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Development of a Candidate Reference Method for the Simultaneous Quantitation of the Boar Taint Compounds Androstenone, 3α-Androstenol, 3β-Androstenol, Skatole, and Indole in Pig Fat by Means of Stable Isotope Dilution Analysis−Headspace Solid-Phase Microextraction−Gas Chromatography/Mass Spectrometry

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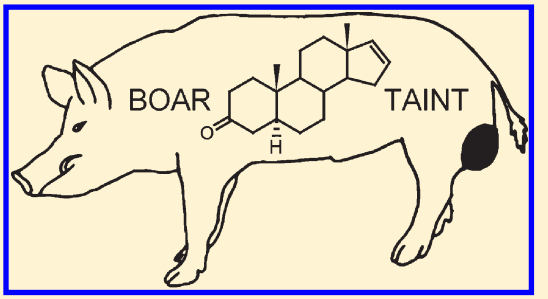
Supporting Information

ABSTRACT: The steroidal pig pheromones androstenone (5α-androst-16-en-3-one), 3α-androstenol (5α-androst-16-en-3α-ol), and 3β-androstenol (5α-androst-16-en-3β-ol) as well as the heterocyclic aromatic amines skatole and indole, originating from microbial degradation of tryptophan in the intestine of pigs, are frequently recognized as the major compounds responsible for boar taint. A new procedure, applying stable isotope dilution analysis (SIDA) and headspace solid-phase microextraction−gas chromatography/mass spectrometry (HS-SPME−GC/MS) for the simultaneous quantitation of these boar taint compounds in pig fat was developed and validated. The deuterated compounds androstenone-d_3, 3β-androstenol-d_3, skatole-d_3, and indole-d_6 were synthesized and successfully employed as internal standards for SIDA. The new procedure is characterized by a fast, simple, and economic sample preparation: methanolic extraction of the melted fat followed by a freezing and an evaporation step allows for extraction and enrichment of all five analytes. Additional time-consuming cleanup steps were not necessary, as HS-SPME sampling overcomes fat-associated injector and column contamination. The method has been validated by determining intra- and interday precision and accuracy as well as the limit of detection (LOD) and limit of quantitation (LOQ). Additionally, a cross-validation for androstenone, skatole, and indole was carried out comparing the results of 25 back fat samples obtained simultaneously by the new SIDA−HS-SPME−GC/MS procedure with those obtained in separate GC/MS and high-performance liquid chromatography fluorescence detection (HPLC-FD) measurements. The cross-validation revealed comparable results and confirms the feasibility of the new SIDA−HS-SPME−GC/MS procedure.



In order to avoid the incidence of boar taint in pork meat surgical castration of male piglets without anesthesia is applied routinely in many European countries.[1] Since it is evident that piglets sustain serious distress when castrated without anesthesia, castration is generally not accepted, particularly by animal welfare organizations.[1−3] Hence, several European countries, e.g., Germany, Norway, and The Netherlands, voluntarily ban the painful castration and start rearing entire male pigs instead, which remains the most practicable alternative.[3−9] Yet, the abandonment of castration has a major drawback influencing the meat quality: the incidence of boar taint in a significant number of carcasses, which is estimated to exceed 10%.[10]

Boar taint is an undesired off-flavor, which is often described as sweaty, musky, urine- or faecal-like and is perceived when fresh meat or meat products are heated prior to consumption.[11−13] The major compound associated with boar taint is the steroidal pig pheromone 5α-androst-16-en-3-one (ANON) that has an unpleasant urine-like and sweaty odor.[11] Being synthesized in the testes, ANON is released into the bloodstream via the testicular vein and subsequently accumulated in adipose tissue due to its

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lipophilic character.[12,13] Next to ANON, the musky-smelling steroidal pig pheromones 5α-androst-16-en-3α-ol (3α-OL) and 5α-androst-16-en-3β-ol (3β-OL), all three belonging to the family of C_{19}-Δ16 steroids, contribute to boar taint.[13−18] Published data concerning the concentrations of 3α-OL and 3β-OL in back fat are rare but are reported to be present in significantly lower concentrations than ANON (ANON/3α-OL/3β-OL = 10/1/0.5).[14,19−21] Nevertheless, the odor thresholds of 3α-OL and 3β-OL are comparable with the threshold of ANON and may have an influence in samples with high concentrations, especially when heated before consumption.[13] Furthermore, skatole (SK) and indole (IND), both microbial degradation products of tryptophan, formed in the intestine by specific bacteria, contribute to boar taint.[22,23] Their odor perceptions are often described as musty and faecal-like.[11] SK and IND are partly absorbed by the intestinal mucosa, distributed via the bloodstream, and finally enriched in adipose tissue and thus also contribute to boar taint.[24]

