Formation mechanisms and aroma potential of 2-aminoacetophenone in boar fat

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Summary

For decades the surgical castration of male piglets has been a common practice to avoid the incidence of boar taint within Europe. Boar taint is known as an offensive off-flavour occurring when meat of uncastrated boars is heated prior to consumption. According to the current state of knowledge the off-flavour's perception by sensitive consumers is mainly caused by high endogenous concentrations of two substances: The boar pheromone androstenone (5α-androst-16-en-3-one) and the heterocyclic amine skatole (3-methylindole). As the surgical castration of male piglets without anaesthesia is known to cause serious pain and distress for the animals this practice is increasingly questioned and refused by the majority of consumers. Driven by this public concerns, in 2010 all European stakeholders along the production chain of pork voluntarily agreed to ban surgical castration without anaesthesia by the beginning of 2018 and search for alternative strategies to prevent consumers from tainting pork.

Since the formulation of this declaration known as "declaration of Brussels" many efforts have been undertaken in order to minimize the levels of androstenone and skatole in boars, *i.e.* immunocastration, boar fattening. However, frequent discrepancies between the sensory evaluation of boar fat and analytically determined levels of androstenone and skatole raised the question whether and to what extend other substances contribute to the off-flavour. In this context compounds of miscellaneous classes have been discussed. Indole, like skatole a degradation product of L-tryptophan in the pig's large intestine, and reduced metabolization products of androstenone, 5α-androsten-ol and 5β-androsten-ol, were proven to have a minor impact on the occurrence of boar taint. Furthermore, fatty acids and their microbiological degradation products as well as phenolic compounds were suggested as additional boar taint agents.

Interestingly, no focus was put on hepatic phase-I-metabolites of skatole although seven products of the hepatic clearance of the amine were already identified almost 20 years ago. Among these metabolites a substance appears, that already attracted attention as a key substance within the origination of off-flavours in white wines and milk products, 2-aminoacetophenone. However, no data about 2-aminoacetophenone levels in boars exist and it's biogenesis in pigs has not yet been elucidated. Thus, the presented thesis addressed the question of a possible contribution of 2-aminoacetophenone to the perception of boar taint and

its formation in pigs. To that end, the thesis consists of four chapters, each one dealing with one aspect of the initial hypothesis.

First of all, back fat levels of 2-aminoacetophenone in Pietrain × Baden Württemberg hybrid boars were determined by the adaption of a recently published HS-SPME-GC/MS method for the determination of boar taint compounds in fat. By the application of the isotopically labelled counterpart of 2-aminoacetophenone in a stable isotope dilution assay (SIDA), d_3-2-aminoacetophenone, the method guaranteed for precise and robust results. Determined average levels of 0.1 ppm confirmed the assumption that an accumulation of 2-aminoacetophenone analogous to androstenone and skatole takes place in the investigated samples. Additionally, the determination of the compound's sensory threshold in fatty matrices revealed a comparable aroma potential to both boar taint agents, emphasizing a potential contribution to the perception of the off-flavour.

As 2-aminoacetophenone was listed as a minor product of the hepatic clearance of skatole in pigs, the high back fat concentrations raised the question whether other endogenous pathways lead to the compound's formation. Therefore the volatile metabolites of hepatic skatole conversion were investigated by a developed HS-SPME-GC/MS method in *in vitro* assays using porcine liver microsomes of Pietrain × Baden Württemberg hybrid boars. Liver microsomes are known to contain high concentrations of CYP 450 monooxygenases, a ubiquitous existing enzyme family highly involved in phase-I-reactions. The determined product profiles revealed 2-aminoacetophenone as the main volatile metabolite, followed by 3-methyloxindole and indole-3-carbinol.

Although incubation experiments with porcine liver microsomes served as an explanation for high 2-aminoacetophenone concentrations found in fat samples exact pathways en route to 2-aminoacetophenone remained unclear. In this context the determination of kinetic isotope effects was applied to gain further information about the reaction mechanism. Kinetic isotope effects are defined as measurable changes in reaction rates when at least one atom is replaced by its isotope. Accordingly, incubations with labelled skatole were conducted next to incubations with genuine skatole. In these incubations mechanistic details of single reaction steps could be elucidated. Furthermore, incubations with possible intermediates revealed the formation of 2-aminoacetophenone via the intermediates 3-methyloxindole and 3-hydroxy-3-methyloxindole.

Finally, the contribution of 2-aminoacetophenone to the overall aroma of boar fat was evaluated by the application of the so-called “sensomics” approach to an aroma distillate of fat from boars, female pigs and castrated male pigs, respectively. The sensomics approach comprises the determination of flavour dilution factors (FD factor) by the measurement of stepwise diluted aroma extracts by gas chromatography coupled to the human nose as a very sensitive biological detector. This concept became known as aroma extract dilution analysis (AEDA). A substance’s FD factor is denoted as the highest dilution at which a substance is still detectable by the human nose. On the basis of the determined FD factors the most important odorants in a given set of volatiles are identified. In the case of boar fat 16 aroma active compounds were identified and later on quantified by the application of SIDA using a published HS-SPME-GC/MS procedure. To verify whether all odorants were correctly identified and quantified, odour activity values (OAVs) of each odorant were calculated by dividing the determined concentrations by the corresponding odour thresholds in fat. Accordingly, highest OAVs were identified for skatole, androstenone, indole and 2-aminoacetophenone verifying that the latter plays an important role within the overall aroma of boar fat from Pietrain × Baden Württemberg hybrid boars. A reconstitution model of all odorants with OAVs above one in deodorized sunflower oil showed good similarities to an authentic boar fat.

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Abbreviations

UK	United Kingdom
EU	European Union
EC	European Commission
CYP 450	cytochrome P450
CYP 2E1	cytochrome P450 subtype 2E1
CO_2	carbon dioxide
GnRH	Gonadotropin releasing hormone
LH	Luteinizing hormone
FSH	Follicle stimulating hormone
PSE	pale, soft and exudative
DFD	dark, firm and dry
ppm	parts per million
NADPH	Nicotinamide adenine dinucleotide phosphate
CO	carbon monoxide
nm	nanometre
SIDA	stable isotope dilution assay
^{2}H	deuterium/hydrogen isotope
^{13}C	heavy carbon/carbon isotope
HS-SPME	headspace-solid phase microextraction
GC/MS	gas chromatography/mass spectrometry
KIE	kinetic isotope effect
C-H bond	carbon-hydrogen bond
C-D bond	carbon-deuterium bond
GC-O	gas chromatography olfactometry
UTA	untypical aging off-flavour
m/z	mass-to-charge ratio

SPE	solid phase extraction
LOD	limit of detection
LOQ	limit of quantification
CV	coefficient of variation
RE	relative error
ppb	parts per billion
mRNA	messenger ribonucleic acid
AhR	aryl hydrocarbon receptor
CAR	constitutive androstane receptor
PXR	pregnane X receptor
DNA	deoxyribonucleic acid
EROD	Etoxyresorufin o-deethylation
MROD	Methoxyresorufin-o-demethylation
EDTA	etylenediaminetetraacetic acid
QC	quality control
k_E	reaction constant of an element
k_I	reaction constant of an isotope
E	enzyme
S	substrate
$(k_H/k_D)_{obs}$	observed ratio of reaction rates comprising hydrogen or deuterium abstraction
ES	enzyme substrate complex
EP	enzyme product complex
AEDA	aroma extract dilution analysis
FD factor	flavour dilution factor
OGA	olfactometry global analysis
cAEDA	comparative aroma extract dilution analysis
OAV	odour activity value

SAFE	solvent assisted flavour evaporation

1 Introduction

1.1 Boar taint: physiological origin

In 1743 a general encyclopaedia published in Germany by Johann Heinrich Zedler, mentions the necessity of castration of piglets for the preservation of pork quality: "*Die Schweine müssen geschnitten seyn, wenn das Fleisch und Speck wohl gerathen soll. Es kann solches noch bey der Muttermilch geschehen, da sie es eher vergessen.*".[1] This lexical entry as well as earlier historical sources refers to an off-flavour occurring in heated meat of uncastrated male pigs, today summed up under the term "boar taint". Typical sensory descriptors of boar taint are "faecal-like", "musky", "sweaty" or "urine-like".[2,3]

In the current state of knowledge the perception of boar taint is mainly based on the interaction of human nasal receptors with two substances: the C_{19}-Δ16 steroid androstenone (5α-androst-16-en-3-one) and the heterocyclic aromatic amine skatole (3-methylindole).[4,5] With the onset of puberty androstenone is synthesized from the precursors pregnenolone and progesterone in the leydig cells of the boar's testes. In contrast to other testicular androgens, *e.g.* testosterone, androstenone shows no anabolic effect but functions as a sex pheromone. Once released from the leydig cells via the testicular vein into the bloodstream, androstenone is transported into the salvia glands where it serves as a sexual attractant when emanating from the boar's salvia.[6,7] However, because of its lipophilic properties, androstenone is also stored in adipose tissues. It has been demonstrated that greatest parts of circulating androstenone are rapidly distributed into storage compartments while the remaining amount is subject to two degradation steps: in phase-I-metabolism hydrogenation to corresponding alcohols (5α-androsten-ol and 5β-androsten-ol) and in phase II metabolism sulfoconjugation to androstenone sulfate. Androstenone levels in porcine fat are therefore influenced by countervailing effects of testicular biosynthesis on the one hand and testicular and hepatic metabolism on the other hand.[8] A steady accumulation of the steroid in lipophilic compartments may therefore lead to androstenone concentrations that can be sensed by sensitive consumers as a pronounced urine-like odour when pork is heated prior to consumption. Interestingly, individual and also gender-related variations can be observed regarding the sensitivity of humans towards androstenone perception. In general, women are more sensitive than men, while a significant part of the consumers is anosmic to androstenone.[9–11]

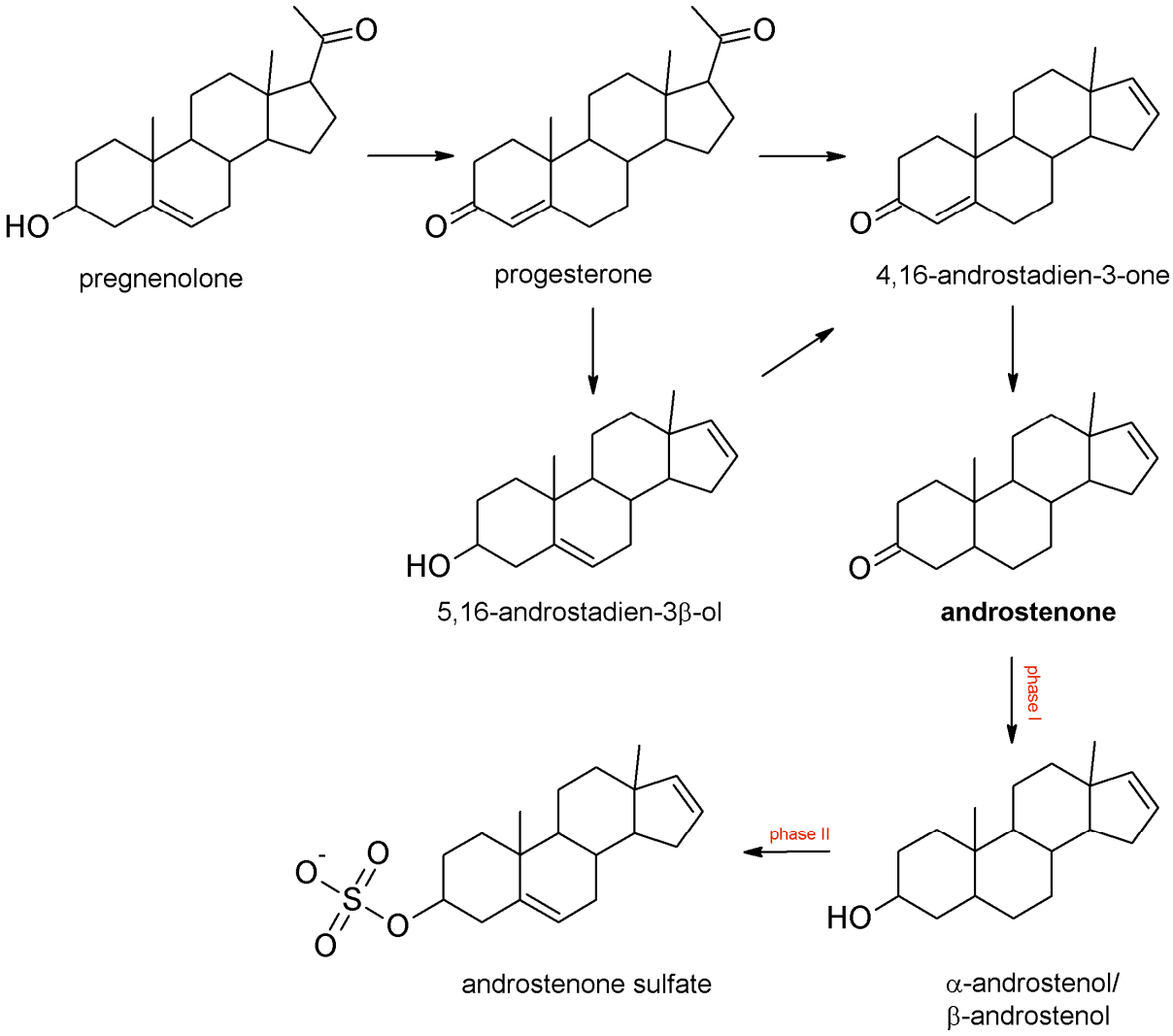

Figure 1 Biosynthesis of androstenone located in the boars testis according to Robic *et al.*.[8]

Besides androstenone another molecule plays a decisive role within the generation of boar taint. Skatole, in contrast, is not formed by the boars' organism, but derives from a two-step microbiological breakdown of the amino acid L-tryptophan in the pigs' intestine: while the conversion into indole-3-acetic acid can be performed by many microbes such as *Escherichia coli*, the following decarboxylation to skatole is restricted to highly specific bacteria. A *lactobacillus* strain was demonstrated to be mainly responsible for skatole formation in the colon of pigs.[7,12–14] Via the intestinal mucosa a part of the skatole reaches the bloodstream, is distributed in the organism and accumulated in adipose tissues analogous to androstenone. Unlike the boar pheromone, skatole is uniformly detected by all persons as the faecal-like note of boar taint, although individual differences in the sensitivity of consumers towards skatole exist.[15,16] As in the case of androstenone, skatole levels in fat are steadily influenced by dynamic processes. On the one hand varying intestinal L-tryptophan levels because of digestion of feed-derived protein and tryptophan release from the apoptosis of mucosa cells

determine the formation rate of skatole. On the other hand skatole is also subject to the CYP 450 mediated hepatic phase-I-metabolism.[7] It was shown that skatole itself induces CYP 450 enzymes catalysing reaction pathways leading to seven known metabolites in the liver. Interestingly the boar taint agent androstenone is able to inhibit the induction of the CYP 450 isoform 2E1 (CYP2E1), an enzyme with major responsibility for the metabolism of skatole in pigs.[17–19]

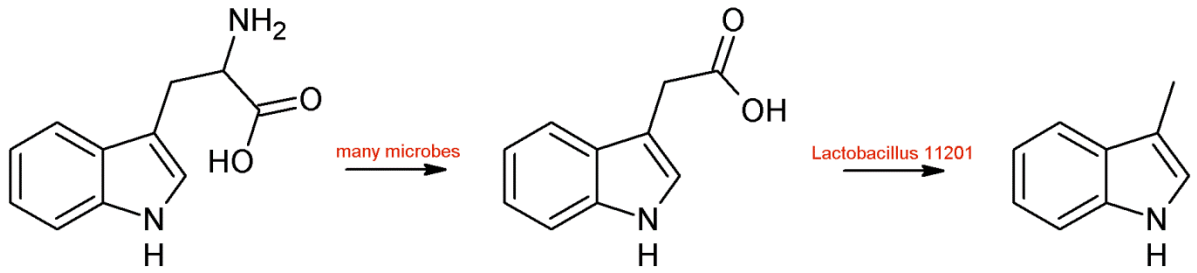

Figure 2 Biosynthesis of skatole located in the boar's large intestine.

1.2 Boar taint: economic significance

In 2016 about 257 million pigs were slaughtered in the European Union. Approximately 50% of these animals came from abattoirs in the five big producer countries Germany, Spain, France, Poland and Denmark.[20] In order to enhance growth performance, to reduce aggressive and sexual behaviour and to prevent the incidence of boar taint, surgical castration of male piglets is and was common practice in most member states, except for Ireland and the UK.[21–24] According to the EU Directive 2001/93/EC this castration can be conducted without the application of anaesthesia before the seventh day of live.[25] Studies focusing on endocrine and behavioural responses of piglets to surgical castration without anaesthesia elucidated that this practice causes serious distress and pain for the animals and therefore impairs animal welfare.[26,27] Thus, already in 2008, German stakeholders formulated their intention to voluntarily ban surgical castration without anaesthesia and establish alternative routines. However, the declaration, known as "*Düsseldorfer Erklärung*", provided no concrete intermediate objectives or time tables.[28] In 2010 representatives of several member states of the EU, including Germany, the Netherlands, Norway and Denmark, agreed to ban castration without anaesthesia or analgesia by the beginning of 2012 and to search for alternatives to surgical castration till the beginning of 2018 to fully end this painful procedure.[29] However, a progress report published in 2014 dealing with the efforts undertaken by each member state came to the result that the European campaign may be on half way, while not half of the goals were achieved.[30] In Germany, the European agreements have already been transposed into national right by a novel of the German Animal Welfare Act in 2013. Following this novel

any surgical castration of piglets without anaesthesia or analgesia will be forbidden by the beginning of 2019.[31] With respect to this legal amendment pork industry faces the question for economic and effective alternatives to the previously common practice. So far, three different procedures have been discussed:

Obviously, the treatment of castrated piglets with anaesthesia and/or prolonged analgesia could be a relevant alternative. However, anaesthesia does neither reduce acute distress for the animals due to the catching procedure before castration nor does it eliminate chronic distress within the first days after the surgery.[32] Additionally, as the use of anaesthetics is restricted to veterinarians, the method suffers from additional cost and effort. While the Netherlands consider the application of CO_2 as appropriate, German stakeholders question the use of the anaesthetics, since it was shown to be ineffective in respect to pain relief.[33,34]

Another often discussed alternative to the practice of surgical castration is referred to as "immunocastration". The aim of this method is the active immunization against gonadotropin-releasing hormone (GnRH), a neuropeptide that is released from the hypothalamus to stimulate the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH), two hormones regulating the production of testicular steroids. By injecting a synthetic analogue of GnRH (Improvac®), which itself has no hormonal effect, the production of antibodies against endogenous GnRH is induced and the concentration of testicular steroids, such as testosterone or androstenone is consecutively reduced.[35,36] As androstenone levels have an influence on the hepatic metabolism of skatole, the procedure also positively effects endogenous concentrations of this boar taint compound in fat.[37] Thus, immunocastration is capable of significantly reducing the incidence of boar taint and testosterone related aggressive behaviour without impairing the meat quality or animal welfare issues.[38] Although the application of the vaccination is generally approved in the EU since 2009, it plays a minor role in practical use due to low market acceptance. A survey with European stakeholders revealed low prospects, since a significant fear of consumer's acceptance exists, although studies performed in Belgium and Germany proofed that well-informed consumers will prefer meat from immunocastrates.[39–41] Regarding the situation in 2017, vaccination of entire male pigs could not prevail against surgical castration of piglets with or without anaesthesia throughout the EU. Only in Belgium immunocastration was successfully implemented as a valuable practice because of retailer's demands since 2011.

Instead, the most practicable alternative to replace surgical castration seems to be the production of entire male pigs, which has been common tradition in the UK and Ireland for

decades. As no invasive procedures are required, rearing of entire male pigs is the method of choice with regard to animal welfare issues. Besides the protection of physical integrity, the practice offers other advantages, such as a better food conversion, a faster growth performance and a superior meat quality (leaner carcasses compared to castrates) due to the presence of natural anabolic steroids.[42,43] However, the hormonal status of entire male pigs entails aggressive behaviour expressed in rank order fights and mounting of littermates. Both, fighting and mounting activity cause painful injuries and exhibit serious stress, which leads to a slightly higher liability to obtain PSE (pale, soft and exudative) or DFD (dark, firm, dry) meat.[21,22] The greatest challenge for a successful application of boar fattening, however, remains in the prevention of boar taint. Worst case scenarios reported 40% tainting carcasses when applying common threshold levels of 1.0 ppm androstenone and 0.2 ppm skatole.[19,44,45] Nevertheless broad agreement exists among German stakeholders with regard to the stepwise implementation of boar fattening as the most reasonable alternative to finally ban castration by 2018. As a first step German retailers announced to solely distribute meat of uncastrated boars by the beginning of 2017.[46] In this context slaughter of boars at younger age and lower weight has been discussed as a strategy to minimise the risk of boar taint, since this approach might ensure, that animals are slaughtered before the onset of puberty.[47] However, as the time of puberty markedly differs between breeds and even between individuals of the same breed, slaughter weight by itself, will not be able to fully overcome the risk of boar taint occurrence. In addition, several scientific studies with regard to interfering factors of boar taint revealed the importance of other aspects such as genetics, hormonal status, biogenesis and metabolic clearance of boar taint compounds. For example, boar taint levels can be manipulated by changing the hormonal status of the animals or dietary composition to reduce intestinal skatole synthesis.[48] However, despite all efforts, the incidence of boar taint is not fully understood and a significant amount of tainting carcasses is still expected.

