the end of the sequence, results shall be checked to verify if any sample should be outside of the working range and, if necessary, the procedure described in 8.3.1 is applied.

8.3 Data Analysis & Reporting

8.3.1 Calibration

Calibration is performed by triplicate 5 µL injections of the calibration solutions, randomized along the sequence. All solutions shall be equilibrated to room temperature prior to injection.
The calibration curve is obtained by plotting the peak area ratio of the ion transition of the target compounds' quantifier and its corresponding labelled standard against the relative concentration of these compounds in the calibration solutions.
The calibration function is determined for each analyte by unweighted linear regression, and can be described by Equation 2.

$$\frac{C_x}{C_{IS_x}} = \alpha_x \frac{A_{Q_1 x}}{A_{Q_1 IS_x}} + \beta_x$$

Equation 2.

with C the mass fraction [ng/g] of x, the compound of interest, or its corresponding labelled standard IS_x
with A the quantifier ion transition peak area (Q_1) relevant for x, respectively IS_x
with α_x and β_x respectively the slope and intercept of the calibration curve.

Prepare appropriate calibration curves in case the content of boar taint compounds in the sample should be outside the calibration range and within the linear range determined during the method validation. A new sample extraction shall be performed, adjusting the test portion weight so to obtain a final concentration of the boar taint compounds in the extract within the calibration range.

8.3.2 Calculation

Equation 3 is used for the calculation of the boar taint compound mass fraction in the sample prepared as described in paragraphs 7.2 to 7.4.

$$[X]_{sample} = \frac{\left(\frac{\left(\frac{A_{Q_1 x}}{A_{Q_1 IS_x}} - \beta_x \right)}{\alpha_x} \right) S_{IS_x}}{W_{sample}}$$

Equation 3

with $[X]_{sample}$ the mass fraction [µg/kg] of *x,* the compound of interest, in the test portion.
with A the quantifier ion transition peak area (Q_1) relevant for *x*, respectively IS_x (the corresponding labelled standard for *x*).
with α_x and β_x respectively the regression coefficients for *x* determined according to paragraph 8.3.1.
with S_{ISx} the mass (µg) of labelled standard IS_x spiked into the test portion (7.2).
with W_{sample} the mass (kg) of test portion (7.2).

8.3.3 Reporting

Analysis results will not be reported if the quality control criteria are not met. (8.1.2).
All results will be reported together with the corresponding expanded measurement uncertainty, as determined during the method validation study.
In case that the analyte content is below LOD or LOQ the result will be reported as below LOD or below LOQ respectively, and the concentration corresponding to the LOD / LOQ of the method will be provided.
If the calculated analyte content exceeds the upper limit of the working range, the sample shall be re-analysed with an adjusted, lower sample intake.

9 Sample analysis by GC-MS

Before starting the sequence a solvent blank - toluene (4.9) - and the standard solution CS2 (see Table 5) have to be injected to verify the instrumental performances for this specific method.
The chromatogram of the solvent blank is applied to evaluate potential carry over.

9.1 GC-MS operating conditions

9.1.1 Injection conditions

Injetion type: splitless (2 min)
Injection volume: 1 µl
Injection speed: 5 µl/s
Pre injection delay: 1 s
Post injection delay: 1 s
Injector temperature: 250 ºC
Purge flow: 50 ml/min
Purge time: 2 min
Total flow: 53.7 ml/min
Gas type: Helium (5.2)

9.1.2 Oven conditions

Initial temperature: 70 ºC
Initial time: 2 min
Ramp at 40 ºC/min up to 180 ºC, static time 0 min
Ramp at 10 ºC/min up to 220 ºC, static time 0 min
Ramp at 40 ºC/min up to 280 ºC, static time 10 min
(GC analysis time 20.25 min)

9.1.3 Column conditions

Carrier gas flow: 1 ml/min (constant flow mode)
Gas type: Helium (5.2)

9.1.4 Transfer line conditions

Temperature: 325 ºC

9.1.5 Mass spectrometer conditions

MS source temperature: 250 ºC
MS Quadrupole temperature: 150 ºC
Solvent delay: 5 min
Electron Ionisation Energy: 70 eV

9.1.6 Mass spectrometer acquisition parameters and peak identification

The analytes are ionised by electron ionization (EI) at 70 eV. The target ions are recorded in Single Ion Monitoring (SIM) mode, and quantified by an isotope dilution method.