As the abandonment of castration increases a growing amount of smelling carcasses will potentially end up in the food chain. Thus, the quantitation of boar taint compounds will be the major challenge in order to keep smelling carcasses out of the food chain and thereby implement boar mast successfully. In the past, several analytical methods have been developed to determine boar taint compounds in pig back fat samples ranging from a number of immunoassays, e.g., radioimmunoassay (RIA),[25] enzyme-linked immunosorbent assay (ELISA),[26] or fluoroimmunoassay (FIA),[27] to several chromatographic applications like high-performance liquid chromatography (HPLC),[28,29] gas chromatography (GC),[19,30] or gas chromatography/mass spectrometry (GC/MS).[31,32] More recently a liquid chromatography−tandem mass spectrometry method for the simultaneous detection of SK, IND, and ANON has been published,[33] but as pointed out by the authors, matrix interferences were encountered when determining androstenone in fat. In addition, an HPLC fluorescence detection (HPLC-FD) procedure for simultaneous detection of SK, IND, and ANON was published but requires a derivatization step for fluorescence detection of ANON.[34] All other aforementioned methods separately determine either the indolic compounds or ANON. The quantitation of 3α-OL and 3β-OL as a cumulative value has been previously achieved by applying a GC flame ionization detection (GC-FID) method.[20] In addition, a more selective GC/MS procedure was developed which allows for separate quantitation of both 3α-OL and 3β-OL.[19]

As the lipophilic boar taint compounds are embedded in an interfering fat matrix, a special focus has to be put on the sample preparation to overcome fat-associated matrix effects like insufficient analyte extraction.[33] The most reliable method to eliminate such matrix effects is the application of a stable isotope dilution assay (SIDA). Because isotopically labeled internal standards and the corresponding analyte show almost identical physicochemical properties, SIDA achieves superior accuracy and precision and thereby delivers reliable results. Hence, SIDA is the method of choice, especially when the quantitation of trace odorants is required.[35−37]

The present work describes the development of a headspace solid-phase microextraction−gas chromatography/mass spectrometry (HS-SPME−GC/MS) procedure for the simultaneous determination of the boar taint compounds SK, IND, ANON, 3α-OL, and 3β-OL in pig back fat samples using deuterium-labeled internal standards for SIDA. The new method applies a fast, simple, and economic sample preparation and is highly valuable for routine analysis of boar taint.

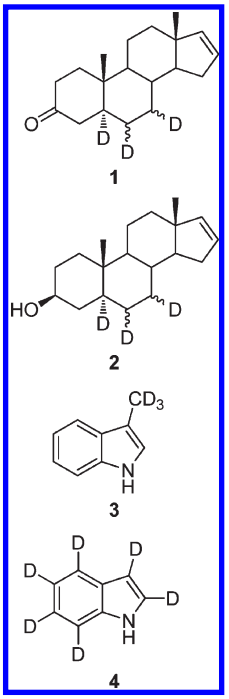

Figure 1. Structures of the four synthesized deuterium-labeled internal standards for boar taint analysis by SIDA−HS-SPME−GC/MS: (**1**) androstenone-d_3, (**2**) 3β-androstenol-d_3, (**3**) skatole-d_3, (**4**) indole-d_6 (ref 38).