1.3 Boar taint: search for additional contributors

Although great efforts have been undertaken to explain and predict the incidence of boar taint by levels of androstenone and skatole, discrepancies between analytically determined levels of both compounds and organoleptic anomalies of boar fat frequently occurred.[45,49,50] In this context, a recently published sensory study revealed the necessity to extend fixed thresholds for androstenone and skatole with a factor related to the interaction of both compounds in the perception of boar taint to estimate the prevalence of tainted samples in the future.[51] However,

fluctuating correlations between androstenone and skatole levels and boar taint led to the assumption that other substances might contribute to the perception of the off-flavour. Namely, the faecal smelling amine indole, as skatole a product of the microbiological breakdown of tryptophan in the large intestine of pigs, the musky smelling boar pheromones 3α-androstenol and 3β-androstenol, both metabolites of phase-I-metabolism of androstenone in the boar's testis, and other C_{19}-Δ16 steroids were discussed as contributors to boar taint.[2,37,52–54] Moreover, phenolic compounds such as *p*-cresol, a product of intestinal tyrosine degradation, and 4-ethylphenol were proposed by Patterson and Ha.[55,56] Rius and Garcia Regueiro could determine high levels of 4-phenyl-3-buten-2-one in back fat samples of boars that were judged as tainted, but with low concentrations of androstenone and skatole. Results of an accompanied sensorial study indicated that the compound was able to promote the perception of androstenone and skatole or to create similar sensory descriptors.[57] Furthermore, short chain or branched fatty acids and their microbiological degradation products were associated with the incidence of boar taint, since these compounds have already been documented to affect meat flavour in various cases. However, recently published results could not explain disagreements between sensory analysis and chemical analysis by fatty acid composition.[58]

While phase-I-metabolites of androstenone have previously been associated with boar taint, no attention was payed to phase-I-metabolites of skatole, although seven products of this metabolism are well-known for more than a decade.[17] As previously mentioned, biogenesis of skatole is located in the large intestine. A part of the produced skatole is excreted via the faeces, while the remaining amount is resorbed through the gut wall into the bloodstream. Via the bloodstream skatole in transported to the liver, where the hydrophobic compound is transformed to more hydrophilic metabolites in two steps. Within phase-I-metabolism, CYP 450 enzymes catalyse several oxidation reactions in order to introduce reactive groups. Diaz identified seven metabolites of skatole in *in vitro* experiments using porcine liver microsomes. Some of these metabolites are transferred to water soluble conjugates by sulphation or glucoronidation in phase-II-reactions to enable excretion via the urine or bile.[8,18,59–61] Interestingly, a compound was identified among these metabolites of skatole that has previously attracted attention as a key compound in off-flavours in white wines and milk products: 2-aminoacetophenone.[62–65] As 2-aminoacetophenone is not transposed in porcine phase-II-metabolism and comparably lipophilic, an accumulation in adipose tissues, analogues to androstenone and skatole, could be assumed. However, no studies regarding

porcine back fat concentrations of this compound have been reported, yet. Typical sensory descriptors of 2-aminoacetophenone are “mothballs” or “leather”.[63]

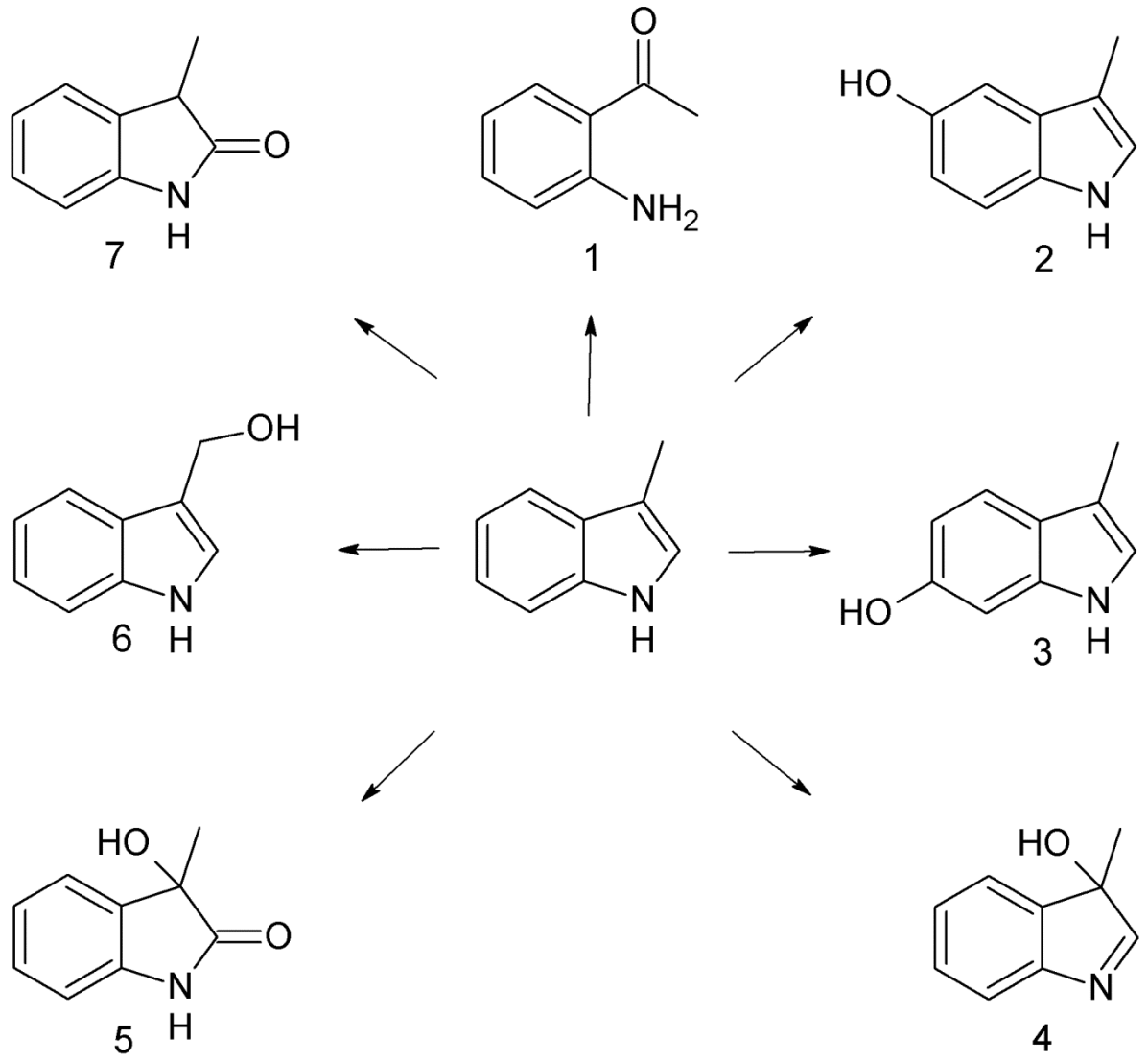

Figure 3 Hepatic phase-I-metabolites of skatole formed in *in vitro* incubations using porcine liver microsomes: 2-aminoacetophenone (1), 6-hydroxyskatole (2), 5-hydroxyskatole (3); 3-hydroxy-3-methylindolenine (4), 3-hydroxy-3methyl-oxindole (5), indole-3-carbinol (6), 3-methyl-oxindole (7).

While exact pathways of the biosynthesis of 2-aminoacetophenone in pigs have not been elucidated yet, many scientific studies have been conducted in terms of CYP 450 skatole metabolism, since the CYP 450 mediated clearance of skatole has great influence on skatole levels in boars. The family of CYP 450s comprises a group of monooxygenases with low substrate specificity known to catalyse oxidizations in collaboration with NADPH. A systematic nomenclature based on genetic relations distinguishes between families, subfamilies and individual isoforms.[66] While CYP 450s occur ubiquitously, highest concentrations can be found in the microsomes of the endoplasmatic reticulum of hepatocytes.

The enzymes belong to the group of haemoproteins, with the prefix “450” referring to a spectrophotometric peak at 450 nm in their reduced state in complex with CO. In contrast, CO complexes of other reduced haemoproteins, *e.g.* haemoglobin, show an absorption maximum at 420 nm, because they comprise histidine as proximal ligand instead of electron rich cysteine.[48,67] Porcine CYP 1A, 2A19, 2C33v4, 2C49, 2E1 and 3A were identified as the major isoforms involved in skatole metabolism. Initially, CYP 2E1 was considered as the main skatole metabolising isoform. Later, Diaz and Squires emphasized the importance of CYP 2A, since skatole levels in pig were more strongly related to CYP 2A levels than to CYP 2E1 levels. Recently, CYP 1A and cofactor CYP B5A were suggested to play a decisive role in the compound’s metabolization.[68] Despite all efforts, there is still confusion about the hepatic clearance of skatole in pigs and the prediction of *in vivo* levels, because various factors, such as breed, genetic variations, feeding and environmental and physiological factors, influence CYP 450 levels. Accordingly, very little is known about in vivo concentrations of skatole metabolites in boars, their formation pathways and possible contributions to boar taint.

1.4 Objective and outline of the study

Thus, the main objectives of the present study were the screening of 2-aminoacetophenone concentrations in boar fat, the elucidation of possible formation pathways and the evaluation of the compound’s possible contribution to the perception of boar taint.

Trace analysis of compounds in complex matrices like fat tend to suffer from undesired matrix effects like insufficient analyte extraction or signal suppression. The application of isotopically labelled internal standards was demonstrated to be the most reliable method to overcome such effects in a stable isotope dilution assay (SIDA).[69–72] In such assays a commonly used internal standard, being only a structural analogue of the target compound with similar physicochemical properties, is replaced by isotopically labelled counterparts of the target compound. In practice, such reference compounds are synthesized by the introduction of naturally scarce isotopes ^{2}H or ^{13}C in defined positions of the desired analyte. In consequence, these labelled standards show higher molecular weights, but almost identical physicochemical properties, such as solubility, polarity and volatility. Furthermore, analysis of fatty matrices bears the danger of contaminations of the analytical system. Thus, sampling out of the headspace above the sample using a fused-silica fibre coated with appropriate adsorbent was another requirement on the quantification method for 2-aminoacetophenone. The sampling technique is commonly referred to as HS-SPME (headspace-solid phase

microextraction).[73] Based on these considerations a screening of 2-aminoacetophenone concentrations in back fat samples of boars was achieved by adapting a published HS-SPME-GC/MS for the analysis of boar taint compounds androstenone, skatole, indole, 3-α-androstenol and 3-β-androstenol.

Average concentrations of 2-aminoacetophenone were found to be 0.1 ppm and arose the question whether this compound could originate exclusively from hepatic skatole metabolism or if other pathways might contribute to the accumulation in adipose tissues. A frequently used technique to investigate product profiles of CYP 450 mediated substrate conversions in toxicological studies is the implementation of liver microsomes in *in vitro* incubations.[68] As the conventionally used high speed centrifugation for the isolation of liver microsomes suffers from the need for expensive centrifuges and time-consuming procedures, calcium aggregation combined with low speed centrifugation was employed for the isolation of porcine liver microsomes.[74] In contrast to similar studies, the focus was put on volatile metabolites of skatole, since a sufficient volatility was considered as a precondition for a possible contribution to the off-flavour boar taint. Thus, a HS-SPME-GC/MS method was elaborated to determine the product profiles of skatole using porcine liver microsomes.

Although high formation rates of 2-aminoacetophenone in these *in vitro* assays served as an explanation for high concentrations in boar fat, exact pathways leading to the compound's biosynthesis in our experiments still remained unclear. The measurement of kinetic isotope effects (KIE) plays a crucial role within the elucidation of reaction mechanisms and biosynthetic pathways. KIEs express changes in reaction rates when at least one atom is replaced by its isotope. The cleavage of a C-H bond, for example, can be revealed by measuring KIEs resulting from different reaction rates at C-H bond versus the analogous C-D bond.[75] However, in terms of enzymatic reactions the interpretation of KIEs must be carried out very carefully, as such reactions represent complex multistep systems and may involve a number of transition states. This is especially valid for the application of microsomal preparations, since these systems represent a complex mixture of enzymatic isoforms instead of individually expressed enzymes. Nevertheless, branched reaction processes, in which more than one product originates from the same substrate, allow the interpretation of KIEs by a concept known as "isotopically sensitive branching". By application of this concept, evidence for a proposed reaction pathway leading to the formation of 2-aminacetophenone in our assays could be demonstrated.[76]

Although the undesired flavour boar taint has been examined from many different angles using various techniques of different scientific disciplines no studies have been conducted employing the concept of "molecular sensory".[77] In this context the final part of this thesis is addressed to the question which compounds in tainted boar fat contribute to the overall aroma of this food and to what extent. Therefore, the concept of gas chromatography-olfactometry (GC-O) combined with mass spectrometric detection was used to investigate aroma distillates of boar fat. The application of GC-O requires a sufficient separation of a complex mixture of volatiles, followed by simultaneous detection at a mass spectrometer and the human nose. Overall, fat from boars, sows and castrated male pigs was analysed using the described methodology. A set of aroma active compounds was identified in all three species, whereas differences were more of quantitative than of qualitative nature.

2 Results and discussion

2.1 Quantification of 2-aminoacetophenone in boar fat

2.1.2 Method requirements

2-Aminoacetophenone is one out of seven identified hepatic phase-I-metabolites of skatole in pigs.[17] So far the compound was never associated with the incidence of boar taint, although it was already identified as a key compound of off-flavours elsewhere. Namely, 2-aminoacetophenone is responsible for the origination of the "untypical aging off-flavour" (UTA) in white wines. If grapes were grown under stress in terms of insufficient water or nitrogen supply, high amounts of the amine caused atypical changes of the bouquet, which were described as "naphthalene-like", "wet wool-like" or "acacia-blossom-like".[62] Studies on the mechanism of 2-aminoacetophenone formation in white wines revealed the oxidative degradation of the phytohormone indole-3-acetic acid to 2-aminoacetophenone via N-formyl-2-aminoacetophenone.[78,79] Furthermore, the compound's key role within the incidence of off-flavours in milk and milk products has been demonstrated, *e.g.* rennet casein, milk protein concentrate, full fat cream.[64,65] Exact formation pathways in these foods remain unclear, although different investigations suggested an oxidative transformation of tryptophan to the amino acid kynurenine as an initial step of 2-aminoacetophenone biosynthesis in milk protein concentrate.[80]

In the light of these findings several methods for the quantification of 2-aminoacetophenone in white wines and milk products were developed throughout the last years. Recently published mass spectrometric methods made use of stably labelled isotopes as internal standards and solid phase microextraction to allow for precise and reliable results.[63,81,82] The use of isotopically labelled standards in flavour research is subsumed under the term "stable isotope dilution assay" (SIDA) and was introduced by Schieberle and Grosch in the late 1980ies.[83] In contrast to commonly used internal standards, being only rough analogues of the target compounds, the implementation of its isotopically labelled counterparts with almost identical physicochemical properties overcomes typical limitations in trace analysis, *e.g.* matrix effects. Usually such standards are synthesized by the introduction of naturally rare isotopes ^{13}C or ^{2}H into the target compounds. While the synthesis of ^{13}C-labelled standards suffers from expensive, time consuming and difficult procedures, the incorporation of ^{2}H can be performed efficiently by simple H/D-exchange reactions or reductive deuteration of appropriate precursors. However, attention has to be paid to the positions in which a target

compound is labelled, since introduction of ^{2}H in α-position to a carbonyl group or attachment to heteroatoms my lead to H/D back exchanges.[84,85] Another drawback for the use of isotopically labelled standards is the natural presence of heavier isotopes, resulting in inevitable overlaps in the mass spectra of analyte and internal standard. Hence, the introduction of at least three deuterium atoms in stable and distinct positions is recommended to provide for a mass increment of at least *m/z* +3.[86] Finally, special emphasis has to be put on the isotopic purity of the labelled standard, as otherwise the internal standard may contribute to the signal of the analyte and therefore cause non-linear calibration curves.[87] Regarding all these requirements, d_3-2-aminoacetophenone was employed as internal standard to quantify 2-aminoacetophenone in boar fat.

To make reliable and statistically significant statements about the distribution of 2-aminoacetophenone in boar fat back fat samples of at least 100 animals should be investigated at least. Hence, regarding the complex matrix fat, the implementation of an automated and clean sampling technique was requested to protect the analytical system from contaminations. A sampling technique already used for 2-aminoacetophenone analysis in white wines, is solid phase microextraction out of the headspace above the samples (HS-SPME). SPME represents a sorbent extraction by exposing a coated fused silica fibre to a gaseous or liquid sample. In contrast to the commonly used solid phase extraction (SPE) the method offers the possibility to completely ban organic solvents. While mainly apolar coated polydimethylsiloxane fibres and polar coated polyacrylate fibres were used at the beginning of SPME fibre applications in flavour analysis, the selection of an appropriate fibre coating represents a main challenge within method optimization, nowadays. In SPME from the headspace, a sample is placed in a sealed vial at a certain temperature till the equilibrium between the sample and the gaseous phase is reached. Subsequently a SPME fibre is exposed to the headspace to achieve equilibrium between the stationary phase and the gaseous phase. The existence of two equilibria bears the advantage to selectively sample target volatiles without extracting large amounts of impurities, since the time to reach the equilibrium between the solid phase and the gas phase is shorter than the time necessary to reach the equilibrium between sample and gas phase, if the concentration of the target compound in the sample is much higher than the concentration of the target compound in the gas phase at equilibrium.[88,89] Following these considerations HS-SPME sampling was another requirement to the quantification method of 2-aminoacetophenone.

2.1.2 The SIDA-HS-SPME-GC/MS procedure

Following the previous considerations a published HS-SPME-GC/MS procedure for the quantification of boar taint compounds was adapted for the determination of 2-aminoacetophenone levels in boar fat.[90] In brief, back fat from the neck region is minced and subsequently heated in a microwave oven in order to separate connective tissue from liquid fat. An aliquot of 500 mg liquid fat is then spiked with a defined level of d_3-2-aminoacetophenone and extracted with 1 mL of methanol. A separation of the resulting fat-methanol emulsion is achieved by centrifugation at -10°C for 10 minutes. Following decantation into a headspace vial, the methanolic supernatant is evaporated to near dryness by a stream of compressed air. Finally the headspace vial is sealed and subjected to HS-SPME-GC/MS measurement. Method calibration was performed by spiking sow fat with defined quantities of 2-aminoacetophenone and the corresponding isotopomer d_3-2-aminoacetophenone. A calibration curve with excellent linearity ($R^2 > 0.99$) was achieved by plotting the peak area ratios (analyte/isotopomer) against the concentration ratios (analyte/isotopomer). As sow fat shows small amounts of the target compound the obtained linear regression was corrected for the genuine amount represented by the x-axis intersection. In terms of method validation several parameters such as the limit of detection (LOD), the limit of quantification (LOQ), accuracy and precision were determined. LOD and LOQ were calculated as the concentration level with a signal to noise ratio of 3:1 and 10:1, respectively. Accuracy and precision were determined by measuring four replicate samples at a low (50 ng/g) and a high (200 ng/g) concentration level within one day (intra-day) and within four days (inter-day). The coefficient of variation (CV) and the relative error (RE) were used as measures for accuracy and precision.

In order to elucidate the potential contribution of 2-aminoacetophenone to the perception of boar taint a screening of 130 back fat samples of boars was conducted employing an adapted HS-SPME-GC/MS procedure. Chromatograms were recorded in full scan mode and subsequently analysed by extracting significant ion traces for the target compound and the isotopomer. The analysis of mass spectra of 2-aminoacetophenone and d_3-2-aminoacetophenone revealed the mass traces *m/z* 135 and *m/z* 138, representing the respective $[M]^+$ fragment of the genuine and the labelled compound, as the only suitable mass traces for calibration and quantification, since the most abundant fragment *m/z* 120 results from a preferred methyl group cleavage and is also present in the spectrum of the partly overlapping internal standard peak. In total, 2-aminoacetophenone concentrations in porcine back fat ranged between 34 ppb and 1178 ppb with a median of 60 ppb and an arithmetic mean of 100

ppb. To evaluate the sensorial relevance of these concentration levels in the context of boar taint origination a determination of the compound's odour threshold in the corresponding matrix was necessary.

2.1.3 Odour threshold determination

A compound's odour threshold is defined as the lowest concentration of an odorant that can reliably be detected by the human nose.[91] Whether the determined 2-aminoacetophenone levels can possibly be perceived by sensitive consumers therefore depends on the substance's odour threshold. Hence, this threshold was determined by a panel familiar with the evaluation of boar taint compounds using a single staircase, triple forced choice paradigm (triangle test).[92] In the single staircase method subjects are asked to identify one divergent stimulus out of three stimuli, the odorant plus two blanks. To that end a stock solution of 2-aminoacetophenone was stepwise diluted in propylene glycol. Subsequently, the dilutions were presented on paper stripes starting with the lowest concentration in the test (9.5×10^{-7} mmol/mL). Concentrations were increased until correct identification was observable in two consecutive trials. In the case of an incorrect answer the staircase was moved one concentration step up. If a correct answer was given the staircase was reversed and concentration levels moved downwards. Finally, the odour threshold was defined as the mean of the last four out of seven reversal points. Although panellists were not trained or preselected according to their olfactory acuity towards 2-aminoacetophenone a comparable odour potential to skatole and androstenone could be revealed. As interactions of an odorant with the food matrix influence the concentration that is present in the air above a food, odour thresholds are matrix depended and must be determined in the matrix that is dominant in a certain food. However, no reports regarding thresholds for the nasal detection of 2-aminoacetophenone in fatty matrices existed. Thus, a putative contribution of 2-aminoacetophenone to the perception of boar taint was further examined by spiking fat with defined quantities of androstenone (3000 ng/g), skatole (250 ng/g) and 2-aminoacetophenone (200 ng/g). In these experiments panellists were as well able to identify 2-aminoacetophenone in sow-fat, which was almost free of skatole and free of androstenone, as they were able to distinguish between boar fat containing all three compounds and boar fat containing androstenone and skatole only. Accordingly, 2-aminoacetophenone affects the odour perception of back fat, even in the presence of high levels of androstenone and skatole.

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2-Aminoacetophenone — A hepatic skatole metabolite as a potential contributor to boar taint

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ABSTRACT

The major objective of the presented study was to evaluate whether the hepatic skatole metabolite 2-aminoacetophenone (2-AAP) is a potential contributor to boar taint, which is an undesired off-flavor in pork. Therefore, backfat samples were screened by HS-SPME–GC/MS revealing a significant accumulation of the hepatic skatole metabolite 2-AAP in boar fat. Subsequently, a stable-isotope dilution assay (SIDA) was elaborated to precisely quantitate 2-AAP in a set of 130 backfat samples. The observed concentrations ranged between 34 ng/g and 1178 ng/g, resulting in a mean value of 100 ng/g. In addition, the odor detection threshold of 2-AAP was evaluated by a trained sensory panel using a single-staircase, triple forced choice paradigm. The determined 2-AAP odor detection threshold is similar to the thresholds of the major boar taint compounds androstenone and skatole. Finally, a sensory evaluation of backfat samples spiked with 2-AAP was performed in a triangle test with untrained testers. Here, the 2-AAP spiked samples were frequently identified as the odd sample independent of their respective androstenone and skatole levels. In conclusion, the hepatic skatole metabolite 2-AAP was identified as a potential contributor to boar taint.

Introduction

Boar taint is known as an unpleasant and offensive off-flavor that impairs the quality of pork. Sensory descriptors such as sweaty, urine- or fecal-like are frequently used to characterize boar taint (Brooks & Pearson, 1989; Dijksterhuis et al., 2000). According to the current state of knowledge, there are two major compounds responsible for boar taint: the boar pheromone androstenone (5α-androst-16-en-3-one) and the indole-related compound skatole (3-methylindole) (Patterson, 1968; Vold, 1970). While androstenone is endogenously synthesized in the boar's testes with the onset of puberty, skatole originates from microbial breakdown of the amino acid tryptophan and is formed in the pig's intestine. Once absorbed and distributed via the blood stream, androstenone and skatole are especially enriched in the fat tissue (Claus, Weiler, & Herzog, 1994). Due to their steady accumulation, androstenone and skatole may reach perceptible concentrations and cause boar taint when sensed during heating or consumption. Backfat concentrations of 1000 ng/g androstenone and 200 ng/g skatole are frequently reported as consumer acceptance thresholds to distinguish between tainted and untainted carcasses (Lundström, Matthews, & Haugen, 2009; Walstra et al., 1999).

As boar taint leads to product rejections and consumer dissatisfaction (Bonneau, Walstra, et al., 2000), the sensory evaluation and chemical analysis of boar taint as well as its formation and reduction in pork have become important tasks for scientists and the pork industry (Frieden, 2013; Haugen, Brunius, & Zamaratskaia, 2012; Meier-Dinkel et al., 2013). Especially, the relationship of a boar taint compound's concentration and the resulting odor impression is of special interest. Several sensory studies revealed a strong positive correlation ($R^2 = 0.53–0.66$) between the concentration of androstenone and skatole and the resulting boar taint perception in backfat samples (Annor-Frempong, 1997; Bejerholm & Barton-Gade, 1992; Lundström et al., 1988). However, trained panelists do frequently judge samples as “tainted” while chemical analysis attests low androstenone and skatole concentrations and vice versa (Bonneau, Kempster, et al., 2000; Bonneau et al., 1992; Mathur et al., 2012; Xue et al., 1996). This discrepancy has led to the assumption that other unknown compounds might contribute to boar taint (Babol, Squires, & Gullett, 1995; Bonneau et al., 1992; Lundström

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et al., 2009; Rius, Hortós, & García Regueiro, 2005; Rius Solé & García Regueiro, 2001). The musky smelling boar pheromones 3α-androstenol and 3β-androstenol as well as the fecal smelling indole, for instance, are known to have minor contribution to the overall perception of boar taint (Bonneau, Kempster, et al., 2000; Xue & Dial, 1997). Moreover, odorants derived from lipid oxidation (e.g. short chain fatty acids, ketones or aldehydes) or intestinal digestion processes (e.g. phenolic compounds) are also suggested by some authors to contribute to boar taint (Rius Solé & García Regueiro, 2001; Rius et al., 2005).