Table 8. Ionisation parameters for Waters Quattro Micro GC

Mode	EI+
Electron Energy	70 eV
Trap Current	200 µA
Repeller	4.8 V
Extraction Lens	14 V
Focus Lens 1	35 V
Focus Lens 3	42 V
Source Temperature	280°C
GC interface Temperature	300°C
Multiplier Voltage	650 V

Table 18. SIM detection parameters for Waters Quattro Micro GC.

Time window (min)	recorded ion (m/z)	Dwell Time (s)	Compound
3.0-16.5	117	0.100	Indole‡
	90	0.100	Indole†
	123	0.100	Indole D_7†
	130	0.100	Skatole‡
	103	0.100	Skatole†
	132	0.100	Skatole D_3†
	151	0.100	5 Chloroindole
16.6-29.0	272	0.200	Androstenone‡
	257	0.200	Androstenone†
	276	0.200	Androstenone D_4†

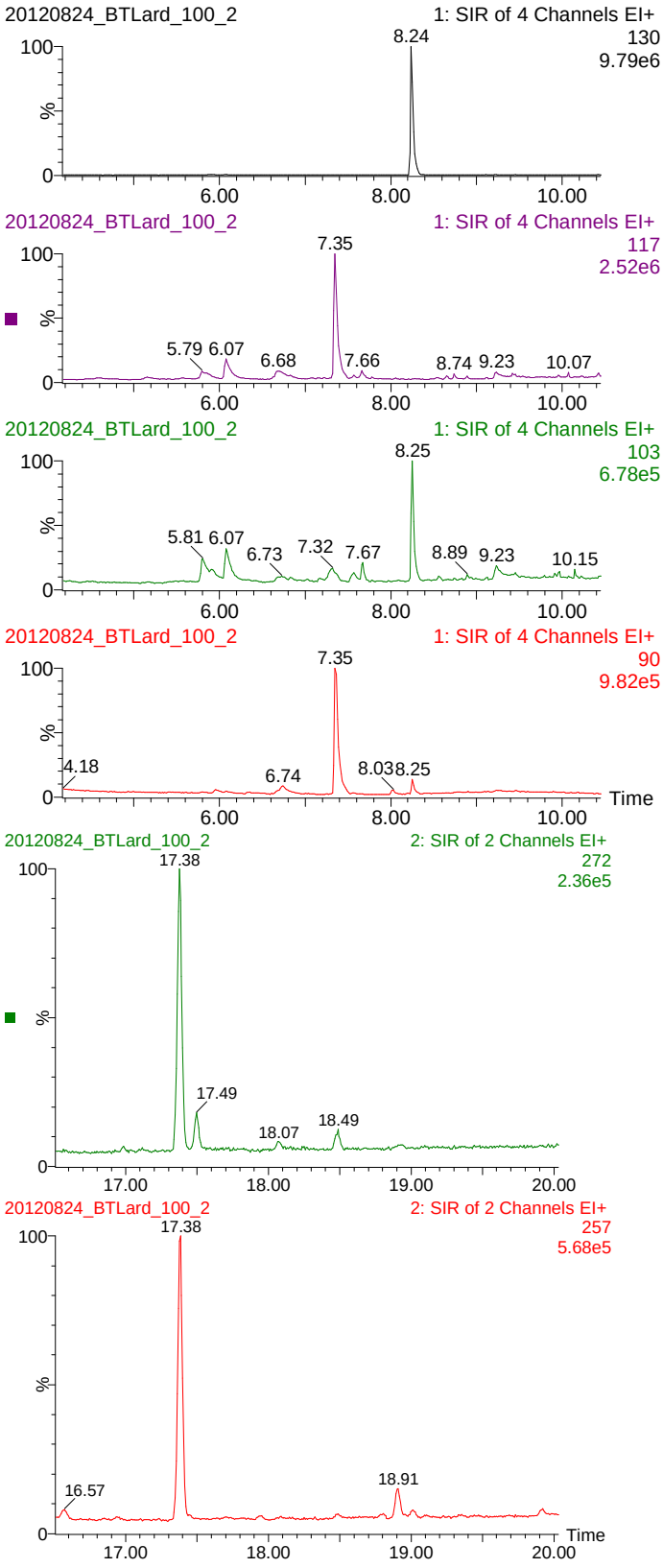


Figure 3 GC-MS (SIM) chromatogram of analysis of lard sample spiked with indole, skatole and androstenone at 100 µg/kg.