EXPERIMENTAL SECTION

Materials. All chemicals were obtained from Sigma-Aldrich (Steinheim, Germany), Roth (Karlsruhe, Germany), VWR (Darmstadt, Germany), or Alfa Aesar (Karlsruhe, Germany) in analytical grade and were used without further purification unless stated otherwise. The deuterium-labeled internal standards (Figure 1) androstenone-d_3 (**1**, ANON-d_3), 3β-androstenol-d_3 (**2**, 3β-OL-d_3), and skatole-d_3 (**3**, SK-d_3) were prepared and characterized in our laboratory as previously reported.[38] In brief, ANON-d_3 was prepared from commercially available 3β-hydroxyandrost-5-en-17-one by selective deuteration of its B-ring double bond using Pd/C and a Mg^0/D_2O/dioxane system, followed by a Shapiro reaction of the ketone to provide 3β-OL-d_3, which was finally oxidized using PDC to obtain ANON-d_3 in 32% overall yield. SK-d_3 was easily obtained by reduction of methyl 1*H*-indole-3-carboxylate using $LiAlD_4$. Aside of these, indole-d_6 (**4**, IND-d_6) was obtained as follows: commercially available perdeuterated indole-d_7 (0.4 mmol, 50 mg, Isotec, Miamisburg, Canada) was refluxed in a methanolic sodium methoxide solution (10 mL, 1%, w/v). After complete exchange (ND to NH) within 24 h as monitored by GC/MS, the solution was evaporated to dryness in vacuo. The remaining residue was redissolved in petroleum ether (10 mL) and subsequently washed with water (5 mL). The organic layer was separated, and the aqueous phase was extracted with petroleum ether (2 × 10 mL). The combined organic layers were dried with anhydrous sodium sulfate, filtered off, and finally evaporated in vacuo to afford IND-d_6 as a slightly red solid (44.1 mg, 88.2%); mp 49−51 °C, lit.[39] 51−52 °C. NMR spectra were recorded on a

Bruker Avance 300 DPX spectrometer operating at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR. Chemical shifts are given in δ values (ppm) referring to the signal center using the solvent peaks for reference (chloroform-d_1: 7.26/77.0). Coupling constants J are reported in hertz (Hz). To characterize the spin multiplicity the following abbreviations are used: bs broad singlet, d doublet. ^{1}H NMR ($CDCl_3$) δ: 6.58 (d, 0.2H, ^{4}J (H, H) = 2.0 Hz), 8.13 (bs, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 102.34 (C-3), 127.60 (C-3a), 135.61 (C-7a). GC/MS: purity 99%. MS CI (m/z, rel int %): 123 ($[M\text{-}d_5 + H]^+$, 28); 124 ($[M\text{-}d_6 + H]^+$, 100). Isotopic purity: d_5 22%, d_6 78%.

Samples. An overall population of 1054 crossbred intact boars of type Piétrain × Baden-Württemberg hybrid sows were either group penned or single penned and feed ad libitum until reaching a slaughter weight between 85 and 95 kg. Back fat samples from the neck region were taken at slaughter, wrapped in aluminum foil, vacuum-packaged, and stored at −20 °C until analysis. Androstenone and skatole levels in all samples were determined by conventional methods.[19,29] For method development and cross-validation 25 samples were selected with respect to their back fat androstenone and skatole levels in order to cover the whole physiologically relevant concentration range including low, medium, and high levels of both compounds.

Sample Preparation and Headspace Solid-Phase Microextraction. The back fat samples were thawed and separated from skin. Subsequently, the fat tissue was diced and heated for 2 min at 180 W in a microwave. The occurring connective tissue was separated from the liquid fat by decanting. An aliquot of 500 mg of the connective-tissue-free fat was transferred into a 2 mL plastic cap and spiked with 250 ng of ANON-d_3, 250 ng of 3β-OL-d_3, 50 ng of SK-d_3, and 50 ng of IND-d_6 to achieve final concentrations of 500 ng/g ANON-d_3 and 3β-OL-d_3 as well as 100 ng/g SK-d_3 and IND-d_6. To allow for equilibration, the sealed cap was thoroughly shaken for 30 s, stored for 10 min at 55 °C, and again mixed for 30 s. Subsequently 1 mL of methanol was added to the liquid fat. Extraction was achieved by a single repetition of the above-mentioned mixing procedure (mix 30 s, store 10 min at 55 °C, mix 30 s). In order to separate the fat phase, a freezing step was carried out by centrifuging the samples (10 min, 6500 rpm, −15 °C). The methanolic supernatant was transferred into a 10 mL headspace vial and evaporated to dryness at 40 °C by a gentle stream of air. The headspace vial was sealed and placed in an autosampler device (Varian Combi Pal, Darmstadt, Germany), operating with a heated agitator and an SPME assembly, using a fused-silica fiber coated with 65 μm poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB) (Supelco, Bellefonte, PA). Automated HS-SPME sampling was carried out as follows: equilibration for 5 min at 100 °C; extraction for 30 min at 100 °C; desorption for 20 min within the injector.