Interestingly, another group of potential boar taint compounds has not yet been investigated: the skatole metabolites. After microbial formation in the intestine, skatole is partly absorbed by the intestinal mucosa. While circulating in the blood stream, the majority of skatole is subject to hepatic CYP2E1 metabolism, resulting in seven known phase-I metabolites (Fig. 1A) (Diaz et al., 1999). Among these, 2-AAP (2) especially attracts immediate attention as a potential boar taint compound, because 2-AAP has already been identified as an off-flavor compound in white wines (Fan, Tsai, & Qian, 2007; Rapp, Versini, & Ullemeyer, 1993; Schmarr, Ganß, Sang, & Potouridis, 2007). Depending on its concentration, aroma descriptors such as "mothballs", "naphthalene", "animal" or "floral" are used to describe the odor of 2-AAP (Fan et al., 2007). These descriptors, in turn, are very similar to those that are frequently used for skatole. Moreover, in vitro studies have revealed that 2-AAP does not underlie any sulfation or glucuronidation in phase-II metabolism (Diaz & Squires, 2003), thus limiting the renal excretion of 2-AAP. In consequence, a certain amount of free 2-AAP will circulate in the blood stream and may accumulate in the fat tissue. To estimate the in vivo partitioning of a boar taint compound between aqueous and lipophilic compartments, the octanol–water partitioning coefficient (K_{ow}) can be used (Fischer, Brinkmann, Elsinghorst, & Wüst, 2012). As skatole and 2-AAP share comparable K_{ow} values (400 vs. 40), one can expect a somewhat lower but still significant tendency for fat accumulation. When accumulated in sufficient concentrations, 2-AAP may then trigger product rejections whenever sensed by the consumer during preparation and consumption of pork.

Thus, the objective of the presented work was the evaluation of 2-AAP as a potential boar taint compound. After a first qualitative analysis of 2-AAP in backfat samples, a novel SIDA–HS-SPME–GC/MS method was developed to precisely quantitate 2-AAP in backfat samples. Finally, the odor detection threshold of 2-AAP was determined and sensory evaluation of backfat samples spiked with 2-AAP was performed.

Materials and methods

Chemicals

All chemicals were obtained from Sigma-Aldrich (Steinheim, Germany), Alfa Aesar (Karlsruhe, Germany) or Steraloids (Newport, USA) in analytical grade. The deuterium labeled internal standards d_3-2-aminoacetophenone (d_3-2-AAP) and d_3-skatole (d_3-SK) were obtained from Dienstleistungszentrum ländlicher Raum (Neustadt/Weinstraße, Germany) or ELFI Analytik (Neufahrn, Germany), respectively.

Animals and samples

Backfat samples from the neck region were collected at slaughter from a group of conventionally fattened intact boars of type Piétrain × Baden-Württemberg Hybrid. The boars were group penned and fed ad libitum until reaching a slaughter weight between 85 kg and 95 kg (around 140 days of age). The fat layer was separated from the skin and meat residues and a piece of approximately 3 × 5 cm pure fat (corresponding to 20–70 g fat, depending on the thickness of the fat layer) was wrapped in aluminum foil, vacuum packed and stored at −20 °C until analysis.

Screening for and quantitation of 2-AAP

Sample preparation and HS-SPME conditions

The screening for 2-AAP in boar backfat samples as well as the subsequent quantitation of 2-AAP were achieved by adapting a previously published SIDA–HS-SPME–GC/MS procedure (Fischer et al., 2011). For sample preparation, backfat samples were thawed, diced and heated for 1.5 min at 700 W in a common household microwave. The remaining connective tissue was separated from the liquid fat by decanting and an aliquot of 500 mg of the warm liquid fat was transferred into a 2 mL plastic cap and filled with 1 mL of methanol for extraction. The sealed cap was immediately mixed for 30 s to give a cloudy emulsion. In order to separate the fat from the methanolic phase, a freezing step was carried out by centrifuging the samples at −10 °C (10 min,

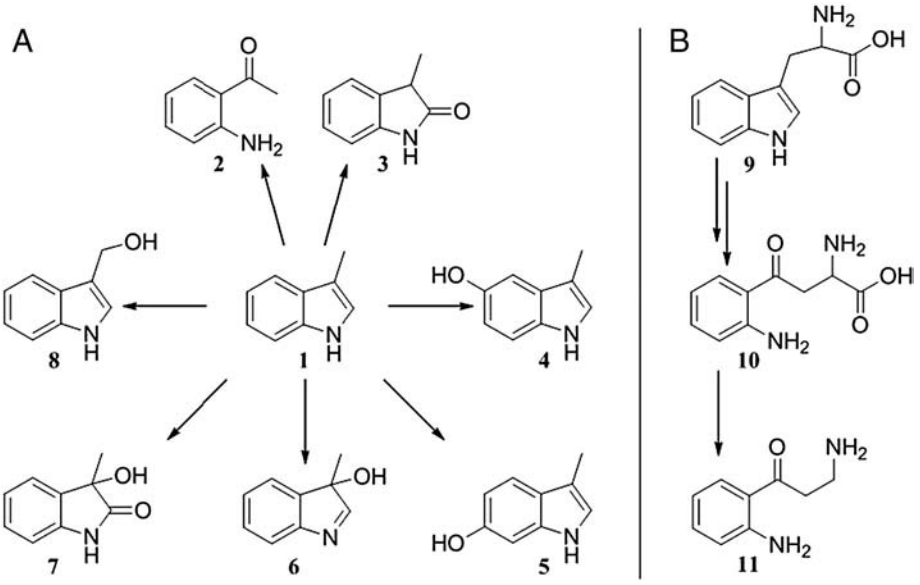

Fig. 1. Hepatic skatole metabolites build during phase-I-metabolism in pigs (A); skatole (1), 2-AAP (2), 3-methyloxindole (3), 5-hydroxyskatole (4), 6-hydroxyskatole (5), 3-hydroxy-3-methylindolenine (6), 3-hydroxy-3-methyloxindole (7), and indole-3-carbinole (8) (Diaz, Skordos, Yost, & Squires, 1999). Intermediates build during hepatic tryptophan metabolism in rats (B); tryptophan (9), kynurenine (10), and kynurenamine (11) (Kaseda, Noguchi, & Kido, 1973).

8500 rpm). Subsequently, the methanolic supernatant was decanted into a 10 mL headspace vial and evaporated to near dryness at 45 °C by a gentle stream of compressed air. Finally the vial was sealed and passed to the automated HS-SPME–GC/MS measurement.

For quantitation of 2-AAP, the described procedure was extended by a spiking step and conducted as follows: after the transfer of 500 mg liquid fat into the plastic cap, the fat was spiked with 50 ng d_3-2-AAP (c = 5.0 μg/mL in MeOH, 10 μL) to achieve a final concentration of 100 ng/g d_3-2-AAP. To allow for standard equilibration, the sealed cap was thoroughly mixed for 30 s and stored for 10 min at 60 °C before adding methanol (1 mL).

Sampling (HS-SPME) was achieved with an automated heating agitator and a polyacrylate coated SPME fiber (PA, 85 μm; Supelco, Bellefonte, USA). Automated sampling conditions were as follows: equilibration for 5 min at 100 °C; extraction for 30 min at 100 °C; and desorption for 20 min within the GC injector at 270 °C.

GC/MS conditions

Gas chromatography and mass spectrometry (GC/MS) 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 to 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, and 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. For both, screening and quantitation, the chromatograms were recorded in full scan mode (*m/z* 50–300) using electron impact ionization (EI). Screening for 2-AAP was performed by extracting the specific mass traces *m/z* 120 + 135 from the obtained full scan chromatograms and comparison of the retention time and mass spectrum of a previously measured authentic sample of 2-AAP (c = 0.01% in MeOH).

For quantitation of 2-AAP, the peak area ratios of analyte and deuterium labeled internal standard (IS) were determined by extracting the specific mass traces of analyte and internal standard, i.e. *m/z* 135 for 2-AAP and *m/z* 138 for d_3-2-AAP. As the intensive mass trace *m/z* 120 occurs in the mass spectra of both, analyte and internal standard, this trace was not considered for quantitation.

Calibration

A four-point matrix calibration was performed in duplicate by spiking sow fat with defined quantities of 2-AAP and its deuterium labeled isotopomer d_3-2-AAP. The added concentration of deuterium labeled d_3-2-AAP was set constant at 100 ng/g for each calibration level. The amount of 2-AAP added to the four calibration levels was variable and as follows: 50, 100, 150 and 250 ng/g. Linear regression was performed after plotting the peak area ratios (2-AAP/d_3-2-AAP) versus the concentration ratios (2-AAP/d_3-2-AAP). As sow fat genuinely contains small amounts of 2-AAP, the obtained linear regression was corrected by the genuine amount which can be obtained from the x-axis intersection of the regression equation.

Validation

As a measure of 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 (Reichenbächer & Einax, 2011). In addition, intra- and inter-day accuracy and precision were determined. For intra-day accuracy and precision, four replicates of a low calibration level (50 ng/g 2-AAP) and four replicates of a high calibration level (200 ng/g 2-AAP) were prepared and subsequently analyzed within one day. Inter-day accuracy and precision were evaluated in the same manner, but analyzing only one replicate of each calibration level per day within four days. The coefficient of variation (C.V.) and the relative error (R.E.) were used as a measure for precision and accuracy of the method.

Correlation between 2-AAP and skatole

To investigate whether the 2-AAP backfat concentrations are correlated to the skatole concentrations, a correlation plot was constructed by plotting the obtained 2-AAP and the previously determined skatole concentrations against each other. Therefore, skatole concentrations were additionally determined in 25 samples using a previously published SIDA–HS-SPME–GC/MS method (Fischer et al., 2011).

In brief, the thawed, skinned and diced backfat was melted in a microwave. After separating from the connective tissue, 500 mg of the melted fat was spiked with 50 ng of the internal standard d_3-skatole (c = 5 μg/mL in MeOH, 10 μL). After equilibration, the liquid fat was extracted with 1 mL methanol. To separate the obtained methanol/fat emulsion, a freezing step was carried out by centrifuging the samples at −10 °C (8500 rpm, 10 min). The obtained methanolic supernatant was then transferred into a headspace vial and evaporated to near dryness. Finally the vial was sealed and passed to the automated HS-SPME–GC/MS measurement. Headspace sampling and the applied GC/MS conditions for backfat analysis were similar to the conditions mentioned in Sections 2.3.1 and 2.3.2. For quantitation the mass traces *m/z* 130 and *m/z* 133 + 134 were used for skatole and d_3-skatole, respectively.

Odor detection threshold determination of 2-AAP

Odorant sample preparation

A stock solution of 2-AAP was prepared in methanol with a concentration of 1 mg/mL. To prepare the odorant dilutions, the stock solution was further diluted in propylene glycol to achieve a concentration of 1.0 mmol/mL (dilution step 0), representing the highest concentration level within the testing procedure. Subsequently, a dilution series (1:1; v/v) was prepared from step 0 (1.0 mmol/mL) to step 20 (9.5×10^{-7} mmol/mL). For preparation of smell strips (240 g/m^2; SSP IDENT, Einbeck, Germany) either the odorant dilution or neat solvent (20 μL) was pipetted on paper strips, which were placed in polystyrene tubes, dried at room temperature for 24 h to visual dryness and sealed before further use.

Threshold testing procedure

Odor detection thresholds were determined by 10 subjects of an experienced panel trained on the evaluation of the boar taint compounds androstenone and skatole in pork fat. Thresholds were determined in two replicates using a single-staircase, triple forced choice paradigm (triangle test) (Hummel, Sekinger, Wolf, Pauli, & Kobal, 1997).

This procedure starts with the presentation of the triangle that includes the lowest odorant concentration, i.e. dilution step 20, and two blanks (propylene glycol only). The subjects were asked to identify the odd sample (odorant strip). Therefore, the experimenter placed the strips for approximately 2 s 1–2 cm in front of the panelists' nostrils. Samples within a triangle were presented only once in an ad hoc randomized order and in a fast sequence (2–3 s). Between each triangle test an interstimulus interval of 10 s was granted to the tester. Subjects were blindfolded to prevent visual identification of the strips. Either two successive correct discriminations of the odd sample or one incorrect assessment triggered a reversal of the staircase paradigm to the next higher or the next lower dilution step, respectively (Fig. 2). The procedure ended as seven reversals were obtained.

Per testing day only one threshold testing was completed per subject. The subject's intra-day individual detection threshold was defined as the mean value of the last four out of seven staircase reversal points (Frasnelli, Livermore, Soiffer, & Hummel, 2002). Subjects' individual thresholds were calculated as the arithmetic mean of the two intra-day individual thresholds. For data analysis the individual thresholds, expressed as the respective dilution step, were used for the calculation of the overall mean, standard deviation, median, minimum and

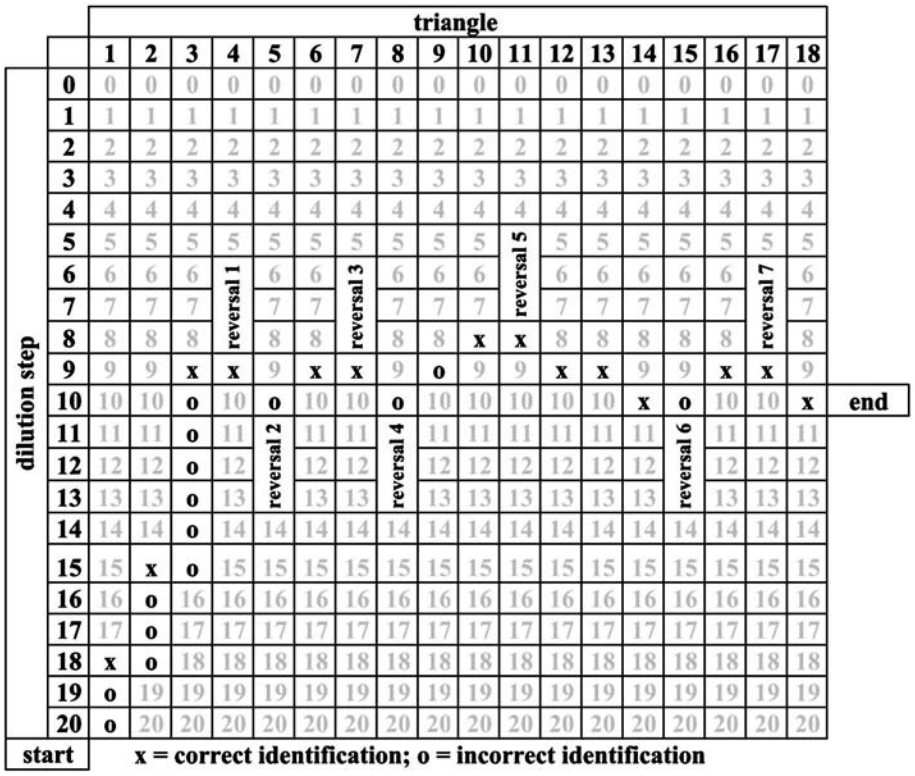

Fig. 2. Filled form of a performed 2-AAP detection threshold test, representing the course of a subject's evaluation during the testing procedure. Testing starts with the lowest concentration, i.e. step 20 and ends after seven reversals.

maximum values of the 10 subjects. In the same manner, odor detection thresholds of skatole and androstenone were determined with the same panelists in two replicates.

Sensory evaluation of 2-AAP in backfat samples

Backfat sample preparation

The backfat samples were heated and separated from the connective tissue as described in Section 2.3.1. An aliquot of 2 g liquid fat was transferred into a 10 mL welted glass with snap-on lid. Methanolic stock solutions of 2-AAP, androstenone and skatole (1 mg/mL each) were diluted with methanol to achieve spiking solutions with the following concentrations: 40 μg/mL 2-AAP, 50 μg/mL skatole and 600 μg/mL androstenone. Depending on the triangle design, 10 μL of the respective spiking solution was added to the liquid fat to achieve the required concentrations. To eliminate any solvent effect on the overall odor perception, spiking volumes were adjusted in each sample by adding neat methanol. After thoroughly mixing for 30 s, the samples were stored for 30 min at 60 °C before presentation.

Triangle testing procedure

The first triangle consisted of two blanks (sow fat) and one odd sample (sow fat spiked with 2-AAP to 200 ng/g). For the second triangle test, the three samples from the first triangle were additionally spiked with high androstenone (3000 ng/g) and skatole (250 ng/g) concentrations. In the third triangle test, two boar fat blanks with high genuine androstenone (3000 ng/g) and skatole (250 ng/g) concentrations were presented along with one boar fat sample that was additionally spiked with 2-AAP (200 ng/g). The testing was performed with 9 untrained subjects (3 males, 6 females) aged between 25 and 55. The testers were asked to identify the odd sample. Repeated sniffing of the samples was granted to the testers within a triangle.

Statistical analysis

Statistical evaluation of the triangle test was performed by using expanded statistical tables for estimating significance of triangle tests (Roessler, Pangborn, Sidel, & Stone, 1978; Stone & Sidel, 2004). Identification of the odd-sample in a triangle test represents a one-tailed ($p = 1/3$) testing procedure.

Results and discussion

In the presented work, boar backfat samples ($n = 10$) were screened for 2-AAP by an established HS-SPME–GC/MS method (Fischer et al., 2011). Stimulated by 100% hits from this initial screening, a stable-isotope dilution assay (SIDA) was developed allowing for the precise quantitation of 2-AAP. A set of 130 backfat samples was subsequently analyzed by the SIDA–HS-SPME–GC/MS method to get a picture of the physiological concentration range of 2-AAP in backfat. A subset of 25 samples was also analyzed for skatole to investigate the correlation between skatole and its metabolite 2-AAP. Finally, the odor detection threshold of 2-AAP was determined by a trained panel using a single-staircase, triple forced choice paradigm (Hummel et al., 1997) and sensory evaluation of backfat samples spiked with 2-AAP was performed by triangle tests with untrained testers.

Screening for and quantitation of 2-AAP

Screening for 2-AAP in backfat samples was achieved by adapting a previously published HS-SPME–GC/MS procedure (Fischer et al., 2011). For this purpose, methanolic fat extracts were obtained from conventionally fattened boars, evaporated to dryness and subsequently extracted by HS-SPME sampling using a polyacrylate SPME fiber. Besides the polyacrylate coating, a DVB/PDMS fiber was also found suitable for 2-AAP extraction, but quantitation measurements revealed that higher precision and accuracy can be obtained with the polyacrylate

coating (data not shown). As HS-SPME sampling affords both extraction and enrichment of volatiles and semi-volatiles from the rather clean gas phase, no further preconcentration or clean-up was necessary. Chromatograms were recorded in full scan mode (Fig. 3A) and subsequently screened for 2-AAP by displaying the specific mass traces, i.e. m/z 120 + 135 (Fig. 3B). To unambiguously identify the 2-AAP peak, a stock solution of authentic 2-AAP was analyzed under the same conditions to obtain its retention time and mass spectrum (Fig. 3C).

In 10 out of the 10 randomly selected boar fat samples, 2-AAP was found with varying peak intensities. This finding prompted us to develop a stable-isotope dilution assay (SIDA) using d_3-2-AAP as internal standard to allow for precise quantitation of 2-AAP in backfat samples. Because the isotopically labeled internal standard d_3-2-AAP with its three-fold deuterium labeled methyl group and the corresponding analyte 2-AAP show almost identical physicochemical properties such as solubility or volatility, the use of such an isotopomer guarantees superior accuracy and precision and thereby delivers reliable results.

The observed regression equation from calibration shows excellent linearity, possessing a coefficient of determination of $R^2 > 0.99$ within the given working range. For quantitation of 2-AAP, chromatograms were recorded in full scan mode (Fig. 4A) and subsequently analyzed by extracting specific mass traces (Fig. 4B + C). However, the formerly used mass traces m/z 120 + 135, albeit being specific for 2-AAP, were unsuitable for calibration/quantitation purposes with the SIDA approach, as the mass trace m/z 120 is also abundant in the mass spectrum of the partly overlapping d_3-2-AAP peak (Fig. 5A + B). The loss of 15 amu (m/z 135 → m/z 120) and 18 amu (m/z 138 → m/z 120) in the mass spectra of 2-AAP and d_3-2-AAP, respectively, reveals the methyl group cleavage ($[M]^+ \rightarrow [M–CH_3/CD_3]^+$) as the preferred fragmentation mechanism (Fig. 5C). As the isotopic labeling is lost upon first fragmentation, the evaluation of the trace chromatograms was performed using the mass traces m/z 135 (2-AAP) and 138 (d_3-2-AAP) only.

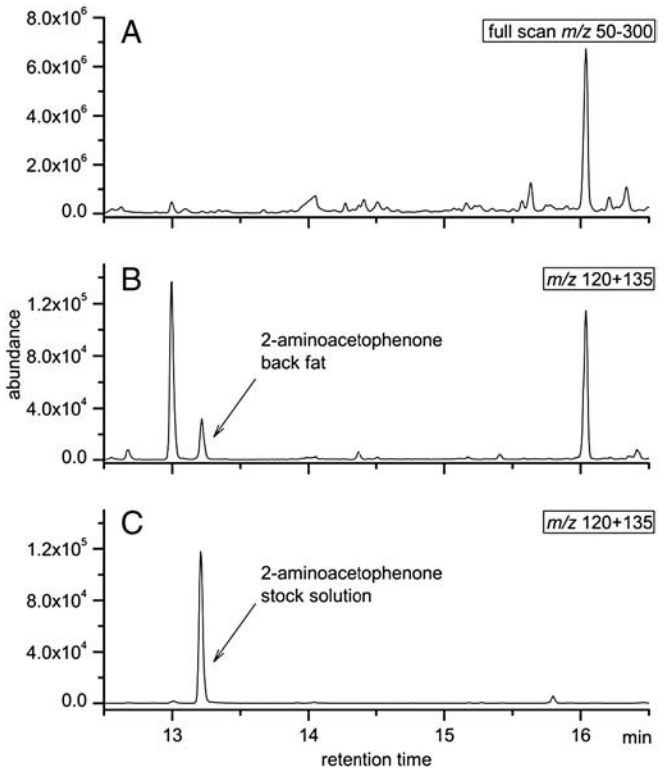

Fig. 3. Screening for 2-AAP in backfat samples was performed by HS-SPME–GC/MS analysis. Extraction of the specific mass traces of 2-AAP from the full scan chromatogram (A) revealed several peaks (B), of which the 2-AAP peak was identified by comparison with the mass spectrum (see also Fig. 5A) and retention time, obtained from the genuine compound (C).

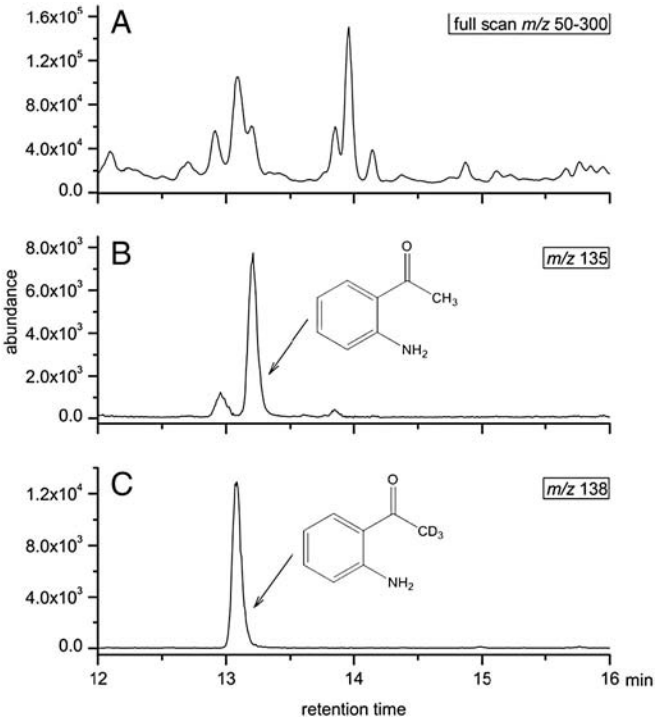

Fig. 4. SIDA–HS-SPME–GC/MS analysis of a backfat sample containing 78 ng/g 2-AAP. Selected ion traces of the full scan chromatogram (A) are displayed for 2-AAP (B) and the internal standard d_3-2-AAP (C).