9.2 Calibration curve

Calibration is performed by triplicate injections of 1 µL of the calibration solutions, randomized along the sequence. All solutions shall be equilibrated to room temperature prior to injection.
The calibration curve is obtained by plotting the peak area ratio of the quantifier ion of the target compounds to its corresponding labelled standard against the relative concentration of these compounds in the calibration solutions.

9.3 Sample analysis

Analogue to chapter 8.2

10 Calculation and reporting

Analogue to chapter 8.3

Annex 1

In principle the analysis can also be performed employing classic HPLC systems, whereas care has to be taken to guarantee suffienct chromatographical resolution and sensitifity.
HPLC conditions:
Column: Waters Symmetry C18 150 x 2.1 mm 3.5 µm
Injection volume: 5 µL
Column temperature: 35 °C
Flow rate: 0.3 mL/min
Gradient:

Time	%LC eluent A (4.15)	Curve
0	50	6
1.5	50	6
10	10	6
13	4	6
13.1	50	6
18	50	6

Indicative retention times are presented in Table 19.

Table 19 Indicative analyte retention times.

Compound	Retention time (min)
Indole	3.31
Indole D_7	3.31
Skatole	4.76
Skatole D_3	4.76
5-Chloroindole	5.61
Androstenone	11.97
Androstenone D_4	11.97

For the MS method only the time windows need to be adjusted all other parameters are identical with those used for UHPLC.

Table 20 SRM detection parameters for Waters Quattro Premier

Time	SRM Transition	Dwell Time (s)	Cone Voltage	Collision Energy (eV)	Compound
0.0-4.0	117.90 > 65.01	0.100	40.00	29.00	Indole‡
	117.90 > 90.97	0.100	40.00	20.00	Indole†
	123.97 > 95.88	0.100	40.00	20.00	Indole D_7†
4.0-8.0	131.98 > 89.80	0.100	34.00	31.00	Skatole‡
	131.98 > 116.90	0.100	34.00	23.00	Skatole†
	134.98 > 116.90	0.100	34.00	23.00	Skatole D_3†
	151.95 > 116.95	0.100	30.00	18.00	5-Chloroindole†
8.0- 18.0	273.23 > 158.37	0.100	30.00	17.00	Androstenone‡
	273.23 > 255.12	0.100	30.00	14.00	Androstenone†
	277.23 > 259.12	0.100	30.00	14.00	Androstenone D_4†

†: quantifier SRM transition
‡: qualifier SRM transition

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Abstract

Meat from male pigs may develop an off-flavour, commonly known as boar taint. For that reason male piglets are surgically castrated at young age to avoid the potential off-flavour formation.
Animal welfare concerns have triggered research into alternatives to surgical castration of male piglets with the long-term goal of abandoning it by 1 January 2018.
The ALCASDE study has shown that the agreement of testing results for androstenone and skatole in pig fat produced in several laboratories was not satisfactory and one of the conclusions of this project was that in a follow-on project a standardised reference method should be elaborated to rectify this shortcoming.
In the suggested reference method, the 3 marker compounds (skatole, andostrenone and indole), for boar taint are quantified in pork fat by isotope dilution liquid chromatography tandem mass spectrometry (LC MS/MS). The fat is separated from the ground pork fat tissue sample via melting and centrifugation thereafter. The fat is spiked with isotopically labelled standards and prepared for size exclusion chromatography (SEC). The SEC purified sample is evaporated nearly to dryness and after addition of an injection standard analysed by LC-MS/MS in selected reaction monitoring mode.
This method has been validated to obtain the method performance characteristics.
When summarising the method performance characteristics and comparing them to requirements for official food control methods in the area of food contaminants it can be concluded that the method is fit for its intended purpose.
The recovery rates are between 106 % and 115% and are therefore within the range of 60 % to 120 %.
The repeatability standard deviation ranges from 7 % to 18 % and the intermediate precision between 9 % and 18 %, all below the set limit of 20 %.
The measurement uncertainty (U) is below 30 % for all analytes. The method has proven to be robust and free from matrix interferences.
The method is sensitive enough to determine the off-flavour compounds at the sensory threshold values.

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