Gas Chromatography/Mass Spectrometry Conditions. GC/MS analysis was carried out using a Varian GC-450, equipped with a Varian VF-5 ms capillary column (30 m × 0.25 mm × 0.25 μm), coupled to a Varian MS-240 ion-trap (EI, scan range 50−300 m/z, Varian, Darmstadt, Germany). The flow was set at 1.0 mL/min using helium as carrier gas. The temperature program was set as follows: start at 50 °C, hold for 3 min, then raise to 160 °C at a rate of 10 °C/min, followed by a rate of 5 °C/min up to 240 °C, hold for 1 min. The injector temperature was kept at 270 °C. Splitless injection was carried out for 3 min, then the split valve was opened to result in a split ratio of 1:100. To allow for quantitation, the mass spectra were recorded in full scan mode using electron impact ionization (EI). Subsequently, the peak area ratios of analyte and internal standard (IS) were determined by displaying the specific mass traces of each analyte and each corresponding IS in selected ion monitoring (SIM) mode. The selected mass traces (m/z) were as follows: SK m/z 130, SK-d_3 m/z 133 + 134, IND m/z 117, IND-$d_{5/6}$ m/z 122 + 123, ANON m/z 257 + 272, ANON-d_3 m/z 260 + 275, 3α-OL m/z 241 + 259 + 274, 3β-OL m/z 241 + 259 + 274, and 3β-OL-d_3 m/z 244 + 262 + 277.

Calibration. Except for 3α-OL, calibration curves were determined for each of the boar taint compounds by applying their previously synthesized isotopomers as internal standards. In the case of 3α-OL no corresponding isotopomer was synthesized as internal standard, but 3β-OL-d_3 was used instead for calibration due to its structural similarity and similar retention time. A seven-point matrix calibration was performed in duplicate by spiking melted sow fat with ANON, 3α-OL, and 3β-OL and palm oil with SK and IND, since sow fat contains small genuine amounts of SK and IND. Defined quantities of deuterium-labeled internal standards and analytes were added to the melted fat. The concentrations of deuterium-labeled internal standards were set constant for each calibration level and were 100 ng/g for SK-d_3/IND-d_6 and 500 ng/g for ANON-d_3/3β-OL-d_3. The amounts of analyte added to the calibration levels were as follows: 0.5, 1, 10, 100, 250, 500, and 1000 ng/g for SK and IND and 50, 250, 500, 750, 1000, 2500, and 5000 ng/g for ANON, 3α-OL, and 3β-OL. The spiked samples were subsequently extracted as described before and analyzed by SIDA−HS-SPME−GC/MS. Linear calibration curves were obtained by plotting the peak area ratios (analyte/IS) versus the concentration ratios (analyte/IS).

Sensitivity. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated as the concentration level with a signal-to-noise ratio of 3:1 and 10:1, respectively.[40]

Accuracy and Precision. To determine intraday accuracy and intraday precision, four replicates of a low calibration level (50 ng/g SK/IND; 500 ng/g ANON/3α-OL/3β-OL) and four replicates of a high calibration level (500 ng/g SK/IND; 2500 ng/g ANON/3α-OL/3β-OL) were prepared and subsequently analyzed within 1 day. The interday accuracy and interday precision were evaluated in the same manner, but analyzing only one replicate of each calibration level per day (within 4 days). The coefficient of variation (CV) and the relative error (RE) were used as a measure for accuracy and precision of the method.

Cross-Validation. A set of 25 back fat samples of entire male pigs was prepared as described above and subsequently analyzed by SIDA−HS-SPME−GC/MS. For comparison, the concentrations of ANON, SK, and IND in the same samples were additionally determined by adapting a previously published reversed-phase (RP)-HPLC procedure[29] with fluorescence detection (FD) for the measurement of SK and IND as well as a previously published GC/MS procedure for the measurement of ANON.[19] The experimental conditions were as follows: RP-HPLC-FD measurement was carried out on a Dionex Ultimate 3000 HPLC system coupled to a Dionex RF 2000 fluorescence detector (Dionex, Idstein, Germany) using a C_{18} column (Hypersil ODS C18, 5 μm, 150 mm × 4.6 mm, MZ Analysentechnik, Mainz, Germany) as well as a C_{18} precolumn (Hypersil ODS C18, 5 μm, 10 mm × 4.6 mm, MZ Analysentechnik, Mainz, Germany) applying isocratic elution (60% 0.02 M acetic acid, 25% acetonitrile, and 15% isopropyl alcohol). The column thermostat temperature was set at 40 °C, and the injection volume was 10 μL. The detection parameters were set

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as follows: excitation wavelength, 270 nm; emission wavelength, 350 nm; emission gain, 4.0; emission response, 0.5 s; emission sensitivity, medium. 2-Methylindole was used as internal standard.