The limit of quantitation (LOQ) of 2-AAP was reached at 15 ng/g, and the corresponding limit of detection (LOD) was 5 ng/g. Accuracy (R.E.) and precision (C.V.) were determined by measuring four replicate samples at a low (50 ng/g) and a high (200 ng/g) 2-AAP level within one day (intra-day) and within four days (inter-day). Satisfactory accuracy was obtained, ranging between 5% and 8%, while none of the precision values exceeded 10% and hence were satisfactory as well (Table 1).

After successful calibration and validation, a set of 130 backfat samples was analyzed using the new SIDA–HS-SPME–GC/MS method. The obtained 2-AAP concentrations within this set ranged between 34 ng/g and 1178 ng/g (median = 60 ng/g, mean = 100 ng/g). A total of 18% and 7% of the samples exceeded a level of 100 ng/g and 200 ng/g, respectively.

Correlation between 2-AAP and skatole

Although 2-AAP is described as a minor metabolite (0.5% on average) in the hepatic skatole metabolism (Diaz et al., 1999), the performed quantitation study revealed a considerable accumulation of 2-AAP in backfat. This finding raises the question, whether 2-AAP does exclusively derive from hepatic skatole metabolism or not. In this context, a correlation plot was constructed, plotting the measured 2-AAP concentrations against the previously determined skatole concentrations. The weak correlation between both compounds ($R^2 = 0.33$) is an indicator for other processes leading to 2-AAP. One possible pathway might be the kynurenine pathway (Fig. 1B). Kynurenine is a key metabolite in the hepatic tryptophan metabolism and was found to be metabolized to 2-AAP via the intermediate kynurenamine in rat liver extracts (Kaseda et al., 1973). Another pathway might be the intestinal formation of 2-AAP by bacteria. A *Pseudomonas putida* strain was found to degrade skatole to 2-AAP in pig slurry under oxygen limited conditions (Li, Tong, Liu, & Wang, 2010). Accordingly, the observed 2-APP concentrations in backfat might be partly derived from microbial breakdown of skatole and subsequent absorption of 2-AAP via the intestinal mucosa. In addition, enzymatic formation of 2-AAP from indole-3-acetic acid by oxygenases has been described in rat liver extract and also in wine (Frydman, Tomaro,

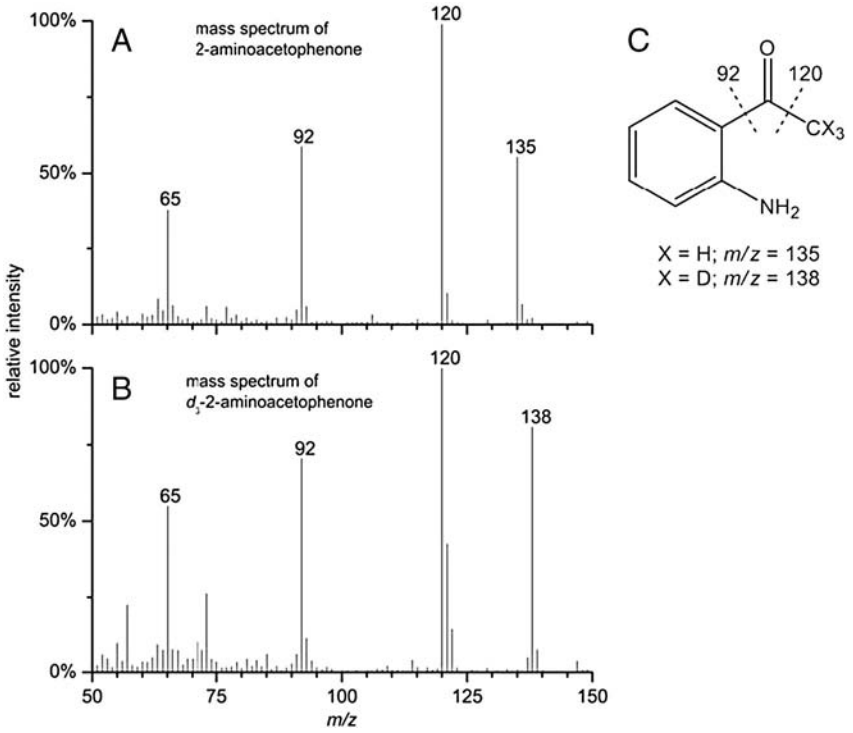

Fig. 5. EI-MS spectra of 2-AAP (A) and d_3-2-AAP (B) and its suggested fragmentation (C).

& Frydman, 1971; Hoenicke, Borchert, Grüning, & Simat, 2002). Indole-3-acetic acid, in turn, is a known intermediate formed during microbial tryptophan breakdown in the pig's intestine (Chung, Anderson, & Fulk, 1975; Whitehead, Price, Drake, & Cotta, 2008). In consequence, 2-AAP may also derive from oxidative degradation of indole-3-acetic acid.

Sensory evaluation

Odor detection thresholds

As summarized in Table 2 the mean threshold values, expressed as the mean dilution steps, are of the same magnitude for all three compounds. The lowest odor threshold was observed for skatole (10.5), followed by androstenone (10.2) and 2-AAP (9.7). A dilution step of 10 corresponds to a concentration of 0.976 μmol/mL of the respective odorant. Due to a spiking volume of 20 μL, a total of 0.01952 μmol 2-AAP is perceptible on the odorant stripe. As can be seen from the standard deviation and the range values (min/max), the highest variation of the individual odor thresholds was observed for 2-AAP. This wide range reveals a strong individual variation in the ability to perceive 2-APP. A maximum value of 13.3, however, still indicates that some subjects are highly sensitive towards 2-AAP. In this context, it should be stated that the panelists were familiar with the evaluation of androstenone and skatole, but not with the evaluation of 2-AAP. In addition, it is noteworthy that the panelists were preselected concerning their olfactory acuity towards androstenone and skatole. Their olfactory acuity was sufficient as the panelist succeeded repeated differentiations of a low concentration of androstenone/skatole (dilution step 9/dilution step 7) in triangle tests. Preselection for their olfactory acuity towards 2-AAP was not performed. In summary, the results from the odor threshold testing reveal an odor potential of 2-AAP, which is comparable to the potential of androstenone and skatole.

Backfat evaluation

Odor detection thresholds were obtained for neat 2-AAP dissolved in an odorless solvent (propylene glycol). To test whether 2-AAP could also be detected in its native matrix, differently spiked backfat samples were evaluated by an untrained panel (n = 9) in a triangle test. Whenever 2-AAP was added, its concentration was set to 200 ng/g as the previous quantitation had revealed a total of 7% of the measured samples above this value, being the group of boars with high physiological 2-AAP concentrations. The first triangle presented to the testers consisted of two blanks containing pure sow fat and one odd sample containing sow fat spiked with 2-AAP. As sow fat is generally free of androstenone and almost free of skatole, the first triangle was designed to evaluate whether 2-AAP was detectable in the absence of these major boar taint compounds. In this first test, 8 out of the 9 assessors identified the 2-AAP spiked sample as the odd sample. The next triangle aimed

Table 1
Intra- and inter-day accuracy (R.E.) and precision (C.V.) for the SIDA–HS-SPME–GC/MS determination of samples spiked with low and high concentrations of 2-AAP. (Values were obtained using a polyacrylate SPME fiber).

Analyte	Added (ng/g)	Found (ng/g) (mean ± S.D.)	C.V. (%)	R.E. (%)
Intra-day (n = 4)				
2-AAP	50	54 ± 3	6.4	8.0
	200	207 ± 7	3.6	6.5
Inter-day (n = 4)				
2-AAP	50	53 ± 5	9.9	6.0
	200	210 ± 15	7.3	5.0

S.D. = standard deviation; C.V. = coefficient of variation; R.E. = relative error.

Table 2
Summary of the odor detection threshold testing for 2-AAP, androstenone and skatole performed with 10 subjects.

Odorant	2-AAP[a]	Androstenone[a]	Skatole[a]
Mean	9.7	10.2	10.5
Median	10.4	10.6	10.6
Standard deviation	2.9	2.3	1.3
Min	5.2	4.4	8.3
Max	13.3	12.3	12.5

[a] Expressed in dilution steps (from 0 = 1 mmol/mL to 20 = 9.5×10^{-7} mmol/mL); serial dilution: 1:1, v/v.

on the influence of 2-AAP on the overall odor perception in the presence of androstenone and skatole. Therefore, all three samples from the first triangle were additionally spiked with high androstenone (3000 ng/g) and skatole (250 ng/g) concentrations. This time, 7 out of the 9 testers still identified the 2-AAP enriched sample as the odd one, although the major boar taint compounds, androstenone and skatole, were present above their frequently given consumer acceptance level of 1000 ng/g and 200 ng/g, respectively (Lundström et al., 2009; Walstra et al., 1999). In the last triangle, native boar fat with naturally high androstenone (3000 ng/g) and skatole (250 ng/g) concentrations was used to overcome possible odor-influencing differences in the fat composition between sow fat and boar fat. Two boar fat blanks were presented along with one 2-AAP spiked boar fat sample. Again, 7 out of the 9 testers judged the 2-AAP spiked sample as the odd one. According to Roessler et al. a minimum number of 6 correct judgments within 9 trails already establish significance at a probability of 0.05, while 7 correct judgments give higher significance at 0.04. A number of 8 correct judgments give significance at a probability of 0.005 (Roessler et al., 1978; Stone & Sidel, 2004).

Conclusion

A considerable accumulation of 2-AAP in backfat was observed, but a weak correlation between 2-AAP and its suggested precursor skatole was observed. It appears reasonable, that 2-AAP is additionally formed by other pathways than the phase-I metabolism of skatole. Sensory evaluation of 2-AAP revealed an odor detection threshold comparable to the detection thresholds of androstenone and skatole. Moreover, 2-AAP was found to affect the odor perception of backfat, even in the presence of high androstenone and skatole concentrations. These simple tests with untrained testers in combination with the determined low odor detection threshold demonstrate the potential of 2-AAP as a contributor to boar taint. Therefore, 2-AAP should be investigated in future studies aiming on the evaluation of boar taint. Research on the fate of other skatole metabolites and a detailed sensory evaluation of backfat extracts by GC-O will be presented in due course.

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2.2 2-Aminoacetophenone formation by porcine CYP 450 enzymes

2.2.1 Regulation mechanisms of porcine CYP 450 enzymes

Although the decisive role of skatole within the origination of boar taint was already demonstrated, little was known about the metabolic fate of the amine in pigs twenty years ago till Diaz and co-workers identified seven metabolites in *in vitro* incubations using porcine liver microsomes.[17] These metabolites included a compound that already attracted attention as a key substance of off-flavours in white wines and milk, 2-aminoacetophenone.[62,64,65,81] However, as only small amounts of 2-aminoacetophenone were determined, the compound was considered to be a minor product of hepatic skatole conversion in pigs. This assumption was later on confirmed by Wiercinska and co-workers in incubation experiments with individually expressed porcine CYP 450 isoforms.[93] However, 2-aminoacetophenone is not transformed to water soluble conjugates in the context of porcine phase-II-metabolism and could therefore accumulate in adipose tissues.[61] Fischer et al. suggested the octanol water partitioning coefficient as a measure for a compound's tendency to accumulate in adipose tissue.[94] Accordingly, an accumulation of the hepatic metabolite is conceivable, as 2-aminoacetophenone and skatole show comparable values (40 vs 400). This assumption is in accordance with the average concentrations of 2-aminoacetophenone in back fat samples of boars that were reported in the previous chapter. However, back fat concentrations of 100 ng/g raised the question for alternative biosynthetic routes of 2-aminoacetophenone formation, as the reported formation rates for this compound were comparably low.[17,93,95]

To the author's best knowledge four biosynthetic routes of 2-aminoacetophenone formation have been reported, yet. Kaseda and co-workers revealed the formation of 2-aminoacetophenone by enzymatic decarboxylation of the amino acid kynurenine in rat liver.[96] In white wines, in which the substance causes the off-flavour "UTA", a formation by oxidative degradation of indole acetic acid was demonstrated.[78,79] In swine manure the microbial breakdown of skatole by pseudomonas putida leads to 2-aminoacetophenone.[97] Finally, the compound was demonstrated to be one out of seven products of skatole conversion by porcine CYP 450 monooxygenases as already mentioned above. Thus, the CYP 450 mediated hepatic clearance of this boar taint agent has direct impact on the accumulation rates of 2-aminoacetophenone in adipose tissues. According to the current state of knowledge, the expression of porcine CYP 450 monooxygenases is regulated by three different processes, namely mRNA expression, protein expression and activity of individual CYP 450 isoforms itself.[68] In this context the CYP 450 isoforms demonstrated to be involved in the hepatic

clearance of skatole in pigs are recalled: CYP 1A, 2A, 2A19, 2C33v4, 2C49 and 2E1.[61,93,95,98,99]

The mRNA expression of these enzymes in pigs is mainly regulated by three receptors that initiate gene expression in the cell nucleus: The Aryl hydrocarbon receptor (AhR), the Constitutive androstane receptor (CAR) and the Pregnane X receptor (PXR). Upon activation by ligand binding in the cytosol, the receptors translocate into the nucleus where they interact with response elements of the DNA. This interaction initiates the gene transcription that finally leads to the gene expression of CYP 450s. Interestingly, skatole itself downregulates the activity of CAR and PXR, while studies using human bronchial epithelium cells could show an upregulation of AhR leading to increased CYP 1A1 expression.[100–103]

Following the central dogma of biology, protein expression is positively correlated to mRNA expression. However, factors like mRNA turnover or protein stabilization influence the amounts of expressed protein that are effectively measurable. In terms of skatole metabolizing CYP 450s literature reports could demonstrate an upregulation of the protein expression of CYP 2E1 by skatole in a time- and dose-dependent manner.[18] However, this effect was eliminated by co-treatment with androstenone. Other androgens like testosterone are also suspected to hamper the protein expression of individual isoforms like CYP 1A. However, this effect is breed-dependent.[104,105]

A crucial point within the investigation of the regulation of porcine CYP 450s is the determination of catalytic activities of individual isoforms. Usually activities are estimated by measuring the metabolization rates of particular substrates in model reactions. The activities of CYP 1A1 and CYP 2A1, for example, are calculated as rates of Etoxyresorufin deethylation (EROD) or Methoxyresorufin demethylation (MROD), respectively.[106] As some of these model reactions were only validated using human CYP 450s and as factors like cross activities, solvents, or reaction conditions can influence the results, the estimation of porcine CYP 450 activities still suffers from the lack of appropriate and harmonised methodologies.[107,108]

Nevertheless, results of previous studies have proven the influence of hormonal status and diet of pigs on CYP 450 regulation. The capability of testicular steroids to downregulate protein expressions of CYP 450s was already mentioned, whereby this effect was shown to be breed-dependent.[104,105,109,110] Additionally, bioactive components of the pig's diet can also effect the regulation of specific enzymes. Increased expression and activity of CYP 1A and

CYP 2A after administration of chicory root are based on an increased mRNA expression induced by secondary metabolites of chicory.[111] In addition, the influence of some flavonoids on the catalytic activities of porcine CYP 450s could be demonstrated, *e.g.* CYP 1A1, 1A2, 2E1.[112]

In summary, the regulation mechanisms of porcine CYP 450s reflect the different levels on which the enzymatic activities of single skatole metabolizing isoforms can be influenced by various factors, *i.e.* genetic variation, environmental or physiological factors. Thus, quantitative results for the hepatic skatole clearance in pigs must not be simply transferred from one breed to another. In our previous study we addressed the question of back fat levels of the porcine hepatic skatole metabolite 2-aminoacetophenone. The investigated boars were of Pietrain × Baden-Württemberg hybrid type, since these animals play an important role in German breeding programs. To the author's best knowledge the hepatic skatole clearance of this breed has not been investigated, yet. Accordingly the aim of the reported study was the determination of the metabolomic profile of skatole under *in vitro* conditions using microsomal preparations of Pietrain × Baden Württemberg hybrid boars. In order to contribute to the perception of boar taint a compound must exhibit sufficient volatility to interact with the human nasal receptors. This is why we have exclusively put our focus on the volatile metabolites of skatole, while similar studies aimed at the identification and quantification of the full metabolome and all involved isoforms. In consequence the formed metabolites were sampled directly out of the headspace above incubation mixtures using a SPME-fibre. To allow for precise quantification of 2-aminoacetophenone the analysis was conducted by stable isotope dilution analysis (SIDA) using gas chromatography-mass spectrometry.

In order to minimize variations of our results 12 liver samples of boars that were raised and fattened under harmonized conditions, were investigated.[113] All samples were taken at the slaughter line in a German abattoir at a weight of approximately 90 kg, subsequently shock frozen using liquid nitrogen and kept at -80 °C till the day of analysis. To elucidate possible differences between tainted and untainted boars nine out of twelve boars were chosen according to elevated back fat concentrations of androstenone and skatole (> 2000 ng/g androstenone, > 300 ng/g skatole), while the three remaining animals showed concentrations of both substances below postulated sensory thresholds.[114]

2.2.2 Isolation of microsomes

The isolation of microsomes from human or porcine tissues is usually realized by application of high speed centrifugation methods. However, recent publications focused on the preparation of porcine liver microsomes could demonstrate the possibility to replace this cost-, time- and material-consuming procedure by a calcium aggregation method without significant losses of isolated enzyme concentrations. However activities of specific CYP 450 isoforms were shown to be reduced when compared to high speed centrifugation, most likely due to enzyme inhibition by calcium.[74] Nevertheless we could identify MROD and EROD activities comparable to literature reports.[106] Additionally, overall CYP 450 concentrations comparable to published data could be determined by recording CO difference spectra.[67,95]

2.2.3 Microsomal incubations

Microsomal incubations were performed in ten mL Headspace-Vials, so that formed skatole metabolites could be sampled directly out of the headspace above the samples. In detail, microsomal protein was suspended in sodium phosphate buffer (50 mM sodium phosphate, pH 7.4, 5 mM magnesium chloride, 1 mM EDTA) and incubated with skatole and co-enzyme NADPH at 37 °C in sealed vials. By cooling to -20 °C enzymatic reactions were stopped and immediately prior to GC/MS analysis the isotopically labeled internal standard was added. To investigate putative concentration- or time-dependent CYP 450 activity reaction mixtures were incubated for different periods of time and with different concentrations of skatole.

To guarantee for reliable results three types of negative controls were implemented next to quality control samples (QC). To proof that the formation of reaction products is based on enzymatic activity, microsomal protein and buffer were kept at 90 °C for five minutes prior to incubation. This type of negative control is termed as boiled blank. In addition, the involvement of NADPH dependent CYP 450 should be verified by omission of NADPH in incubation mixtures. Finally, to demonstrate that product formation arises solely from skatole conversion, incubations were conducted without addition of skatole. QC samples were produced by spiking boiled blanks with defined quantities of 2-aminoacetophenone, 3-methyloxindole and indole-3-carbinol.

2.2.4 Quantification of skatole metabolites

With respect to 2-aminoacetophenone a five point calibration was performed in triplicate by spiking sodium phosphate buffer (50 mM, pH 7.4) with defined quantities of 2-aminoacetophenone and d_3-2-aminoacetophenone. The spiked calibration standards were subsequently treated as described before and analyzed by SIDA-HS-GC/MS. A linear

calibration curve showing excellent linearity ($R^2 > 0.99$) was obtained by plotting the peak area ratios (analyte/isotopomer) against the concentration ratios (analyte/isotopomer). In the cases of 3-methyloxindole and indole-3-carbinol, response factors were recorded by spiking sodium phosphate buffer with the equal concentrations of the analytes and d_3-2-aminoacetophenone in a concentration range of 1-1.5 µg/mL.

2.2.5 Metabolomic profile of microsomal incubations

In total, three volatile metabolites of skatole could be identified in *in vitro* assays using hepatic porcine microsomes: 2-aminoacetophenone, 3-methyloxindole and indole-3-carbinol. The analysis of negative controls indicated that the formation of all three metabolites rests upon enzymatic conversion of skatole by NADPH dependent enzymes. In order to produce comparable results the amounts of each metabolite were normalized with respect to incubation duration and protein content. Accordingly, formation rates for each metabolite are expressed as pmol of analyte formed in a certain incubation time in an incubation mixture containing a certain amount of protein (pmol/min/mg). Highest formation rates were determined for 2-aminoacetophenone, followed by 3-methyloxindole and indole-3-carbinol. Taking into account the amount of skatole that was actually converted by CYP 450 enzymes approximately 50 % of the formed metabolites were not identified, most likely because of being non-volatile. In consequence these metabolites will probably not contribute to the perception of boar taint and were no objects of the presented study.

When comparing the formation rates of the three metabolites in tainted and untainted boars significantly lower values were observable for 2-aminoacetophenone in the latter, while no significant differences could be determined for 3-methyloxindole and indole-3-carbinol. This data can serve as an explanation for high concentrations of 2-aminoacetophenone found in back fat samples of Pietrain x Baden-Württemberg hybrid boars (see publication *Food Research Int*., chapter 1). However, these results are inconsistent with a weak correlation between back fat concentrations of 2-aminoacetophenone and skatole that initially raised the question for alternative routes of the compound's biosynthesis in pigs. This inconsistency could otherwise indicate that a simple correlation plot of back fat levels is inappropriate to elucidate coherences of *in vivo* reactions mediated by CYP 450s. Obviously, individual effects such as intestinal absorption, fat composition, CYP 450 concentration and activity will influence skatole and 2-aminoacetophenone levels. Additionally, a closer look on the chemical structures of skatole and 2-aminoacetophenone reveals that the formation of the latter compound requires different steps, *i.e.* oxidation, ring-opening and deformylation. Thus,

the involvement of more than one CYP 450 isoform in a multi-step reaction seems likely. This assumption is supported by the results of inhibition experiments using the specific CYP 1A inhibitor α-naphtoflavone. In microsomal incubations pretreated with this inhibitor the 2-aminoacetophenone formation declined by approximately 90% indicating a substantial involvement of CYP 1A in our experiments. However, similar studies investigating the conversion of skatole by porcine CYP 450s reported a minor role of this isoform within 2-aminoacetophenone formation.[93] Furthermore, female pigs, which show only low back fat concentrations of 2-aminoacetophenone, show even higher concentrations of CYP 1A and CYP 2A together with increased EROD activities compared to boars.[115] A substantial role of CYP 1A within the hepatic formation of 2-aminoacetophenone in pigs should therefore lead to even higher levels of this compound in female pigs. Otherwise, these results underline the assumption that other isoforms could be involved in a multistep reaction leading to 2-aminoacetophenone formation in the present *in vitro* experiments.

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2-Aminoacetophenone Is the Main Volatile Phase I Skatole Metabolite in Pietrain × Baden-Württemberg Hybrid Type Boars

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ABSTRACT: Skatole metabolites have been considered as putative contributors to boar taint. Recently, 2-aminoacetophenone, a volatile phase I skatole metabolite, was identified in back fat samples from boars of Pietrain × Baden-Württemberg hybrid type. This paper addresses the question of the physiological origin of the observed 2-aminoacetophenone in these pigs. Microsomal fractions from nine boars were isolated, and formation of skatole metabolites was subsequently analyzed by stable-isotope dilution analysis (SIDA) using headspace solid-phase microextraction gas chromatography−mass spectrometry (HS-SPME/GC-MS). Significant breed-related differences in phase I skatole metabolism were observed, explaining the high levels of 2-aminoacetophenone in Pietrain × Baden-Württemberg hybrid type boars.

KEYWORDS: *SIDA, HS-SPME/GC-MS, skatole, microsomes, metabolism*

INTRODUCTION

Boar taint is a well-known offensive off-flavor that negatively affects the quality of meat from boars.[1] For decades, surgical castration of male piglets was the common procedure within Europe and other countries to prevent the occurrence of boar taint.[2] However, as castration without anesthesia causes serious pain and distress to the animals, several European countries decided to voluntarily ban this practice.[3] Reasonable alternatives to castration currently considered are either the rearing of entire male pigs or the vaccination with antibodies against a neuropeptide that itself regulates puberty.[4] In addition to improved animal welfare the abandonment of painful surgical castration offers also economic benefits: entire boars show a higher nutrient uptake efficiency and superior meat quality when compared to castrates.[5,6] Yet, high numbers of boars within the food chain bear the risk of tainted carcasses with expected quotas ranging from 6 to 44%.[7] As the occurrence of tainted meat will lead to considerable consumer rejections, boar taint appears as an important problem in the production of fresh pork meat.

On the basis of today's knowledge, two compounds are mainly responsible for the occurrence of boar taint: the boar pheromone androstenone (5α-androst-16-en-3-one) and the aromatic amine skatole (3-methylindole).[1] Whereas androstenone is synthesized in the boar's testes with the onset of puberty, skatole originates from the microbiological breakdown of tryptophan in the pig's intestine. After resorption and distribution via the bloodstream, androstenone and skatole are enriched in fat tissue due to their lipophilic character.[8] As a steady accumulation takes place, both boar taint compounds can reach perceptible concentrations, causing consumer rejections when sensed during heating and consumption of pork meat. Androstenone concentrations of 3000 ng/g liquid fat and skatole concentrations of 200 ng/g liquid fat have recently been reported as thresholds for consumer acceptance of tainted meat.[9]

Although trained sensory panels demonstrate a strong correlation between the perception of boar taint and high levels of skatole and androstenone, occasionally samples with low concentrations of both substances are judged as tainted.[10,11] These observations suggest that further compounds may contribute to the overall aroma of boar taint.[11−14] As such, 3-α-androstenol and 3-β-androstenol, the reduced forms of the ketone androstenone, and the aromatic amine indole were shown to have minor impact on boar taint perception.[6,15] Frequently, products of lipid oxidation, such as aldehydes, ketones, or short-chain fatty acids, are considered as additional contributors to the off-flavor. Some authors also suggest boar taint compounds derived from intestinal digestion processes, fpr example, *p*-cresol from tyrosine degradation.[13,14,16]

Recently, we have focused on skatole phase I metabolites as another group of putative boar taint contributors.[17] According to literature reports, hepatic clearance of skatole in pigs is mainly mediated by cytochrome P450 enzymes (CYPs, the term P450 relating to their reduced complex with carbon monoxide giving an absorption maximum at 450 nm).[18,19] CYPs comprise a diverse group of monooxygenases heavily involved in phase I metabolism because of their ability to oxidize a wide variety of substrates. A systematic nomenclature based on genetic relation distinguishes between families

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(CYP1), subfamilies (CYP1A), and individual isoforms (CYP1A1).[20]

Although CYP2E1 was initially considered to play a major role in porcine hepatic skatole clearance, recent studies have also emphasized the role of CYP1A and CYP2A.[21] Diaz et al. were the first to identify seven major skatole metabolites through in vitro assays with liver microsomes (artificial, vesicle-like fragments of the endoplasmic reticulum containing high CYP amounts).[22] These liver microsomes originated from pigs that were obtained by backcrossing F3 European Wild Pig × Swedish Yorkshire boars with Swedish Yorkshire sows. These metabolites included one compound that had already been identified as a key compound of an off-flavor in white wines: 2-aminoacetophenone.[23−25] Depending on its concentration, aroma descriptors similar to skatole are attributed to 2-aminoacetophenone such as "mothballs" or "leather".[23] Interestingly, 2-aminoacetophenone is neither sulfated nor glucuronidated during porcine phase II metabolism and may therefore accumulate in fat tissue.[26] Considering the octanol−water partitioning coefficient (K_{OW}) to estimate the in vivo partitioning between aqueous and lipophilic compartments, a tendency for fat accumulation is expected as 2-aminoacetophenone and skatole share comparable K_{OW} values (40 vs 400).[27,28] Moreover, we have detected substantial amounts of 2-aminoacetophenone in back fat samples of boars with a comparable odor potential to that of skatole and androstenone.[17]

Several other in vitro studies have investigated the influence of specific CYP isoforms on skatole clearance and resulting product profiles. However, in all studies conducted, 2-aminoacetophenone, if identified at all, was found to be a minor product of skatole conversion by porcine CYPs.[29−31] Although various factors (feeding, castration, breed) might influence expression levels of individual CYP isoforms and thus metabolism,[21,32−34] it has not been clarified why high amounts of 2-aminoacetophenone can be observed in back fat samples of boars, especially as only a small percentage of skatole is converted into this specific metabolite according to previous studies.

To further elucidate the contribution of 2-aminoacetophenone and other porcine skatole phase I metabolites to boar taint, we evaluated the volatile products formed by porcine liver microsomes. Microsomal fractions from nine pigs of Pietrain × Baden-Württemberg hybrid type were isolated, and formation of skatole metabolites was analyzed by stable-isotope dilution analysis (SIDA) using headspace solid-phase microextraction gas chromatography−mass spectrometry (HS-SPME/GC-MS).

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma-Aldrich (Steinheim, Germany), Alfa Aesar (Karlsruhe, Germany), VWR (Darmstadt, Germany), abcr (Karlsruhe, Germany), or Carl Roth (Karlsruhe, Germany). The deuterium-labeled internal standards d_3-skatole and d_3-2-aminoacetophenone were from ELFI Analytik (Neufahrn, Germany) or DLR (Neustadt/Weinstraße, Germany), respectively.

Preparation of Liver Microsomes. Liver samples from 12 pigs of type Pietrain × Baden-Württemberg hybrid were obtained from the Institute of Animal Science (University of Bonn, Germany). These animals were chosen according to elevated back fat levels of skatole and androstenone (skatole > 300 ng/g, androstenone > 2000 ng/g) as determined by a reported GC-MS method.[35] Additionally, three liver samples from boars with back fat levels of skatole and androstenone below the postulated sensory thresholds were analyzed. All samples were shock-frozen using liquid nitrogen after slaughter at a weight of 90 kg (around 140 days of age) and kept at −80 °C prior to analysis.

Preparation of microsomes was achieved following a reported procedure.[36] Briefly, porcine liver tissue (4 g) was finely minced in Tris−sucrose buffer (12 mL, 10 mM Tris-HCl, 250 mM sucrose, pH 7.4) using an Ultra-Turrax homogenizer (IKA Labortechnik, Staufen, Germany) while ice-cooling to prevent protein denaturation. Subsequently, the homogenate was centrifuged (10 min, 8000*g*, 4 °C), and the resulting supernatant was decanted and diluted to a total volume of 30 mL by addition of precipitation buffer (10 mM Tris-HCl, 250 mM sucrose, 8 mM calcium chloride, pH 7.4). After another centrifugation step (30 min, at 18000*g*, 4 °C) the resulting pellets were suspended again in Tris−sucrose buffer (0.25 M Tris-HCl, 1 mM EDTA, 20% glycerol, pH 7.4) to a final concentration of 15 mg protein/mL. Protein concentrations were determined using a commercially available Bradford assay purchased from Bio-Rad (Hercules, CA, USA).

CYP Activity and Inhibition Assays. Overall CYP content of the prepared liver microsomes was evaluated by recording carbon monoxide difference spectra using a Varian Cary 100 spectrophotometer (Darmstadt, Germany).[19] Additionally, catalytic activities of individual CYP isoforms were estimated from the metabolism rates of specific substrates. In particular, *O*-demethylation of 7-methoxyresorufin (MROD) and *O*-deethylation of 7-ethoxyresorufin (EROD) were measured as markers for CYP1A1 and CYP1A2 activity, respectively. The product of both reactions, resorufin, was quantitated following a reported HPLC procedure.[37] Chromatographic separation was achieved using a Gemini-NX column (4 μm, 250 × 3 mm, Phenomenex, Aschaffenburg, Germany) and an isocratic mobile phase consisting of 52% sodium phosphate buffer (20 mM, pH 6.8), 45% methanol, and 3% acetonitrile with a flow of 0.8 mL/min at 20 °C. The HPLC system was equipped with a pump (ProStar 210, Varian), an autosampler (ProStar 210, Varian), and a fluorescence detector (excitation, 560 nm; emission, 586 nm; LaChrom Elite, Hitachi, San Jose, CA, USA).

To calculate the individual contribution of CYP1A isoforms to the overall biotransformation of skatole, inhibition experiments were carried out adapting a reported methodology.[37] Briefly, microsomal protein (2 mg) was suspended in buffer (0.5 mL, 50 mM sodium phosphate, pH 7.4, 5 mM magnesium chloride, 1 mM EDTA) and pre-incubated with NADPH (4 mM) and α-naphthoflavone (0.02 mM, a CYP1A specific inhibitor) in 10 mL headspace vials at 37 °C for 10 min. Afterward, skatole (4 μM) was added, the reaction mixture was again incubated at 37 °C, and samples were drawn at different time points (30, 60, 90 min). After the addition of the internal standard (d_3-2-aminoacetophenone, 1.5 μg/mL), HS-SPME/GC-MS analysis was carried out as described below.

Microsomal Incubations. Microsomal protein (1 mg) was suspended in buffer (0.5 mL, 50 mM sodium phosphate, pH 7.4, 5 mM magnesium chloride, 1 mM EDTA) and pre-incubated with skatole (4 μM) for 3 min in 10 mL headspace vials at 37 °C. Afterward, NADPH (4 mM) was added, and the mixture was incubated at 37 °C for defined periods of time (30, 60, 90, 120, 150, 180 min). Incubation was stopped by cooling to −20 °C prior to GC-MS analysis. To investigate a putative concentration-depended CYP activity, higher concentrations of skatole were used as well (40 μM and 0.4 mM). All experiments were carried out in duplicate, and three types of negative controls were implemented, where (i) protein inactivation was achieved by heating mixtures of microsomal protein and buffer to 90 °C for 5 min before the actual incubation experiment or where (ii) CYP activity was suppressed by omission of the reducing agent NADPH or where (iii) skatole as the substrate of enzymatic conversion was omitted. Prior to final HS-SPME/GC-MS analysis, the internal standards d_3-skatole and d_3-2-aminoacetophenone were added to all samples at final concentrations of 0.1 and 1.5 μg/mL, respectively. QC samples with defined quantities of 2-aminoacetophenone, 2-methyloxindole, and indole-3-carbinol were used throughout the study to ensure reliable results. For this purpose incubation mixtures that had been previously heated to achieve protein

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inactivation were spiked with the target compounds and d_3-2-aminoacetophenone.

HS-SPME/GC-MS. HS-SPME sampling was achieved using an automated heating agitator and a divinylbenzene/polydimethylsiloxane coated SPME fiber (DVB/PDMS, 85 μm, Supelco, Bellefonte, PA, USA) applying the following sampling conditions: equilibration for 5 min at 55 °C, extraction for 10 min at 55 °C, desorption for 10 min at 270 °C within the GC injector. GC-MS analysis was performed 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. The carrier gas flow (helium) was set to 1 mL/min, and the temperature program was set as follows: start at 40 °C, hold for 3 min, then raise to 230 °C at a heating rate of 8 °C/min. The injector temperature was kept at 270 °C, and splitless injection was carried out for 3 min before the split valve was opened, resulting in a split ratio of 1:100. Chromatograms were recorded in full scan mode (EI, m/z 50−300) using electron impact ionization (EI). Quantitation of skatole metabolites was achieved by extraction of specific mass traces of the analytes and internal standards: m/z 135 for 2-aminoacetophenone, m/z 138 for d_3-2-aminoacetophenone, m/z 147 for 3-methyl-2-oxindole, m/z 161 for indole-3-carbinol, m/z 130 for skatole, and m/z 133 + 134 for d_3-skatole. All analytes were identified on the basis of comparison of retention time and mass spectra to authentic samples.

Calibration. A five-point calibration was obtained in triplicate by spiking sodium phosphate buffer (50 mM, pH 7.4) with defined quantities of 2-aminoacetophenone (0.3−3.0 μg/mL) and d_3-2-aminoacetophenone as the internal standard (1.5 μg/mL). Linear regression was used to describe the relationship between peak area (2-aminoacetophenone/d_3-2-aminoacetophenone) and concentration ratios (2-aminoacetophenone/d_3-2-aminoacetophenone). Additionally, a five-point calibration of skatole was prepared using d_3-skatole as the internal standard to estimate the overall amount of skatole that was converted during incubation. Quantitation of 3-methyl-2-oxindole and indole-3-carbinol was based on individual response factors obtained by spiking sodium phosphate buffer (50 mM, pH 7.4) with equal concentrations of 2-aminoacetophenone, 3-methyl-2-oxindole, and indole-3-carbinol. Division of the concentration ratios (d_3-2-aminoacetophenone/3-methyl-2-oxindole, d_3-2-aminoacetophenone/indole-3-carbinol) by the observed peak area ratios (d_3-2-aminoacetophenone/3-methyl-2-oxindole, d_3-2-aminoacetophenone/indole-3-carbinol) provided response factors in a concentration range of 1.0−1.5 μg/mL. The response factors were not significantly altered when phosphate buffer was supplemented with microsomal protein as described before for the microsomal incubations.

RESULTS AND DISCUSSION

Liver microsomes from 12 different boars of Pietrain × Baden-Württemberg hybrid type were prepared and successfully employed for subsequent in vitro identification of volatile skatole metabolites by HS-SPME/GC-MS analysis. Overall CYP levels and activities of specific isoforms known to affect phase I skatole metabolism in pigs were verified by carbon monoxide difference spectra and chromatographic analyses, respectively. Activities of CYP1A1 and CYP1A2, measured as rates of *O*-demethylation of 7-methoxyresorufin (MROD, CYP1A1) or *O*-deethylation of 7-ethoxyresorufin (EROD, CYP1A2), were found to be 2 or 23 pmol/min/mg protein, respectively, and are comparable to literature values.[37] All experiments were carried out in duplicate, and two types of negative controls were implemented. On the one hand, protein inactivation was achieved by heating mixtures of microsomal protein and buffer to 90 °C for 5 min before the actual incubation experiment. This sort of negative control is referred to as a boiled blank in literature papers.[38] On the other hand, CYP activity was suppressed by omission of the reducing agent NADPH. To verify whether product formation arises solely from skatole conversion or also from metabolism of other compounds, an additional third type of negative control was implemented by omitting skatole addition. A putative concentration-dependent CYP activity was tested by using various concentrations of skatole (0.4 mM, 40 μM, 4 μM). The resulting product profiles and formation rates did not differ significantly.

Chromatograms obtained by HS-SPME/GC-MS from a typical assay and a boiled blank are shown in Figure 1. Whereas

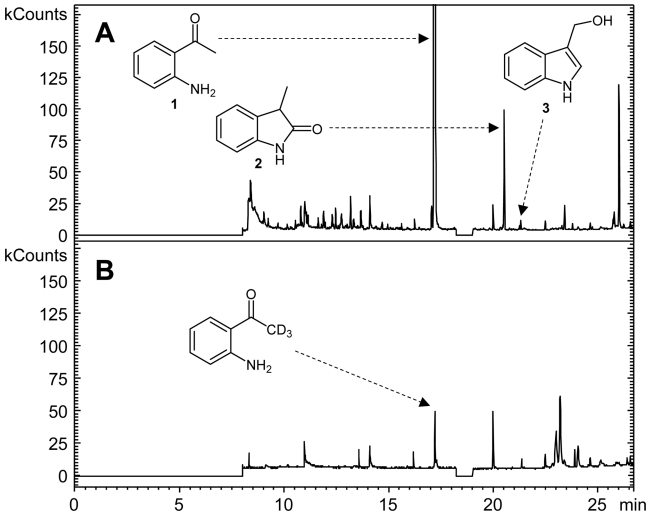

Figure 1. Comparing the chromatograms from a microsomal incubate (A) reveals the formation of 2-aminoacetophenone (**1**), 3-methyl-2-oxindole (**2**), and indole-3-carbinol (**3**), whereas the boiled blank (B) shows no metabolites. During elution of skatole (t_R = 18.6 min) the multiplier was turned off to avoid saturating effects. Chromatograms were recorded in full scan mode, m/z 50−300.

no metabolites were observed in the boiled blanks, 2-aminoacetophenone (**1**), 3-methyl-2-oxindole (**2**), and indole-3-carbinol (**3**) were formed by microsomal incubation. The absence of any metabolites in the boiled blank clearly indicates that formation of the observed products relies on enzyme activity. Results of those control incubations where NADPH was omitted demonstrate that NADPH-dependent enzymes such as CYPs must be involved. However, in some incubations, a small amount of 2-aminoacetophenone (<4%) appears to arise from an enzyme activity that is not NADPH-dependent (data not shown). Blanks without addition of the substrate skatole showed no product formation at all.

To obtain comparable results, amounts of each metabolite were normalized with respect to protein content and incubation duration. Product quantitation after incubation at 37 °C for defined periods of time allowed for the determination of formation rates by plotting peak area ratios of product and internal standard versus incubation time and subsequent linear regression. QC-samples that were used showed variations (accuracy/precision) below 10%. Figure 2 shows the formation rates of nine different boars, which were classified as tainted according to their back fat levels of skatole and androstenone, in comparison to samples from boars (n = 3) with subthreshold back fat levels of skatole and androstenone. 2-Aminoacetophenone was found as the volatile product with the highest formation rate followed by 3-methyl-2-oxindole and indole-3-carbinol, giving 58 ± 2% 2-aminoacetophenone (**1**), 31 ± 1.5% 3-methyl-2-oxindole (**2**), and 11 ± 3% indole-3-carbinol (**3**), respectively (Figure 3). Boars with subthreshold

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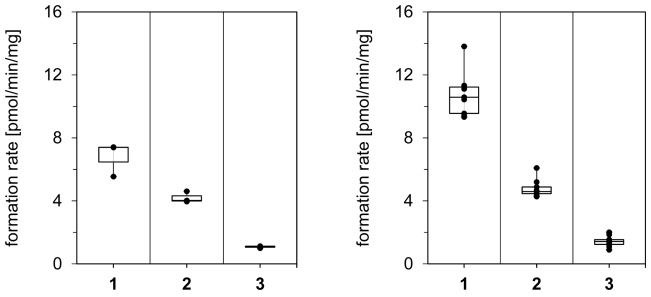

Figure 2. Box plots of untainted (left; $n = 3$) and tainted (right; $n = 9$) boars showing 2-aminoacetophenone (**1**) as the volatile skatole metabolite with the highest formation rate during incubation experiments, followed by 3-methyl-2-oxindole (**2**) and indole-3-carbinol (**3**). The formation rate of 2-aminoacetophenone is significantly higher in tainted boars, whereas formation rates of the other two metabolites appear the same.

back fat levels of skatole and androstenone showed significantly lower formation rates of 2-aminoacetophenone, but similar formation rates of 3-methyl-2-oxindole and indole-3-carbinol, giving 50 ± 1% 2-aminoacetophenone, 35 ± 1% 3-methyl-2-oxindole, and 15 ± 0.1% indole-3-carbinol. These product profiles did not differ significantly when higher concentrations of skatole were used. In contrast, Diaz et al. found 0.5% 2-aminoacetophenone, 28% 3-methyl-2-oxindole, and 3% indole-3-carbinol when performing similar incubation experiments with boars obtained by backcrossing F3 European Wild Pig × Swedish Yorkshire boars with Swedish Yorkshire sows.[22]

Considering overall skatole conversion, 48% of the added skatole was converted into compounds that could not be identified in this study, most probably because they were nonvolatile. However, 30 ± 5.5% of the added skatole was transformed into 2-aminoacetophenone, 16 ± 3% into 3-methyl-2-oxindole, and 6 ± 1% into indole-3-carbinol. Thus, 2-aminoacetophenone appears as a major volatile product of CYP mediated phase I skatole metabolism in microsomal fractions from boars of Pietrain × Baden-Württemberg hybrid type. As 2-aminoacetophenone is neither sulfated nor glucuronidated during porcine phase II metabolism, high formation rates of 2-aminoacetophenone will result in elevated back fat concentrations measured in boars.[26]

In this respect our results differ considerably from literature papers, where 2-aminoacetophenone was either a minor product of skatole conversion or absent.[22,31,39,40] They provide an explanation for the high amounts of 2-aminoacetophenone observed in back fat samples of Pietrain × Baden-Württemberg hybrid type boars,[17] where average 2-aminoacetophenone concentrations in back fat of 100 ng/g and a weak correlation between concentrations of skatole and 2-aminoacetophenone (R^2 = 0.33) raised the question at to whether these high amounts of 2-aminoacetophenone originate exclusively from hepatic skatole metabolism or if other processes lead to the formation of 2-aminoacetophenone, for example, the kynurenine pathway.[17]

In light of our findings, the hypothesis that other pathways lead to in vivo formation of 2-aminoacetophenone in boars appears unlikely. Furthermore, correlations of 2-aminoacetophenone and skatole concentrations might be of limited use to explain the origin of 2-aminoacetophenone in vivo, because individual effects such as intestinal absorption and CYP concentration and activity will influence skatole and 2-aminoacetophenone levels.[17] Thus, when these results are compared to published data, the investigated breeds must be considered: Pietrain × Baden-Württemberg hybrid, Swedish Yorkshire, Swedish Landrace, or Duroc.[22,31,41]

Microsomal assays that included the addition of the specific inhibitor α-naphthoflavone indicated a significant contribution of CYP1A to 2-aminoacetophenone formation, as concentrations declined by approximately 90% after treatment of the incubation mixtures with α-naphthoflavone. Although Wiercinska et al. showed a capability of CYP1A1 for 2-aminoacetophenone formation, this isoform produced only small amounts of 2-aminoacetophenone when compared to CYP2E1.[39] Furthermore, increased activities of CYP1A1 and CYP1A2 in female pigs as well as increased EROD activities in castrates have been reported.[42,43] Considering a direct correlation between CYP1A1 levels and elevated formation rates of 2-aminoacetophenone, female pigs should show even higher back fat levels when compared to boars. However, as we have reported before, female pigs show only low back fat amounts of 2-aminoacetophenone.[17] Thus, it appears that next to CYP1A1 other enzymes must contribute to the formation of

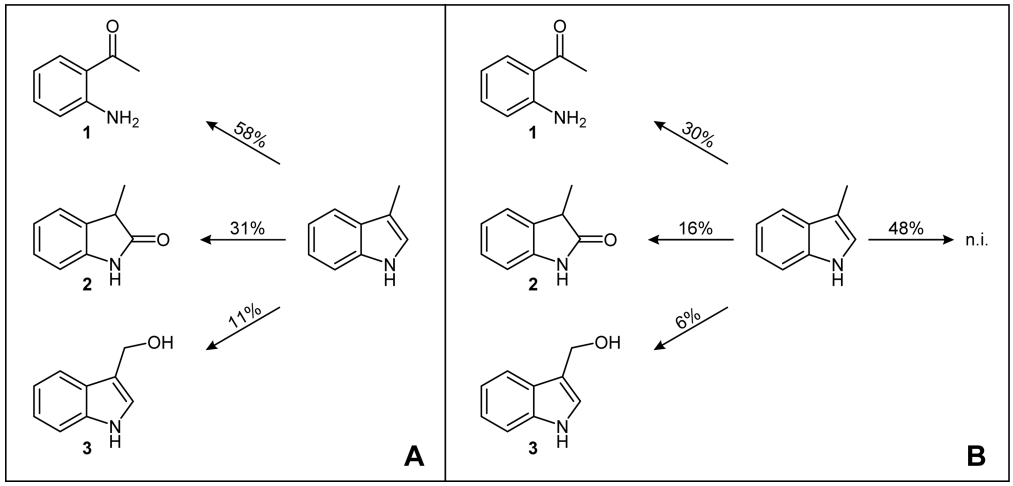

Figure 3. Relative ratios of the volatile skatole metabolites detected by HS-SPME/GC-MS (A) and with respect to the nonvolatiles estimated from the overall skatole conversion (B).

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2-aminoacetophenone because oxidation, ring-opening, and deformylation of skatole are required.

Surprisingly, formation rates of 2-aminoacetophenone were higher in boars with high back fat levels of skatole and androstenone compared to boars with low back fat levels of both substances. In general, mature pigs with high levels of testicular steroids show lower CYP activities, and regulation of porcine CYPs by testicular steroids has been shown to be breed-dependent.[44,45] In particular, androstenone is capable of a direct inhibition of CYP2E1, an isoform that is highly involved in skatole metabolism.[42] Thus, it appears very important to carefully distinguish between the investigated breeds.