GC/MS analysis was carried out using an Agilent GC 6890 (Agilent Technology, Waldbronn, Germany), equipped with a Restek Rxi-5HT capillary column (30 m × 0.25 mm ×0.25 μm; flow, 1 mL/min using helium as carrier gas; Restek GmbH, Bad Homburg, Germany), coupled to an Agilent MSD 5973 mass spectrometer (Agilent Technology, Waldbronn, Germany) operating with a fast scan upgrade (software version E 02.00 SP1, Agilent Technology, Waldbronn, Germany). The temperature program was as follows: start 130 °C, hold for 1 min, then raise to 265 at 30 °C/min, subsequently raise to 300 at 5 °C/min, hold for 1 min, finally raise to 360 at 20 °C/min, hold for 10 min. Androstanone was used as internal standard.

RESULTS AND DISCUSSION

Internal Standards. A SIDA−HS-SPME−GC/MS procedure for the simultaneous quantitation of boar taint compounds in pig back fat samples is presented for the first time. As the application of SIDA requires a set of deuterium-labeled internal standards, ANON-d_3, β-OL-d_3, and SK-d_3 were synthesized in our laboratory as previously reported (Figure 1).[38] In addition, IND-d_6 was obtained from commercially available indole-d_7 by an H/D exchange in methanolic sodium methoxide solution. This step was necessary since the deuterium atom attached to the nitrogen atom of indole-d_7 is labile to H/D-exchange whenever indole-d_7 is dissolved in a protic solvent, e.g., methanol (data not shown). When the recorded NMR spectra of the synthesized IND-d_6 were analyzed, an additional but tolerable H/D-exchange was observed, as the spectra show signals at δ 6.58 (^{1}H NMR) and at δ 102.34 (^{13}C NMR), indicating the presence of hydrogen at carbon 3. The extent of the d_5 species, possessing hydrogens at carbon 3 and attached to the nitrogen atom, was determined to be 22% as calculated from the fragmentation patterns of the labeled and the corresponding unlabeled compound obtained from chemical ionization (CI) MS spectra.

EI-MS spectra of the internal standards and the corresponding boar taint compounds are presented in Figure S-1 (see the Supporting Information). When the mass cluster of ANON-d_3 is inspected, the ions m/z 242, 260, and 275 show an m/z value that is three mass units higher than the respective ions m/z 239, 257, and 272 of genuine ANON. The same differences were evident between 3β-OL-d_3 (m/z 244, 262, 277) and the genuine analytes 3α-OL and 3β-OL (m/z 241, 259, 274). When the EI-MS spectra of SK and SK-d_3 are analyzed, the ions m/z 130 and m/z 132 are the most abundant. As the ion m/z 130 is assigned to the $[M - H]^+$ fragment of SK, the corresponding ion m/z 132 originates from the $[M - D]^+$ fragment of SK-d_3 and thereby reveals the methyl group as the preferred place of fragmentation.[41] Nevertheless, the less abundant ions m/z 133 and m/z 134 of SK-d_3 were selected and summed for quantitation, since a significant overlap of mass signals between SK and SK-d_3 was detected, when using the ion m/z 132 for quantitation. Because of the sixfold labeling of IND-d_6, the most abundant ion m/z 123 of IND-d_6 has a six mass units higher m/z value than the corresponding ion m/z 117 of the unlabeled isotopomer IND. Hence, all synthesized internal standards were found suitable for SIDA, when selecting the appropriate mass traces for quantitation in SIM mode.