Although many studies of CYP-mediated skatole conversion in pigs have been performed, there is still confusion with respect to quantitative prediction of in vivo skatole metabolism in pigs.[46] As boars of type Pietrain play an important role in German breeding programs, skatole conversion in these animals needs further investigation.[47] Especially the in vivo pathways leading to 2-aminoacetophenone formation are not fully understood yet. A detailed sensory evaluation of back fat extracts by gas chromatography−olfactometry (GC-O) might further elucidate the question of whether and to what extent other compounds contribute to boar taint. Results from such investigations will be reported in due course.

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Notes

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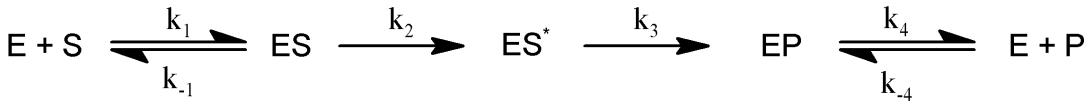
2.3 Mechanism of CYP 450 catalyzed formation of 2-aminoacetophenone

2.3.1 Kinetic isotope effects – theoretical background

As remarked in the previous chapter the biosynthesis of 2-aminoacetophenone in pigs, in contrast to wine, has not been elucidated, yet. To that end, published research predominately focused on the identification of CYP 450 isoforms involved in the phase-I-clearance of skatole. In consequence several enzymes have been suggested as markers for enhanced metabolization rates of skatole, *i.e.* CYP 1A, 2A, 2A19, 2C33v4, 2C49 and 2E1.[61,95,98,99,116] The number of CYP 450s that have been discussed underlines that this practice is limited with respect to the prediction of hepatic metabolization rates of skatole and the associated formation rate of 2-aminoacetophenone in pigs. Although previous investigations demonstrated the capability of these isoforms to form 2-aminoacetophenone without the involvement of other enzymes, the contribution of different CYP 450 isoforms and other enzymes to the compounds *in vivo* generation is considerable. A possible generation pathway in pigs according to literature reports comprises the intermediates 3-hydroxy-3-methylindolenine (2), 3-hydroxy-3-methyloxindole (3) and N-formyl-2-aminoacetophenone (4) (see publication *J. Agric. Food Chem.,* chapter 3). An aldehydoxidase oxidizing **2** to **3** and a formamidase hydrolysing **4** to 2-aminoacetophenone have already been described in pig liver.[117,118]

The previous considerations emphasize the necessity to clarify the mechanism of 2-aminoacethophenone generation to enable a precise understanding of the endogenous factors influencing the metabolization of skatole. A common practice within the elucidation of reaction mechanisms is the determination of kinetic isotope effects (KIEs).[76,119] In general, isotope effects describe the changes in chemical and physical properties of compounds that are based on the substitution of at least one atom by its isotope. In the case of KIEs this substitution is expressed as the measurable change of the reaction rate. When the bond to an element (E) or its isotope (I) is broken within the rate determining step of a reaction, the reaction constant k_E exceeds the constant k_I. This effect is caused by lower zero point energies of heavier isotopes, as an element's ground state vibration at absolute zero is negatively correlated to its mass. In consequence, more energy has to be afforded for bond dissociation.[120,121] Because of the relatively high mass difference between hydrogen and deuterium kinetic isotope effects can extensively be observed when a compound is labelled with deuterium. Mathematically this effect can be expressed as the ratio of reaction constants with and without the presence of deuterium: k_H/k_D.[76]

While the investigation of KIEs has been successfully applied as a powerful tool for the investigation of reaction mechanisms in the field of organic chemistry, a transfer of the concept to enzymatically mediated reactions suffers from complications. These complications are based on the fact that enzymatic reactions, in general, comprise a number of binding and de-binding steps that all contribute to an overall reaction rate. Accordingly, the terminology for these reactions differentiates between the experimentally observable isotope effect $(k_H/k_D)_{obs}$ and the isotope effect associated with the bond breaking step (k_H/k_D), also named intrinsic isotope effect. Considering a simplified scheme of an enzymatic reaction between enzyme (E) and substrate (S) the difference between the both numbers becomes evident:



In this reaction sequence the irreversible, rate determining step leading to the generation of an activated enzyme-substrate complex (ES*, reaction constant k_2) occurs prior to the isotopically sensitive step leading to the enzyme-product complex (EP, reaction constant k_3). A decrease of k_3 via deuterium substitution would instantly lead to an increase in the concentration of the activated enzyme-substrate complex for the deuterated substrate, such that $k_3[ES^*]_D = k_3[ES^*]_H$. The observable isotope effect for this reaction will consequently be lowered or masked, so that $(k_H/k_D)_{obs}$ can only be less or equal to the intrinsic isotope effect.[122]

The ability of CYP 450 enzymes to oxidise a broad variety of substrates was already mentioned above. Prior to the transfer of oxygen to the substrate, an activated heme-iron bound perferryloxy heme species originates under involvement of CYP 450 reductase. This reduction step associated with the formation of active oxygen is known to be rate limiting for CYP 450s, suggesting that isotope effects should not occur for reactions of this enzyme family. However in terms of CYP 450 mediated reactions, KIEs can be unmasked by particular reaction designs. Since the energy required for catalysis is already incorporated within the activated oxygen species, CYP 450s show broad substrate specificity and consequently show the capacity to form multiple products from only one substrate. If a substrate can be oxidised in different positions of which only one position requires deuterium abstraction, a decrease in the formation rate of one product will cause an increase of the formation rate of another product. In this case a so-called intramolecular KIE expressed as the

change of the ratio of formation rates, becomes measurable and can be used to estimate the intrinsic isotope effect.[76,123]

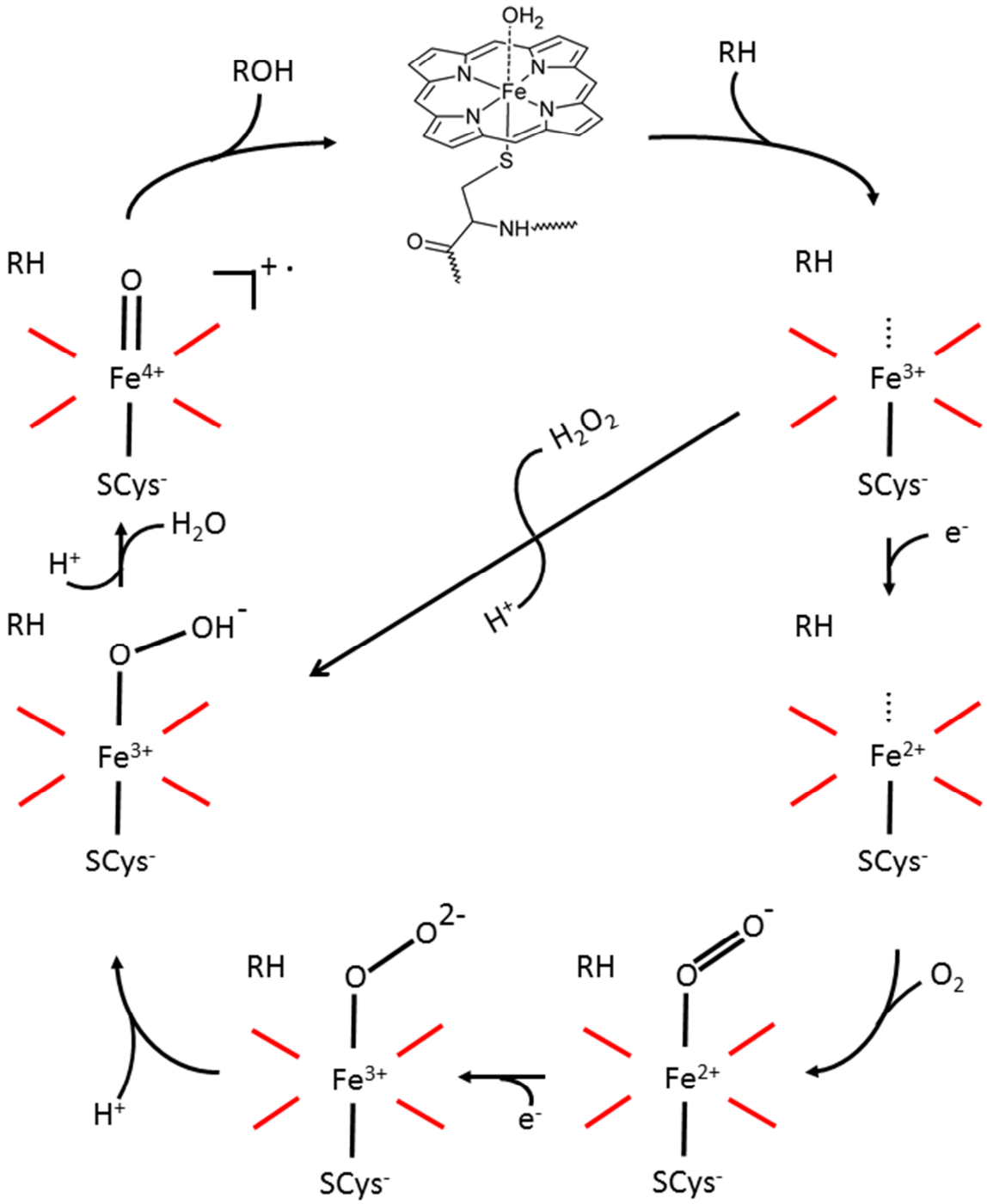


Figure 5 The catalytic cycle of cytochrome P450. The porphyrin macrocycle is illustrated by four bold red lines. Cysteinate as proximal ligand is symbolized by the abbreviation SCys.

2.3.2 Isotopically sensitive branching

In the presented study the intramolecular KIE for the CYP 450 mediated conversion of skatole was investigated in a non-competitive approach by performing incubations of porcine liver microsomes with skatole as substrate to enzymatic activity next to incubations with d_3-skatole as substrate (see figure 6). As already shown in the previous chapter, three volatile metabolites of skatole could be detected in the headspace above the samples by GC/MS: 2-aminoacetophenone, indole-3-carbinol and 3-methyloxindole.

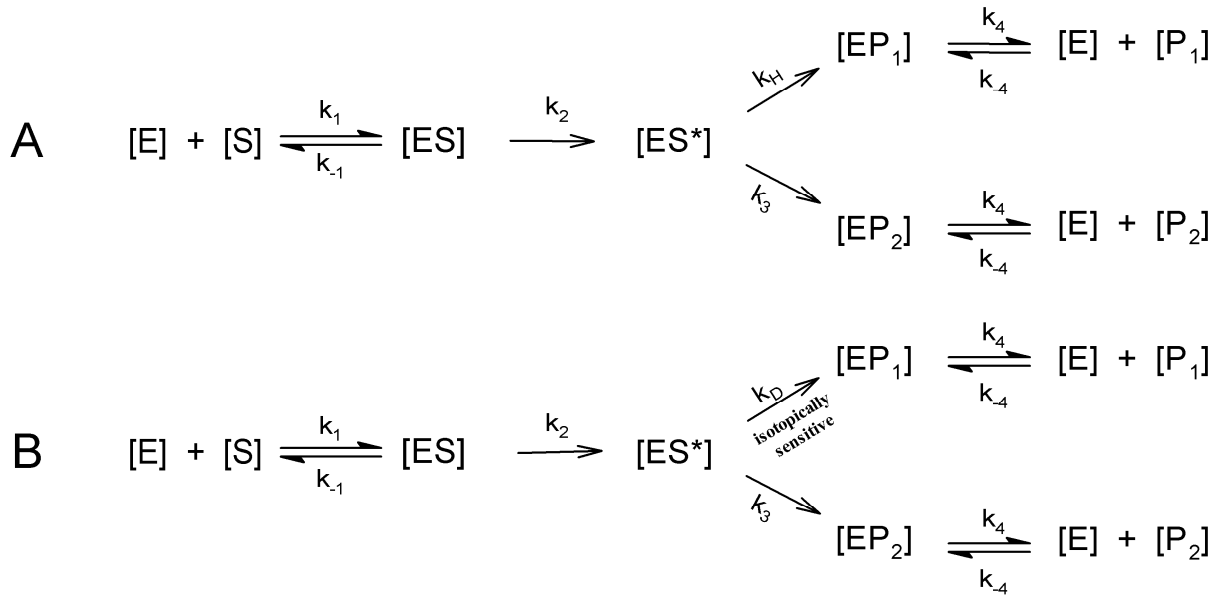


Figure 6 Kinetic model for a non-competitive enzymatically catalysed reaction that includes isotopically sensitive branching. Pathway A illustrates the CYP 450 mediated conversion of skatole, while *d_3*-skatole served as substrate in pathway B.

When labelled substrate was used, the detected products were *d_3*-2-aminoacetophenone, *d_2*-indole-3-carbinol and *d_3*-3-methyloxindole. A comparison of the formation rates determined for both incubation experiments revealed that 2-aminoacetophenone generation is isotopically sensitive, while the generation of both other metabolites showed no measurable isotope effect. However, the absence of a an intramolecular KIE for the performed *in vitro* incubations still allows to draw conclusions in terms of possible reaction steps. Following the previous statements a decrease of the formation rate of *d_2*-indole-3-carbinol should be detectable if the same CYP 450 isoform was responsible for the formation of 2-aminoacetophenone and indole-3-carbinol. Hence, the presented results indicate the opposite. As no deuterium abstraction occurs within the generation of 3-methyloxindole comparable statements cannot be made for this product. Nevertheless other insights into the formation pathways leading to this compound can be deduced from the full retention of the deuterium label. Research by

Skrodos and co-workers addressed the mechanism of the biotransformation of skatole to 3-methyloxindole.[124] The study focused on two possible reaction routes, one including the generation of an epoxide and the other pathway including the generation of an indolenine. In accordance with the results of this study our data indicates the existence of an epoxide structure, since a dehydrogenation to the corresponding indolenine would lead to an abstraction of deuterium, resulting in d_2-3-methyloxindole, a species that was not observed in our experiments (see publication *J. Agric. Food Chem.*, Figure 3). Analogue to these considerations details of 2-aminoacetophenone generation can be deduced from the full retention of the deuterium label. Recently published research by Yoshimoto and co-workers investigated mechanistic details of C-C bond cleavage by CYP 450s. As a result three different reaction pathways for CYP 17A1 catalysed 17-α, 20-lyase reaction of 17-alpha-hydroxypregnonelone were suggested (see publication *J. Agric. Food Chem.*, Figure 4, pathways A, B, C).[125] The results of our measurements are in accordance with pathway A and B. Pathway C seems unlikely, as d_2-aminoacetophenone could not be detected in our incubations. Furthermore, a deuterium abstraction should result in a lower formation rate for d_3-aminoacetophenone compared to the genuine compound, while the presented data shows the opposite.

2.3.3 The formation mechanism of 2-aminoacetophenone

Next to incubations with labelled skatole we conducted incubations in which possible intermediates en route to 2-aminoacetophenone were used as substrates to gain further insight into the reaction mechanism. As stated above, several possible intermediates of 2-aminoacetophenone are conceivable with regard to previous studies, *i.e.* 3-hydroxy-3-methylindolenine, 3-hydroxy-3-methyloxindole, N-formyl-2-aminoacetophenone. As the decomposition of the unstable indolenine to 3-hydroxy-3methyloxindole was demonstrated at room temperature only the latter was synthesized by oxidation of 3-methyloxindole according to a published procedure and subsequently used as substrate in incubations with porcine liver microsomes.[95,126] In addition, incubations in which 3-methyloxindole served as substrate were performed, as the formation of 3-hydroxy-3methyloxindole from 3-methyloxindole was demonstrated in experiments with human liver microsomes.[127] In both cases 2-aminoacetophenone generation was detectable with formation rates similar to incubations with skatole. In contrast to the reaction mechanism that was formulated as a hypothesis at the beginning of this chapter suggesting the generation of an N-formyl structure, the proposed pathway comprises a carbamate structure. Carboxylesterases capable of the scission of carbamates have already been characterized in microsomal fractions of pig liver.[128] However,

a resulting carbamoyl structure was not observed in the presented study, most likely due to a high carboxylesterase activity in our incubation mixtures or due to the inherent instability of carbamates, which have been demonstrated to decompose easily at room temperature.[129]

In summary, 3-methyloxindole and 3-hydroxy-3-methyloxindole were identified as degradation products of skatole en route to 2-aminoacetophenone. Furthermore, mechanistic details of single reaction steps could be deduced from experiments with labelled substrate. While our experiments deliver substantial evidence for the CYP 450 dependent formation of 2-aminoacetophenone in *in vitro* experiments with porcine liver microsomes, it must not be forgotten that such preparations are complex mixtures of CYP 450s and thus the involvement of single isoforms can only be proven indirectly. In addition, the CYP 450 mediated metabolism of skatole in pigs is well-known to be influenced by breed-related genetic differences. This has to be considered when transferring our results to other breeds.

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Deuterium-Labeling Studies Reveal the Mechanism of Cytochrome P450-Catalyzed Formation of 2-Aminoacetophenone from 3-Methylindole (Skatole) in Porcine Liver Microsomes

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ABSTRACT: 2-Aminoacetophenone, a product of the hepatic 3-methylindole (skatole) clearance in pigs, contributes to the specific aroma of fat from tainted boar meat. Surprisingly, high formation rates for 2-aminoacetophenone from skatole in microsomal preparations from Pietrain × Baden−Württemberg hybrid type boars have been previously demonstrated, but the mechanism of this cytochrome P450-mediated reaction remained unknown. Therefore, microsomal fractions from boars were incubated with deuterium-labeled skatole and with possible reaction intermediates. 3-Methyloxindole and 3-hydroxy-3-methyloxindole were identified as degradation products of skatole en route to 2-aminoacetophenone. Additionally, the labeling studies provided further evidence for a cytochrome P450-mediated lyase reaction that leads to the cleavage of the indole heterocyclic ring system in 3-hydroxy-3-methyloxindole and demonstrated the involvement of several cytochrome P450-isoforms by employing isotopically sensitive branching experiments.

KEYWORDS: *skatole metabolism, CYP 450, boar taint*

INTRODUCTION

2-Aminoacetophenone is a well-known off-flavor compound in food, such as wine and milk.[1−3] Furthermore, 2-aminoacetophenone was identified as a product of the hepatic phase I metabolism of skatole in boars catalyzed by cytochrome P450 enzymes (CYPs).[4] CYPs comprise a diverse group of monooxygenases heavily involved in phase I metabolism because of their ability to oxidize a wide variety of substrates. A systematic nomenclature based on genetic relation distinguishes between families (CYP1), subfamilies (CYP1A), and individual isoforms (CYP1A1).[5] On the basis of today's knowledge, boar taint is mainly caused by two compounds, the pheromone androstenone (5α-androst-16-en-3-one) and skatole, a product of microbiological degradation of tryptophan in the pigs' intestine.[6] However, trained sensory panels frequently judge samples with low amounts of both compounds as tainted and samples with high amounts as nontainted, which leads to the assumption that there are more compounds contributing to the perception of boar taint.[7,8] In a recently published screening study, we analyzed 2-aminoacetophenone levels in back fat samples of boars using headspace solid-phase microextraction gas chromatography−mass spectrometry (HS-SPME-GC/MS). Average amounts of 100 ng/g liquid fat revealed a significant accumulation in porcine fat tissue. The sensory threshold of 2-aminoacetophenone in fat matrixes demonstrated that such amounts are perceivable by sensitive consumers.[9] These findings raise the question by which formation mechanism the surprisingly high concentrations of this potent odorant are generated, since other publications listed the compound only as a minor product of porcine skatole metabolism.[4,10] In this context, we investigated the product profile of skatole conversion by porcine liver CYPs via HS-SPME-GC/MS in in-vitro assays and identified 2-aminoacetophneone as the major volatile metabolite in Pietrain × Baden−Württemberg hybrid type boars formed by liver microsomes.[11] Although many studies focused on skatole conversion in pigs, the pathway leading to 2-aminoacetophenone is still unknown.[12] 3-Hydroxy-3-methylindolenine (**2**), 3-hydroxy-3-methyloxindole (**3**), and *N*-formyl-2-aminoacetophenone (**4**) are possible intermediates of 2-aminoacetophenone (**5**) formation in pigs (Figure 1). An aldehyde oxidase catalyzing the oxidation of compound **2** to compound **3** and a formamidase catalyzing the hydrolysis of compound **4** to compound **5** have been already described in porcine liver.[13,14] A powerful tool for elucidating reaction mechanisms in organic chemistry and biochemistry is the evaluation of kinetic isotope effects (KIEs), which express the change of reaction rates in chemical reactions when at least one atom is replaced by its isotope.[15] However, enzymatic reactions, especially with the use of microsomal preparations, represent complex multistep systems, and thus, the application of isotope effects to these reactions has proven to be less successful. Nevertheless, in branched reactions, in which more than one product can derive from an enzyme−substrate complex, an isotope effect can become measurable by a phenomenon named "isotopic sensitive branching" or "metabolic switching".[16] If two products of an enzymatic reaction result from the same intermediate or the same substrate−enzyme complex, a change in their relative product ratios will be observable because of isotopic substitution.[17]

To gain further insights into the formation of 2-aminoacetophenone in porcine in vitro assays, we evaluated the volatile product profiles formed by porcine liver microsomes using various substrates in microsomal incubations, that is,

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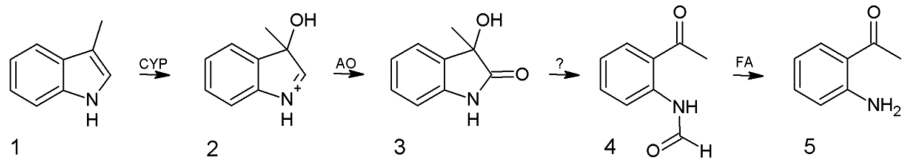

Figure 1. Possible in vivo generation of 2-aminoacetophenone (**5**) by CYP-, aldehyde oxidase (AO)-, and formamidase (FA)-mediated degradation of skatole (**1**) according to literature reports.[4,13,14] The pathway comprises the formation of 3-hydroxy-3-methylindolenine (**2**), 3-hydroxy-3-methyloxindole (**3**), and *N*-formyl-2-aminoacetophenone (**4**).

skatole, d_3-skatole, 3-methyloxindole, and 3-hydroxy-3-methyloxindole. Product formation in microsomal incubations from seven different boars of Pietrain × Baden−Württemberg hybrid type were analyzed by stable isotope dilution anaylsis (SIDA) using HS-SPME-GC/MS. Additionally, the involvement of several cytochrome P450-isoforms could be demonstrated by employing isotopically sensitive branching experiments.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma-Aldrich (Steinheim, Germany), Alfa Aesar (Karlsruhe, Germany), VWR (Darmstadt, Germany), abcr (Karlsruhe, Germany), or Carl Roth (Karlsruhe, Germany). The deuterium labeled internal standards d_3-skatole and d_3-2-aminoacetophenone were from ELFI Analytik (Neufahrn, Germany) or DLR (Neustadt/Weinstraße, Germany), respectively. Following a published protocol, 3-hydroxy-3-methyloxindole was prepared via oxidation of skatole using pyridinium chlorochromate as oxidizing agent and polyaniline salt as catalyst.[18] Products were purified by flash chromatography on silica gel with cyclohexane/EtOAc (3:1 v/v). Chemical purity was >95% (by GC-MS). MS: (EI,70 eV) *m*/*z* (rel int.) 163 $[M]^+$ (33), 148 (16), 146 (2), 135 (78), 120 (100), 117 (5), 90 (71).

Animals. Liver samples of seven boars of type Pietrain × Baden−Württemberg hybrid were collected immediately after slaughter at a weight of 90 kg (around 140 days of age). The samples were shock-frozen using liquid nitrogen and were kept at −80 °C until the day of analysis. All animals were raised using a standardized diet (3.4 MJ ME, 16% crude protein,1% lysine, 0.6% cysteine and methionine, 0.6% threonine) according to the *Zentralverband der Deutschen Schweineproduktion*.[19] Boars were chosen according to elevated back fat levels of androstenone and skatole (skatole > 300 ng/g, androstenone > 2000 ng/g).

Preparation of Liver Microsomes. Microsomal fractions were previously prepared according to a published protocol.[11] In brief, liver tissue was homogenized in Tris-sucrose buffer (10 mM Tris-HCL, 250 mM sucrose, pH 7.4) using an Ultra Turrax homogenizer (IKA Labortechnik, Staufen, Germany). Following centrifugation (8000*g*, 4 °C), the resulting supernatant was decanted and diluted in precipitation buffer (10 mM Tris-HCl, 250 mM sucrose, 8 mM calcium chloride, pH 7.4). After another centrifugation step, the microsomal pellets were suspended in Tris-sucrose buffer and were kept at −80 °C until GC/MS analysis.

Microsomal Incubations. Incubation experiments were conducted as previously reported.[11] Skatole, 3-methyloxindole, and 3-hydroxy-3-methyloxindole were applied as substrates in individual microsomal assays to identify possible intermediates leading to 2-aminoacetophenone formation. All experiments were carried out in duplicate, and three types of negative controls were used throughout the study: first, samples in which CYP activity was eliminated by heating microsomal protein up to 90 °C for 5 min prior to incubation to ensure that product formation is based on enzymatic activity, second, samples in which the reducing agent NADPH was omitted to ensure that NADPH dependent CYP-enzymes are indeed responsible for any conversion of the substrates and to reveal eventually the presence of other non-NADPH dependent enzymes like pyrrol dioxygenases, and third, samples in which the substrates themselves were omitted to ensure that there are no other interfering substrates present in the reaction mixture.

Microsomal Incubations with Labeled Substrate. To investigate possible isotope effects via isotopic sensitive branching, we also used d_3-skatole as a substrate in microsomal incubations. All steps were performed according to incubations with unlabeled substrate, except for use of indole as internal standard instead of d_3-skatole and d_3-2-aminoacetophenone. Seven incubations per animal were performed using labeled substrate and unlabeled substrate.

HS-SPME-GC/MS. HS-SPME sampling was achieved according to a published protocol.[11] In brief, sampling was conducted in an automated heating agitator using a divinylbenzene/polydimethylsiloxane coated SPME fiber (DVB/PDMS, 85 μm, Supelco, Bellefonte, USA). Sampling conditions were as follows: equilibration for 5 min at 55 °C, extraction for 10 min at 55 °C, and desorption for 10 min at 270 °C within the GC injector. GC/MS analysis was performed 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. Quantification of skatole metabolites was achieved by extraction of specific mass traces of the analytes and internal standards: *m*/*z* 135 for 2-aminoacetophenone, *m*/*z* 138 for d_3-2-aminoacetophenone, *m*/*z* 147 for 3-methyloxindole, *m*/*z* 163 for 3-hydroxy-3-methyloxindole, *m*/*z* 150 for d_3-3-methyloxindole, *m*/*z* 147 for indole-3-carbinol, *m*/*z* 149 for d_2-indole-3-carbinol, *m*/*z* 130 for skatole, and *m*/*z* 133 + 134 for d_3-skatole. All analytes were identified on the basis of comparison of retention time and mass spectra to authentic samples.

RESULTS AND DISCUSSION

Preparations of porcine liver microsomes from seven boars were used to investigate the conversion of skatole to 2-aminoacetophenone by SIDA-HS-GC/MS and previously implemented in in-vitro experiments. Overall activity of CYPs and the presence of specific isoforms that are known to influence the conversion of 3-metylindole in pigs were verified by recording carbon monoxide difference spectra and chromatographic analysis as previously reported.[11] Each incubation experiment was accompanied by three types of negative controls: First, samples in which CYP activity was eliminated by heating microsomal protein up to 90 °C for 5 min prior to incubation to ensure that product formation is based on enzymatic activity, second, samples in which the reducing agent reduced nicotinamide adenine dinucleotide phosphate (NADPH) was omitted to ensure that NADPH dependent CYP-enzymes are indeed responsible for any conversion of the substrates and to reveal eventually the presence of other non-NADPH dependent enzymes like pyrrol dioxygenases, and third, samples in which the substrates themselves were omitted to ensure that there are no other interfering substrates present in the reaction mixture. To generate results comparable to literature reports, the amounts of each metabolite were normalized with respect to protein content and incubation time (pmol/min/mg).

Results of the analysis of product profiles of microsomal incubations with skatole by GC/MS were in accordance to previous reports.[11] In brief, three volatile metabolites were identified, whereby 2-aminoacetophenone showed the highest formation rate (11.3 ± 1.8 pmol/mg/min), followed by 3-

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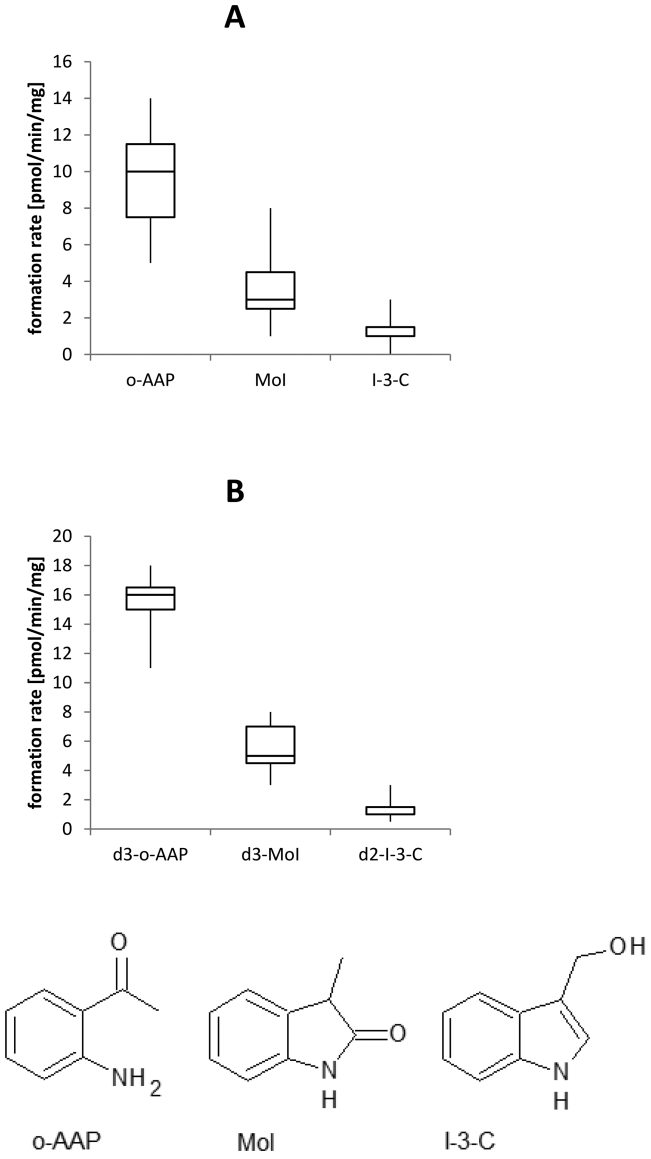

Figure 2. Boxplot of porcine microsomal incubations using genuine (A, top) and labeled (B, bottom) skatole as substrate to CYP mediated conversion (n = 7 boars). We identified 2-aminoacetophenone (o-AAP), 3-methyloxindole (MoI), and indole-3-carbinol (I-3-C) as volatile products of the enzymatic conversion. The formation rate of 2-aminoacetophenone is significantly higher when the labeled substrate is converted while formation rates of the other two metabolites appear the same.

methyloxindole (5.3 ± 0.8 pmol/mg/min) and indole-3-carbinol (2.4 ± 0.63 pmol/mg/min). Taking the overall skatole conversion into account, only 52% ± 2.4% of the added skatole was converted into compounds that could not be identified via HS-sampling, most likely because of not being volatile. However, as the high formation rates for 2-aminoacetophenone differ markedly from results of other similar studies, the question for possible generation pathways in in-vitro assays remains unanswered.[4,10] In this context, it is necessary to state that the investigated breed has to be considered because of individual differences in intestinal absorption, CYP concentration, and activity.

To elucidate these pathways, we used 3-methyloxindole and 3-hydroxy-3-methyloxindole as substrates in microsomal incubations, since both compounds have been reported as

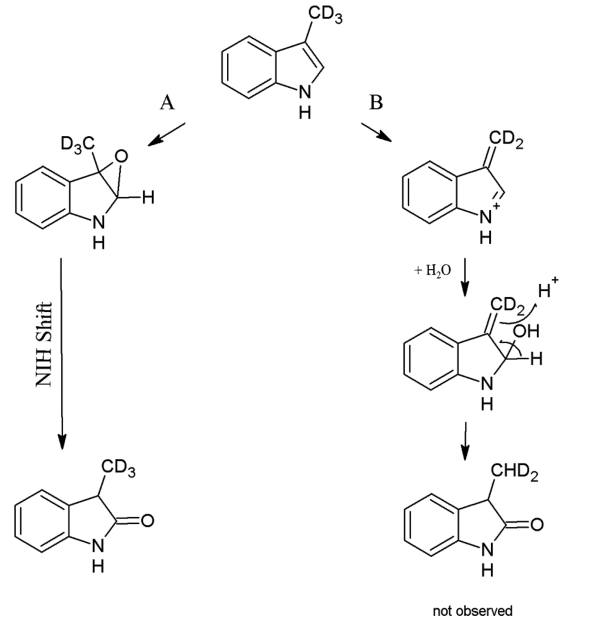

Figure 3. Possible conversion mechanisms of skatole to 3-methyloxindole by CYPs according to recently published data.[23] The results of this study are in accordance with pathway A, since abstraction of deuterium label was not observed.

products of CYP catalyzed skatole conversion. In both cases, 2-aminoacetophenone formation was observable, while no other volatile products could be identified. Product formation rates were 9.6 ± 1.5 pmol/mg/min when 3-methyloxindole served as substrate and 10.4 ± 1.2 pmol/mg/min when 3-hydroxy-3-methyloxindole was added to microsomal incubations. No activity was observed in the absence of the cofactor NADPH. These findings suggest that 2-aminoacetophenone derives from subsequent oxidation of both compounds by CYPs in in-vitro incubations. Additionally, isotopically sensitive branching experiments were conducted, in which d_3-skatole was used as substrate in microsomal incubations in order to investigate whether a KIE is observable for formed products and to gain further insight in the dynamics of possible hydrogen abstraction steps. In these experiments, d_3-methyloxindole, d_2-indole-3-carbinol, and d_3-aminoacetophenone were detected as volatile products of microsomal activity. Comparison of the formation rates for the individual metabolites reveals that 2-aminoacetophenone generation is isotopically sensitive while 3-methyloxindole and indole-3-carbinol generation show no isotope effect (Figure 2). Evaluation of this data indicates the activity of different CYP isoforms for the generation of 2-aminoacetophenone and indole-3-carbinol in our experiments. Since one product is formed by a pathway that requires abstraction of deuterium from the labeled substrate (d_2-indole-3-carbinol), while for the other product (d_3-2-aminoacetophenone) the pathway does not require abstraction of deuterium from the labeled substrate, a rise in the formation rate of d_3-aminoacetophenone would result in a decrease in the formation rate of d_2-indole-3-carbinol if both metabolites had been generated by the same CYP-isoform. As no deuterium abstraction occurs during the generation of d_3-methyloxindole, similar conclusions can be drawn for this compound (see later). Interestingly, the complete retention of the deuterium labels in d_3-methyloxindole allows us to gain more insight into its formation mechanism. Skordos et al. examined its possible

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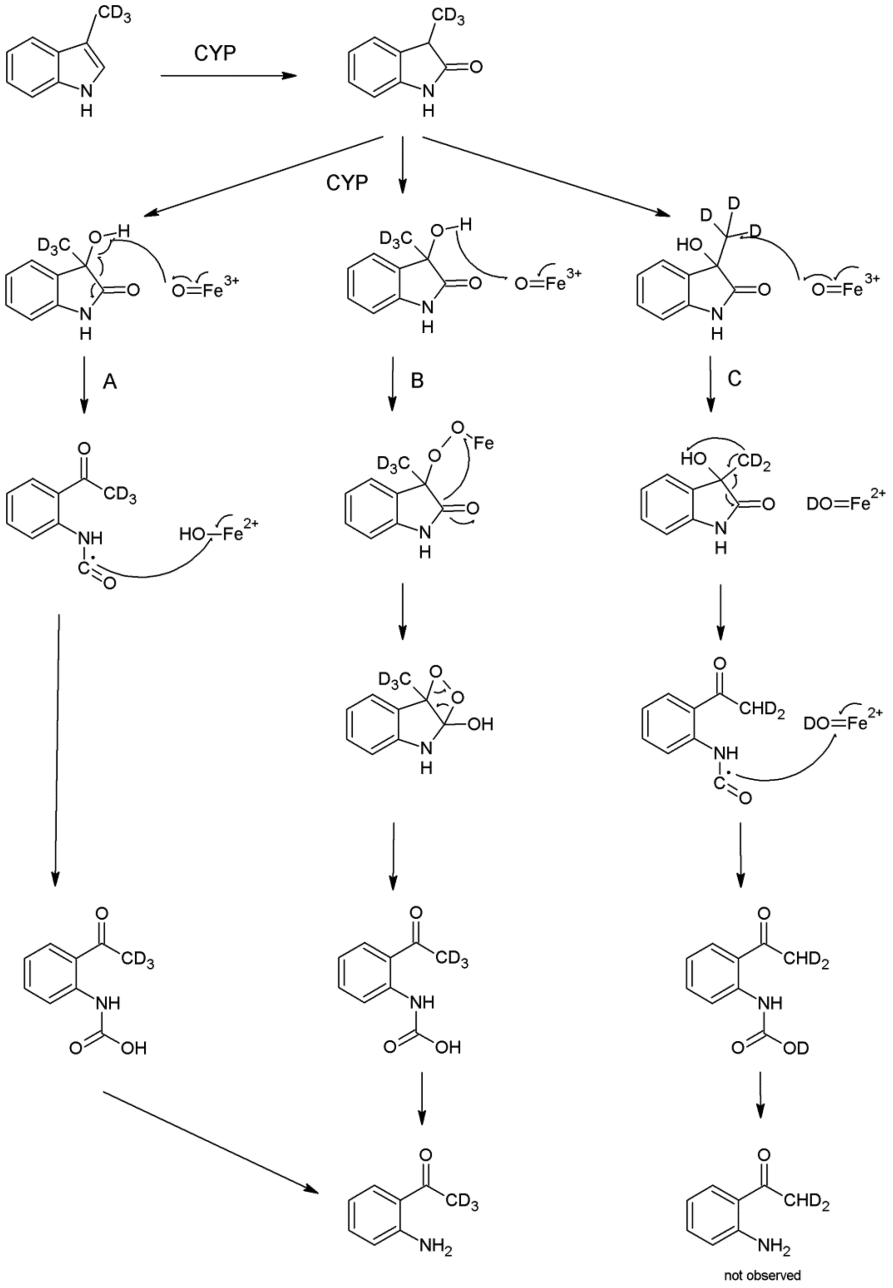

Figure 4. Recently published research formulates three different mechanisms for CYP mediated lyase reactions (pathways A–C).[23] As no abstraction of deuterium could be observed in incubations with labeled skatole, only pathways A and B are in accordance with the results of this study. Pathway C would lead to the generation of d_2-aminoacetophenone, a compound that was not observed in our experiments. Figure adapted from ref 23.

formation pathways using microsomal preparations from goat lungs in labeling studies.[20] Possible reaction mechanisms include either epoxidation and subsequent ring opening with or without hydride shift (Figure 3, pathway A) or dehydrogenation and subsequent hydration at C2 (Figure 3, pathway B). As pathway B would result in a deuterium abstraction from the methyl group, our results indicate the presence of the epoxide followed by a hydrogen shift, which is in accordance with published data. Furthermore, the ratio of ions m/z 133 and m/z 132 assigned to the $[M]^+$ and the $[M-D]^+$ fragment of d_3-skatole and the ratio of m/z 150 and m/z 149 assigned to the corresponding species of d_3-methyloxindole were found to be 2.2 ± 0.3 and 1.9 ± 0.5, respectively, giving evidence that no deuterium abstraction takes place during the formation of 3-methyloxindole.

Yan et al. already described the conversion of 3-methyloxindole into 3-hydroxy-3-methyloxindole using human liver microsomes.[21] However, no 2-aminoacetophenone formation was reported in this study, most likely because microsomes from a different species were employed. The ability of CYPs to catalyze a required carbon carbon cleavage between oxygenated carbons during the transformation of an alpha-hydroxy-oxo compound to 2-aminoacetophenone has extensively been described in literature related to steroid metabolism. Interestingly, an example for a CYP catalyzed cleavage of keto alcohols is the lysis of the C17–C20 bond in pregnenolone by CYP17A1 leading to 5,16-androstadien-3 beta-ol, an intermediate product of the biosynthesis of the pheromone androstenone in boars.[22] Different mechanisms were postulated for this reaction: Akhtar et al. proposed the addition of a ferric

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peroxide species to the C20 carbonyl followed by free radical fragmentation of a peroxy-hemiacetal, whereas recently published research formulates three different mechanisms by ^{18}O-labeling studies (Figure 4).[22,23] As no abstraction of deuterium could be observed in incubations with labeled skatole, only pathways A and B in Figure 4 are consistent with our results. Pathway C would lead to the generation of d_2-aminoacetophenone, a compound that was not observed in our experiments. Additionally, a deuterium abstraction step should result in a decrease of the measured d_3-2-aminoacetophone formation rate compared to the genuine compound, while our isotopically sensitive branching experiments showed the contrary. Finally, the proposed reaction mechanism of 2-aminoacetophenone formation would require the scission of a resulting carbamate structure in the last reaction step. Carboxylesterases, capable of catalyzing the scission of carbamates, have been described in bacteria and in several species including pigs.[24] However, a carbamoyl structure could not be detected in the present study, which is probably due to a high carboxylesterase activity in our microsomal preparations or due to the inherent instability of carbamates, which easily decompose at room temperature.

In this HS-SPME-GC/MS analysis of skatole conversion to 2-aminoacetophenone in porcine microsomal preparations, we presented evidence for a formation mechanism including the generation of 3-methyloxindole, 3-hydroxy-3-methyloxindole, and a labile carbamoyl structure. As it is well-known that breed related genetic differences have large influences on CYP mediated metabolism of skatole in pigs, it is necessary to verify our results for other breeds. In this context, incubation experiments with different genders should also be performed to gain further information about the incidence of boar taint, since a contribution of 2-aminoacetophenone to the overall aroma of boar fat has been already demonstrated.[25] Moreover, microsomal preparations are a complex mixture of enzymes, and statements about the involvement of single CYP isoforms were only made on the basis of labeling experiments and deduction, that is, indirectly. To that end, incubations with individually expressed CYP isoforms should be performed in future studies. Our findings concerning the CYP-dependent formation of the potent odorant 2-aminoacetophenone from skatole might be also of interest for nasal perireceptor processes that include the CYP-dependent metabolism of volatiles in the olfactory mucosa, which has an impact on the quality and the intensity of odor stimuli.[26]

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Notes

The authors declare no competing financial interest.

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2.4 Comparative Aroma Extract Dilution Analysis (cAEDA) of porcine fat

2.4.1 Aroma analysis – the "sensomics" approach

As described in the introduction to this work knowledge about the existence of potentially malodorous meat from uncastrated male pigs exists for several hundred years. The long-time routinely practised castration of young piglets without anaesthesia prevented the so-called boar taint from getting a relevant factor for the commercial acceptance of meat from male pigs. It was only the rising consciousness for animal welfare issues that led to criticism to the painful routine of castration without anaesthesia. Due to this criticism, by 2010, stakeholders of the pig industry voluntarily declared to ban castration and search for alternative strategies to produce untainted meat from male pigs.[29] This decision emphasized the necessity to develop a deeper understanding of the generation of the off-flavour boar taint and its perception by humans.

The term off-flavour implicates that boar taint is as well associated with a negative odour as with a negative taste, since flavour describes the overall impression of a single food generated in the human brain on the basis by both attributes.[130,131] In contrast, previous studies could demonstrate that the problem mainly occurs when boar meat is heated prior to consumption, suggesting that nasal detection of odorous molecules dominates the perception of boar taint.[2,4,7] Thus, the last chapter of this work addresses the question of the overall aroma of boar fat in comparison to the fat of castrated and female pigs. The hunt for odorous molecules in our food started with the development of GC by the 1960ies. While by the beginning of aroma research the assumption was predominant that the entire set of volatiles in a food was responsible for the smell, first failures in the reconstitution of complex foods by volatiles that were previously identified led to a paradigm shift, *e.g.* orange juice.[77] The then practised methodology became known as "sensomics" approach and is based on the idea that only the volatile compounds in our diet will contribute to the overall aroma, which are able to interact with the human nasal receptors.[132] In order to identify these molecules the technique of GC-olfactometry (GC-O) was developed to locate odorous spots in the effluent of a gas-chromatograph by using the highly sensitive and selective human nose as biological detector. By consecutive conduction of GC-O experiments with stepwise diluted extracts of food it is therefore not only possible to identify potent, medium and lower volatile odour-active compounds, but also to evaluate their contribution to the over-all aroma on relative basis.[91]

2.4.2 Experimental design of the aroma analysis

Two techniques for the application of such screening experiments are commonly used: Aroma extract dilution analysis (AEDA) and charm analysis. In the case of AEDA, a flavour dilution (FD) factor is assigned to every volatile odorant, defined as the highest dilution at which a compound can still be sensed. Charm analysis constructs chromatographic peaks similar to peaks produced by a chemical detector, whereby the peak area is proportional to the amount of odorant in the extract. The difference between both methods is that charm analysis determines the dilution over the whole time the compounds elute, while AEDA only determines the maximum dilution value.[91,133] Further GC-O techniques next to the two mentioned ones exist, *i.e.* Osme (derived from the Greek word for smell) and olfactometry global analysis (OGA), but are rarely applied.[134,135] More recently an approach referred to as comparative aroma extract dilution analysis (cAEDA) was used to elucidate differences in the aroma profiles of different samples of a single food, *i.e.* different sorts of rum. If the same amount of food is extracted and the same volumes of extract are injected into the GC direct comparisons of FD factors can be made on semi quantitative basis.[136] Although AEDA and charm analysis are important methods to focus scientific studies on the most important aroma-active compounds in a given set of volatiles they do not allow for statements about a compound's contribution to the overall aroma of a food, since both are based on their threshold in air and not in the respective food matrix. In order to investigate an individual contribution of an odour-active volatile to the overall aroma scientist developed the concept of odour activity values (OAVs), defined as the ratio between the concentration of an odorant and its odour threshold in the predominant food matrix. Thus, the determination of OAVs requires as well the determination of sensory thresholds in diverse matrices as the accurate and precise quantification of the target analytes. Due to the complexity of the volatile fraction and the wide range of volatility, concentration and reactivity of the odorous compounds, precise quantifications require the application of labelled internal standards in stable isotope dilution assays to guarantee for deviations below ten percent. Finally, verification for the developed aroma model is achieved by reconstitution experiments. Therefore synthetic blends of odorants are prepared on the basis of the analytical data and their aromas are compared with the originals by trained panellists.[132] Although reconstitution experiments are a valuable approach for evaluating the quality of aroma models, they suffer from the fact that the aroma impact of volatiles is evaluated individually after GC separation. However, perpetual interactions of odorants like suppression, inhibition and reinforcement are well-known and were recently demonstrated for the additive effect of androstenone and skatole.[51,137]

Therefore, the question to what extent a single odorant contributes to the aroma has to be answered by omission experiments. Accordingly, the following scheme for the evaluation of key odorants in porcine fat resulted:

1. The gentile isolation of volatiles from porcine back fat samples by solvent-assisted flavour evaporation (SAFE).[138] The technique allows for the extraction of volatiles from either diverse matrices, such as solvent extracts, fruit pulps or even foods with high fat content by the application of high vacuum distillation in an especially designed apparatus. As the distillation requires only low temperatures thermal induced artefact formation can be avoided.
2. The identification of the most potent odorants in the produced fat extract by the application of AEDA. To ensure for reliable and reproducible results identification of odorants was based on the comparison of odour impressions (I), retention indices (II) determined on two capillary columns of orthogonal polarity and mass spectra (III) to authentic standards.
3. The determination of OAVs by SIDAs. After spiking the back fat extract with known amounts of labelled standard the extract was distilled off and subsequently analysed by GC/MS. In order to distinguish between labelled and unlabelled odorants the quantification of distinct ions was performed on their individual ion traces.
4. The reconstitution of the investigated boar fat aroma by spiking deodorized sunflower oil with the odorants in their determined concentrations. Subsequently the reconstitute was presented to a panel familiar with the perception of boar taint.