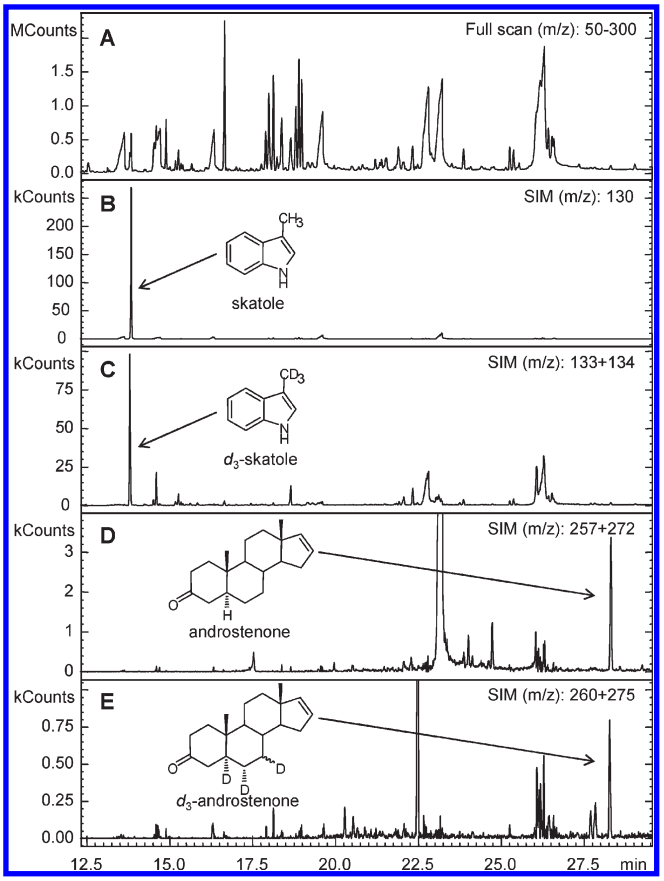

Figure 2. HS-SPME−GC/MS analysis of a back fat sample of an entire male pig containing 1273 ng/g androstenone, 142 ng/g 3β-androstenol, 295 ng/g 3α-androstenol, 181 ng/g skatole, and 60 ng/g indole. Selective ion extraction of the full scan chromatogram (A) is exemplarily displayed for skatole and androstenone determination. The arrows indicate the peaks of skatole (B), androstenone (D) and their corresponding isotopomers skatole-d_3 (C) and androstenone-d_3 (E).

Sample Preparation, SPME Sampling, and GC/MS Analysis. Sample preparation was carried out adapting a previously reported procedure.[34] After methanolic extraction of the boar taint compounds and subsequent separation of interfering fat components by a freezing step, the procedure was extended by an evaporation step: the methanolic supernatant was decanted into a headspace vial and finally evaporated to dryness in order to increase the vapor pressure of the analyte within the headspace vial. No further cleanup was necessary, as HS-SPME sampling avoids injector and column contamination, which is often observed with direct injection of fat containing extracts. In order to find optimum extraction parameters for HS-SPME sampling, various sampling conditions were tested. For this purpose, different extraction temperatures (50, 70, 90, 100, 110 °C) and extraction times (10, 20, 30, 40, 50 min), as well as different fiber coatings (DVB/PDMS 65 μm, polyacrylate (PA) 85 μm, PDMS 7 μm, DVB/carboxen (CAR)/PDMS 50/30 μm) were applied. In addition, different amounts of fat (250, 500, 750, 1000 mg) and different methanol volumes (1, 2, 3, 5 mL) were tested. The decision whether a parameter was adequate or not was based on the respective peak areas applying a univariate optimization design. In the beginning, the optimal fiber coating was selected by analyzing replicates with the four above-mentioned fibers. From this experiment it was evident that the PDMS fiber was the

Table 1. Calibration Data of the Developed SIDA−HS-SPME−GC/MS Method

analyte	linearity (R^2)	concn range (ng/g)[a]	LOD (ng/g)[a,b]	LOQ (ng/g)[a,c]
skatole	0.9996	0.5−800	0.1	0.5
indole	0.9998	1.0−800	0.5	1.0
androstenone	0.9993	50−5000	35	60
3α-androstenol	0.9929	70−5000	50	70
3β-androstenol	0.9932	65−5000	45	65

[a] ng analyte per gram melted fat. [b] LOD = limit of detection (special samples were further diluted for LOD determination). [c] LOQ = limit of quantitation.

most suitable for the pig pheromones ANON, 3α-OL, and 3β-OL, but not suitable for the indolic compounds SK and IND. For simultaneous determination of all five boar taint compounds, both PDMS/DVB and PA fiber were suitable, but PDMS/DVB gave slightly higher peak areas for SK and IND and thus was selected as the fiber of choice. Nevertheless the PDMS fiber is recommended, when the exclusive detection of ANON, 3α-OL, and 3β-OL is required. When the extraction times (10, 20, 30, 40, 50 min) are considered, the pheromones and the indolic compounds showed opposite tendencies: prolonged extraction times lead to increased pheromone peak areas, whereas the peak areas of the indolic compounds were decreasing. As the resulting peak areas of the pheromones are generally less intensive than the resulting peak areas of the indolic compounds, an extraction temperature of 30 min was selected to get sufficient pheromone peak areas. For the same reason, the extraction temperature (50, 70, 90, 100 °C) was set at 100 °C, even though SK and IND showed higher peak areas when temperatures were set at 70 or 90 °C. No significant differences were found when adding different volumes of methanol (1, 2, 3, 5 mL). Hence, 1 mL of methanol was added to all samples. When the sample amount used for extraction is considered, it can be generally stated that an increased amount of fat lowers the pig pheromone peak areas. The opposite effect was found for the indolic compounds. Finally, 500 mg of fat was applied in all analysis.

All GC/MS chromatograms were recorded in full scan mode and subsequently displayed in SIM mode for quantitation. With respect to sensitivity, quantitation of ANON, 3β-OL, and 3α-OL was carried out by summing peak areas of either two or three specific ions. For the same reason, the selective ions m/z 133 and m/z 134 of SK-d_3 were used, when quantifying SK. In the case of IND-d_6, the ions m/z 122 and m/z 123 were suitable for quantitation. A full scan GC/MS chromatogram of a boar back fat sample, containing 1273 ng/g ANON, 142 ng/g 3β-OL, 295 ng/g 3α-OL, 181 ng/g SK, and 60 ng/g IND is presented in Figure 2. Exemplarily, the specific mass traces of SK (m/z 130), SK-d_3 (m/z 133 + 134), ANON (m/z 257 + 272), and ANON-d_3 (m/z 260 + 275) are additionally displayed in SIM mode in Figure 2.

Calibration. Matrix calibration was performed by spiking either sow fat (ANON, 3α-OL, and 3β-OL) or palm oil (SK, IND) with defined quantities of deuterium-labeled internal standards and analytes. Calibration curves were constructed by plotting the peak area ratios (analyte/IS) versus the concentration ratios (analyte/IS). As illustrated in Table 1 the obtained regressions show excellent linearity, possessing coefficients of determination of >0.99 (n = 7) within the given concentration ranges. Concentrations exceeding the highest calibration level of each compound were not tested, since the applied concentrations cover almost all reported physiological concentrations. The LOQs of SK and IND were found to be 0.5 and 1.0 ng/g. The corresponding LOD was 0.1 ng/g for SK and 0.5 ng/g for IND. Higher LOQ (60 ng/g) and LOD (35 ng/g) values were found for ANON, but are still satisfactory for boar taint analysis, when taking into account that the frequently mentioned ANON limit of consumer acceptance in back fat samples was found at either 500 or 1000 ng/g.[7,42,43] The determined LOQ and LOD values for 3α-OL and 3β-OL were as follows: LOQ (3α-OL), 70 ng/g; LOD (3α-OL), 50 ng/g; LOQ (3β-OL), 65 ng/g; LOD (3β-OL), 45 ng/g (Table 1). The values of precision (CV) and accuracy (RE) were determined for each boar taint constituent by measuring four replicate samples at a low and a high concentration level within 1 day (intraday) and within 4 days (interday), respectively. Satisfactory RE values were obtained for all analytes, ranging between −8.6% and 7.0%. When the precision of the method is considered, it can be stated that none of the CV values exceeded 8.4% and hence were satisfactory as

Table 2. Concentrations of Androstenone, 3α-Androstenol, 3β-Androstenol in 25 Back Fat Samples of Entire Male Pigs Determined by SIDA−HS-SPME−GC/MS[a]

sample	androstenone (ng/g)	3α-androstenol (ng/g)	3β-androstenol (ng/g)
1	90	<50	<45
2	91	<50	<45
3	298	87	<65
4	339	117	195
5	366	113	<65
6	388	118	<65
7	452	226	68
8	461	295	76
9	469	90	<65
10	658	179	129
11	699	376	120
12	724	225	123
13	853	194	245
14	862	441	174
15	921	352	160
16	1608	253	171
17	1713	488	185
18	1773	820	341
19	2060	289	159
20	2506	453	275
21	2870	362	263
22	3126	644	375
23	3172	562	351
24	3790	655	331
25	4978	815	453
mean	1410	330	181
range	90−4978	50−820	45−453

[a] Odor thresholds in cottonseed oil (μg/g) (ref 13): androstenone, 0.6; 3α-androstenol, 0.9; 3β-androstenol, 1.2. Frequently mentioned consumer acceptance limit for androstenone (ref 46) in back fat: 500 ng/g. Data not available for 3α-androstenol and 3β-androstenol.

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Table 3. Intra- and Interday Accuracy (RE) and Precision (CV) of SIDA−HS-SPME−GC/MS Determination for Samples Spiked with Low and High Concentrations of Each Boar Taint Compound

analyte	added (ng/g)	found (ng/g) (mean ± SD)	CV (%)[a]	RE (%)[b]
		intraday (n = 4)		
skatole	50	54 ± 1	3.0	−8.1
	500	490 ± 24	5.0	1.8
indole	50	48 ± 1	2.8	3.5
	500	488 ± 5	1.0	2.2
androstenone	500	476 ± 19	4.0	4.4
	2500	2482 ± 132	5.3	0.3
3α-androstenol	500	518 ± 27	5.4	−5.2
	2500	2289 ± 173	7.5	7.0
3β-androstenol	500	505 ± 33	6.7	−2.5
	2500	2381 ± 132	5.5	3.3
		interday (n = 4)		
skatole	50	54 ± 1	1.3	−8.0
	500	470 ± 10	2.1	5.4
indole	50	48 ± 2	5.0	3.8
	500	485 ± 4	0.8	2.9
androstenone	500	493 ± 20	4.1	0.9
	2500	2477 ± 96	4.0	0.5
3α-androstenol	500	527 ± 39	7.4	−7.1
	2500	2355 ± 166	7.0	3.9
3β-androstenol	500	535 ± 45	8.4	−8.6
	2500	2417 ± 118	4.8	1.3

[a] CV = coefficient of variation. [b] RE = relative error.

well (Table 3). Moreover, all determined values are within the guideline ranges recommended by Commission Decision concerning the performance of analytical methods and the interpretation of results.[44]

Cross-Validation and Pheromone Levels. To further validate the novel SIDA−HS-SPME−GC/MS procedure a cross-validation experiment for androstenone, skatole, and indole was carried out. Therefore, the results of 25 back fat samples obtained by SIDA−HS-SPME−GC/MS were compared with the results obtained from the same samples by RP-HPLC-FD measurement of SK and IND[29] and GC/MS measurement of ANON.[19] For comparison, the SIDA results were plotted against their corresponding GC and HPLC results and were analyzed by orthogonal distance regression.[40] The obtained correlation plots are shown in Figure S-2 (see the Supporting Information). For androstenone and skatole no proportional systematic error (P = 95%) and no constant systematic error (P = 99%) were observed. However, for indole a small, but statistically significant, constant systematic error (P = 99%) was observed. Hence, the SIDA method gave lower results when compared with those obtained by HPLC-FD. Nevertheless, it is evident that the SIDA−GC/MS method presented here delivers reliable results.

All measured concentrations for ANON, 3α-OL, and 3β-OL in 25 real samples using the novel SIDA−HS-SPME−GC/MS are presented in Table 2. As can be seen, the concentrations of the pheromones are highly variable with maximum values of 4978 ng/g ANON, 820 ng/g 3α-OL, and 453 ng/g 3β-OL. When the determined pheromone concentrations of ANON, 3α-OL, and 3β-OL are considered in detail, it is obvious that the ANON values exceed the 3α-OL and 3β-OL values in every case. Figure S-3 (see the Supporting Information) shows the correlation between the found concentrations of 3α-OL (Supporting Information, Figure S-3A) and 3β-OL (Supporting Information, Figure S-3B), respectively, and the concentrations of ANON. The coefficients of determination, 0.68 and 0.80, in both linear regression plots indicate that increased ANON levels are accompanied by increased 3α-OL and 3β-OL levels. This finding is in good agreement with their biosynthesis, as 3α-OL and 3β-OL originate from enzymatic reduction of ANON in the boar testes.[45] When only the determined androstenol concentrations are compared, 3α-OL was found in higher concentrations than 3β-OL in most samples. The mean concentration ratio of 3α-OL/3β-OL was found to be 1.7 (Supporting Information, Figure S-3C).

CONCLUSION

A novel and reliable SIDA−HS-SPME−GC/MS procedure for the simultaneous quantitation of ANON, 3α-OL, 3β-OL, SK, and IND in pig fat has been developed. The method validation, including a cross-validation experiment, demonstrates excellent performance, accuracy, and precision within the concentration ranges of interest when investigating boar taint. Hence, the novel SIDA−HS-SPME−GC/MS procedure is recommended as a candidate reference method for future boar taint studies in order to ensure reliable results.

ASSOCIATED CONTENT

Supporting Information. Figures S-1−S-3. This material is available free of charge via the Internet at http://pubs.acs.org.

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