In summary, 15 aroma active compounds were identified and quantified in boar fat, whereas 12 aroma active compounds were found in fat from female pigs and 12 in fat of castrated males. Interestingly, the generation of nearly all potent odorants in porcine fat was heat induced and could only be observed when back fat samples were heated in a microwave oven prior to analysis.

In the case of boar fat the calculation of OAVs revealed a major impact of androstenone, skatole, indole and 2-aminoacetophenone for the overall aroma. As the samples were preselected according to elevated levels of these boar taint causing agents this result was expectable. An aroma simulation of melted boar fat showed good similarities to the original. Additionally, results of preliminary omission experiments with two trained assessors indicated a significant impact of 2-aminoacetophenone for the overall aroma impression of fat from Pietrain × Baden-Württemberg hybrid boars. However, such experiments have to be

conducted with trained panellists under standardized conditions on a large scale. In the case of female fat highest OAVs were obtained for 2,5-dimethylpyrazine, followed by 2,4-decadienal and δ-decalactone, whereas highest OAVs were assigned to skatole, indole, 1-octen-3-ol and methional in the case of castrated males. Especially the significant contribution of skatole and indole to the determined aroma of fat derived from castrates seems surprising, as surgical castration is the commonly applied to avoid the incidence of boar taint. However, absolute OAVs and their ratios differ markedly to the pattern in boar fat and synergistic effects of odorants resulting in divergent and even new odour impressions have already been observed. The application of cAEDA to fat of the three genders revealed significant differences in the aroma constitution, although these differences were mostly based on quantitative than of qualitative nature. With regard to the incidence of boar taint and an evaluation of the importance of single odorants it has to be kept in mind, that multiple effects will influence the constitution of the present aroma compounds in porcine fat, *i.e.* breed, dietary status, keeping conditions, age. Further studies therefore will have to investigate more animals of different breeds. Additionally, in the case of 2-aminoacetophenone, the importance of this compound for the perception of boar taint has to evaluated in broad consumer studies.

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Comparative Aroma Extract Dilution Analysis (cAEDA) of Fat from Tainted Boars, Castrated Male Pigs, and Female Pigs

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S *Supporting Information*

ABSTRACT: The aroma profile of porcine fat from tainted boars, female pigs, and castrated male pigs was investigated by application of comparative aroma extract dilution analysis (cAEDA) on a SAFE distillate of volatiles prepared from porcine back fat samples. The AEDA resulted in a total of 16 aroma active compounds for boar fat with flavor dilution (FD) factors ranging from 2 to 2048, whereas 12 aroma active compounds were found in fat of female pigs and 14 in fat of castrated male pigs, both with FD factors ranging from 2 to 32. Odor activity values (OAVs) of key components for each fat were identified: In boar fat androstenone, skatole, indole, and 2-aminoacetophenone showed highest OAVs, whereas 2,5-dimethylpyrazine, 2,4-decadienal, and δ-decalactone showed highest OAVs in fat of female pigs. Fat of castrated male pigs showed highest OAVs for skatole, indole, 1-octen-3-ol and methional. Finally, the off-flavor attributes of boar fat were successfully simulated by a recombinant of all odorants at their natural concentration level in deodorized sunflower oil.

KEYWORDS: *aroma extract dilution analysis, boar taint, odor activity values, aroma recombination*

INTRODUCTION

The sensory quality of boar meat is negatively influenced by perceptible concentrations of several compounds. Trained sensory panels showed a strong positive correlation between high levels of androstenone (5α-androst-16-en-3-one) and skatole (3-methylindole) and the occurrence of an off-flavor called "boar taint".[1] Whereas androstenone is endogenously synthesized in the boar's testis with the onset of puberty, skatole originates from the microbiological breakdown of the amino acid tryptophan in the pig's intestine. According to their lipophilic character, both substances can be enriched in the fat tissue after resorption and distribution via the bloodstream. As a steady accumulation takes place, both substances can reach concentrations that may be sensed by consumers during the heating and consumption of pork.[2] Varying androstenone levels between 500 and 3000 ng/g liquid fat and skatole levels between 150 and 250 ng/g liquid fat have recently been reported as thresholds for consumer acceptance.[3−5]

Albeit androstenone and skatole have been shown to play a major role in the occurrence of boar taint, other substances are suggested as contributors to the overall aroma, because occasionally samples with low concentrations of both substances are judged as tainted by sensory panels.[6,7] The aromatic amine indole and the reduced forms of the ketone androstenone, 3-α-androstenol and 3-β-androstenol, were demonstrated to have a minor impact on the perception of boar taint.[8] In addition, some authors propose products of lipid oxidation, for example, aldehydes, ketones, and short-chain fatty acids, and products of intestinal digestion, for example, *p*-cresol, as further boar taint compounds.[7,9,10] Lately, we have focused on hepatic phase I metabolites as possible contributors to boar taint, in particular, the contribution of 2-aminoacetophenone.[19,20]

Wagenberg et al. searched for boar taint compounds besides androstenone and skatole by means of gas chromatography−olfactometry (GC-O).[11] Within this preliminary study several volatiles, which were extracted from fried meat of tainted and untainted boars using dynamic headspace sampling, were postulated to be associated with boar taint. However, the differentiation between the two experimental groups was not based on analytical data but on a human nose scoring system, and no quantitation experiments were performed. Interestingly, application of aroma extract dilution analysis (AEDA) on a distillate of volatiles from boar fat to account for the composition of boar taint is still missing. The concept includes consecutive GC-O experiments with a stepwise-diluted aroma extract.[12] The highest dilution at which a substance can still be smelled is denoted its flavor dilution (FD) factor. FD factors are relative measures and can be used as guidelines to estimate the relative importance of a substance for the overall aroma of food. However, AEDA is only a screening method used to identify the most important odorants among a given set of substances that can interact with human nasal receptors. To

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Table 1. Comparison of FD Factors of Aroma Active Compounds Found in Fat of Boars, Female Pigs, and Castrated Male Pigs

			RI		FD factor		
no.	compound	odor quality	DB-1	Carbo-Wax	boar	female pig	castrated pig
1	methional[a]	potato	894	1465	4	2	2
2	2,5-dimethylpyrazine	roasty	927	1312	nd	16	nd
3	1-octen-3-ol	mushroom	973	1396	16	nd[b]	16
4	octanal	flower	990	1282	4	nd	4
5	(*E*,*E*)-2,4-heptadienal	fatty	992	1373	8	nd	2
6	2-ethylhexanol[a]	phenolic	1019	1488	4	4	2
7	nonanal	fruity	1085	1385	8	8	4
8	*trans*-2-nonenal	cucumber	1137	1511	4	4	2
9	*trans*-2-decenal	fatty	1237	1557	2	2	2
10	indole[a]	fecal	1253	2450	64	4	8
11	2-aminoacetophenone	foxy	1261	2224	32	nd	8
12	(*E*,*E*)-2,4-decadienal	fatty	1288	1635	4	8	8
13	skatole[a]	fecal	1347	2484	2048	16	32
14	*δ*-decalactone	coconut	1450	2216	8	16	16
15	1-dodecanol	sweet	1455	1803	4	2	2
16	2-pentadecanone[a]	fatty	1677	2020	4	4	4

[a]Perceivable in GC-O **without** melting the fat in a microwave oven (instead, the fat was melted under gentler conditions at 60 °C for 2 h). [b]nd, not detectable.

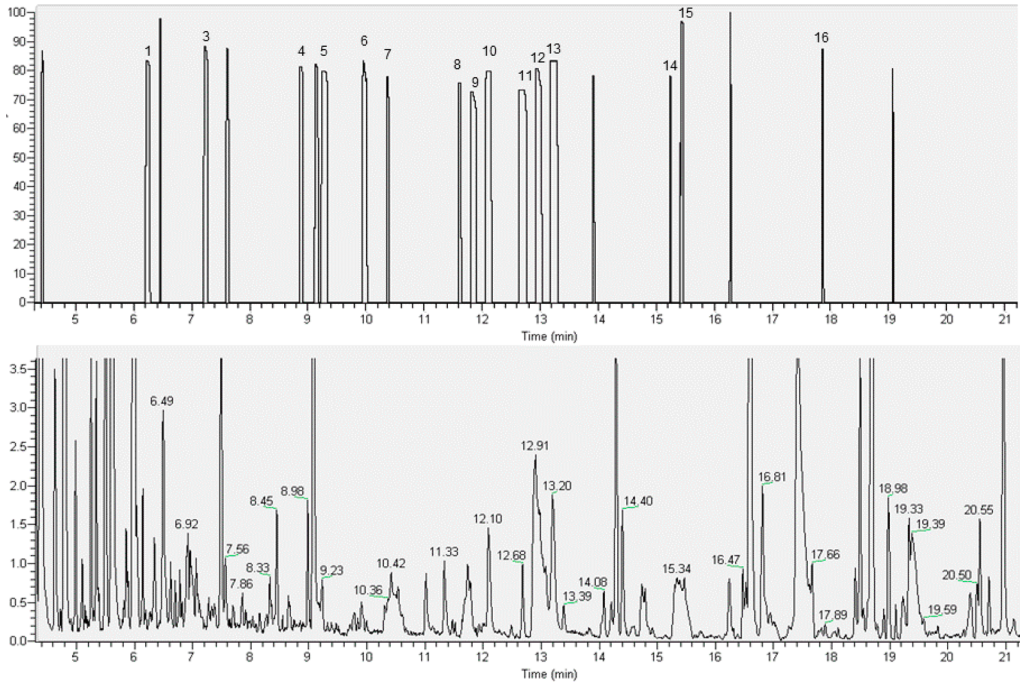

Figure 1. Zoom of a chromatogram (bottom) and corresponding aromagram (top) obtained by application of AEDA on a distillate of boar fat. Odorants are identified by numbers. Numbering is identical with that in Table 1. Peaks without numbers were identified as compounds originating from the chromatographic system.

what extent a certain compound contributes to the overall aroma of a food is based on its interactions with the food matrix, because these effects mainly influence the concentration that is present in the air above a food. The concept of odor activity values (OAVs) accounts for these considerations by dividing the concentration of a compound by its odor threshold. Finally, to verify whether all aroma contributors were correctly identified and quantitated, a reconstitution of the investigated aroma in food matrix is necessary. Therefore, trained panelists develop single odor impressions describing the respective aroma as a basis for the comparison between the aroma profiles of the original and recombinant.[12]

The aim of the present study was thus (i) the identification of the most potent odorants in fat of boars, female pigs, and castrated male pigs based on AEDA, (ii) the performance of quantitation experiments to calculate the OAVs, and finally (iii) the simulation of the aroma of tainted boar fat by recombination experiments.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma-Aldrich (Steinheim, Germany), Alfa Aesar (Karlsruhe, Germany), VWR (Darmstadt, Germany), abcr (Karlsruhe, Germany), or Carl Roth (Karlsruhe, Germany). The deuterium-labeled internal standards d_3-

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skatole, d_3-androstenone, and d_3-2-aminoacetophenone were from ELFI Analytik (Neufahrn, Germany) or DLR (Neustadt/Weinstraße, Germany), respectively.

Fat Samples. Back fat samples from the neck region of intact boars and castrated males, both of type Pietrain × Baden-Württemberg hybrid, together with back fat samples from Baden-Württemberg hybrid female pigs were collected standardized at the slaughter line 12−16 h post-mortem. All animals were raised, penned, and fed ad libitum at the same commercial abattoir. Subsequently, samples were wrapped in aluminum foil, vacuum packaged, and stored at −20 °C until analysis. Among the boar fat samples a preselection was made according to elevated levels of indole, skatole (>0.5 ppm), and androstenone (>2 ppm) by means of stable isotope dilution analysis (SIDA) using headspace solid-phase microextraction gas chromatography−mass spectrometry (HS-SPME-GC-MS).[13] Back fat of three individual animals for each group (boar, female pig, castrated male pig) was pooled and homogenized using a Moulinette to overcome individual physiological differences.

Isolation of the Volatiles. Homogenized porcine back fat was transferred into a 250 mL flask and heated for 2 min at 180 W in a microwave. The occurring liquid fat was separated from the connective tissue by decanting, and subsequently 50 g was transferred into a 250 mL flask. Following extraction with methanol (2 × 100 mL) and a freezing step at −20 °C to separate the crystallized fat by filtration, solvent-assisted flavor evaporation (SAFE) was applied to obtain the volatile fraction.[14] The resulting distillate was concentrated using a Vigreux column (50 cm × 1 cm) to a final volume of approximately 1 mL. In parallel, pure methanol was concentrated as a negative control to ensure that odor active compounds did not originate from the solvent used during extraction.

Gas Chromatography−Olfactometry. GC-O was performed using a Trace GC Ultra (ThermoFisher Scientific, Dreieich, Germany) coupled to a time-of-flight mass spectrometer (BenchTOF-dx, ALMSCO, Llantrisant, UK). GC analysis was conducted with two types of fused silica capillaries: Zebron ZB-1 (30 m × 0.32 mm × 0.25 μm) (Phenomenex, Torrance, CA, USA) and Mega Wax-MS (30 m × 0.32 mm × 0.25 μm) (Mega, Legnano, Italy) at a carrier gas (helium) flow of 1 mL/min. Application of the cool-on-column technique allowed injection temperatures of 40 °C to avoid thermal degradation of the volatiles. The temperature program was set as follows: start at 40 °C, held for 5 min, then raised to 250 °C at a heating rate of 10 °C/min, and held for 5 min. To split the effluent in variable ratios, a microfluidic Deans' switch device (SilFlow, SGE, Ringwood, Australia) was installed, connecting the analytical columns with two deactivated fused-silica capillaries, of which one was directed to the sniffing port (200 °C) and the other was directed to the mass spectrometer. Chromatograms were recorded in full scan mode (m/z 50−600) using electron impact ionization. All odorants were identified on the basis of comparison of retention time and mass spectra to authentic samples. Additionally, a series of *n*-alkanes, C6−C18, was used to determine linear retention indices for each compound. Quantitation was achieved by extraction of specific mass traces of analytes and internal standards (Table 1).

Comparative Aroma Extract Dilution Analysis (cAEDA). The same amounts of back fat (50 g) from the three experimental groups (boar, castrated male pigs, female pigs) were extracted, distilled according to the SAFE method, concentrated to the same volume, and analyzed by GC-O. As the injection volume (1 μL) was the same for all experiments, a direct comparison of FD factors is possible on a semiquantitative basis. Therefore, the concentrated extracts were diluted stepwise 1:1 (by volume) using methanol. The original distillate and each dilution step were evaluated in separate chromatographic runs by three experienced panelists, who were especially trained to detect and identify known boar taint compounds using a commercially available sensory kit (*sense trainer pro*, ELFI Analytik GbR, Neufahrn, Germany). The AEDA results differing by not more than two FD factors were averaged.

Quantitation by Stable Isotope Dilution Analysis. Following the addition of 50 μL of a solution of the isotopically labeled standards dissolved in toluene (d_3-skatole, d_7-indole, d_3-2-aminoacetophenone, d_3-androstenone) to 50 g of liquid fat (concentrations used were determined in previous experiments), the mixture was stirred at a temperature of 60 °C for 60 min to allow for equilibration. Subsequently, the fat was treated as described above for the isolation of volatiles. To obtain the respective response factors, defined ratios of unlabeled and labeled standards (1:5, 1:3, 1:1, 3:1, 5:1) were prepared in fat of female pigs (previous analysis by a published SIDA-HS-GC/MS method showed negligible amounts of the targeted analytes) and were subjected to GC-MS analysis as described below.[13]

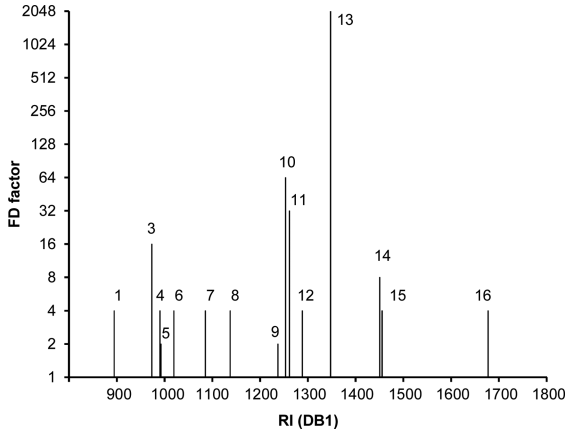

Figure 2. Flavor dilution chromatogram obtained by application of aroma extract dilution analysis (AEDA) on an aroma extract of boar fat. Numbers displayed refer to Table 1.

Quantitation by Standard Addition. For odorants of which no labeled standard was available, we used standard addition as quantitation method. Therefore, different levels of each odorant were spiked to 125 mg of fat. Each level was produced in duplicate. According to preliminary experiments the odorants were divided in two groups according to their expected concentrations. For the first group (δ-decalactone, 2-pentadecanone, 1-octen-3-ol, *trans*-2-nonenal, *trans*-2-decenal, 2,5-dimethylpyrazine, 2,4-decadienal, 2,4-hepatadienal) standard addition ranged from 0 to 12 mg/kg; for the second group (methional, octanal, 2-dodecanol, 2-ethylhexanol, nonanal) standard addition ranged from 0 to 6 μg/kg. Quantitation was performed on 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. The carrier gas flow (helium) was set to 1 mL/min, and the temperature program was set as follows: start at 40 °C, held for 3 min, then raised to 100 °C at a heating rate of 10 °C/min, held for 1 min, then raised to 140 °C at a heating rate of 5 °C/min, and in a final step raised to 240 °C at a heating rate of 10 °C/min. If odorants coeluted with nonsmelling compounds, deconvolution (Target View ver. 5, Markes Int., Neu-Isenburg, Germany) was performed to achieve separation of signals.

Quantitation of Androstenone. Quantitation of the highly lipophilic boar taint compound androstenone was achieved by HS-SPME sampling of aroma extracts (see Isolation of the Volatiles). In this context 0.5 mL of each aroma extract (boar, castrated male pig, and female pig) together with 10 μL of d_3-androstenone, yielding a final concentration of 0.5 μg/mL, was transferred into 10 mL HS vials and then subjected to GC-MS analysis. HS-SPME sampling was performed using an automated heating agitator and a divinylbenzene/polydimethylsiloxane-coated SPME fiber (DVB/PDMS, 85 μm, Supelco, Bellefonte, PA, USA). Sampling conditions were as follows: equilibration for 5 min at 55 °C, extraction for 15 min at 55 °C, desorption for 10 min at 270 °C within the GC injector. The temperature program was set as follows: start at 40 °C, held for 3 min, then raised to 180 °C at a heating rate of 15 °C/min, and finally raised to 270 °C at a heating rate of 5 °C/min. Chromatograms were recorded in full scan mode (EI, m/z 50−300) using electron impact ionization (EI). Quantitation of androstenone was achieved by extraction of specific mass traces of natural and labeled androstenone: m/z 257 and 272 for androstenone and m/z 260 and 275 for d_3-

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Table 2. Odor Activity Values (OAVs) and Orthonasal Odor Thresholds of Aroma Active Compounds Found in Fat of Boars, Female Pigs, and Castrated Male Pigs

					OAV		
no.	compound	coeff of determination[i]	R_f	odor threshold (μg/L)	boar	female pig	castrated pig
1	methional[j]	0.789	25[h]	0.2[a]	21	15	20
2	2,5-dimethylpyrazine[j]	0.997	89[h]	8[b]	nd[k]	29	nd
3	1-octen-3-ol	0.998	154[h]	34[a]	22	nd	50
4	octanal	0.959	22[h]	56[a]	>1	nd	1
5	2,4-heptadienal[j]	0.992	123[h]	4000[a]	2	nd	3
6	2-ethylhexanol[j]	0.990	81[h]	0.4[c]	>1	>1	>1
7	nonanal	0.996	100[h]	1000[a]	2	3	>1
8	*trans*-2-nonenal	0.998	62[h]	900[a]	1	1	>1
9	*trans*-2-decenal	0.998	54[h]	33800[a]	>1	>1	>1
10	indole	0.999	0.82[g]	25	35	2	40
11	2-aminoacetophenone	0.998	0.79[g]	150[d]	30	nd	1
12	2,4-decadienal	0.927	101[h]	180[a]	1	14	12
13	skatole	0.999	0.84[g]	15.6[e]	40	>1	8
14	δ-decalactone[j]	0.855	39[h]	400[a]	15	21	17
15	1-dodecanol[j]	0.898	32[h]	110	3	3	3
16	2-pentadecanone[j]	0.959	46[h]	1800	>1	>1	>1
17	androstenone	0.998	0.44[g]	426[f]	25	nd	nd

[a]Odor thresholds reported in ref 42. [b]Odor threshold reported in ref 43. [c]Odor threshold in air reported in ref 44. [d]Odor threshold reported in ref 19. [e]Odor threshold reported in ref 18. [f]Odor threshold reported in ref 45. [g]Response factor determined by analyzing defined mixtures of analyte and internal standard. [h]Response factor defined as slope^{-1} × 10^{-3}. [i]Coefficient of determination obtained by linear regression analysis of the calibration data. [j]Compound described in pig fat for the first time. [k]nd, not detectable.

androstenone. Identification was based on the comparison of retention time and mass spectrum to an authentic standard.

Aroma Profile Analysis. Trained sensory panelists conducted aroma profile analysis by rating seven selected aroma attributes (roasty, rancy, urinous, fecal, floral, synthetic, fatty) on a seven-point linear scale from 0 (not perceivable) to 3 (strongly perceivable). The panel consisted of seven assessors, who were especially trained in weekly training sessions to recognize and describe different aroma qualities. Sensory analysis were performed in a room at 21 ± 1 °C equipped with a single sensory booth. The fat samples (10 mL) were presented in covered glass vessels (25 mm i.d., total volume = 45 mL) at a temperature of 90 ± 5 °C.

Aroma Recombination. In terms of aroma recombination, solutions of all compounds with an OAV ≥ 1 were prepared in methanol. Prior to the aroma profile analysis conducted as described above, the aroma compounds were added to deodorized sunflower oil (Wesermühle, Bremen, Germany) in the concentration levels found in boar fat. The original boar fat and the recombinate were evaluated by a trained sensory panel using a seven-point scale from 0 (not identical) to 3 (identical).

RESULTS AND DISCUSSION

Identification of Key Odorants in Pork Fat. Previously microwave-melted porcine back fat of three different genders (boar, female pig, castrated male pig) of Piétrain × Baden-Württemberg hybrid type was extracted using methanol and stored at −20 °C to separate the fat phase. Subsequently, the methanolic extract was subjected to SAFE distillation. The resulting aroma extracts were then examined by means of AEDA. All experiments were carried out in duplicate, and two types of negative control were performed to exclude odorants that would originate from solvents: First, the same amount of methanol that was used during extraction was concentrated to the final volume of the aroma extracts. Injection of such controls showed no odor active compounds in GC-O experiments. Second, the separation of liquid fat of connecting tissue was realized under gentler conditions by melting the minced fat at 60 °C for 2 h to test if the generation of odorants was heat-induced. In these experiments it turned out that 10 of 16 odorants were at least partially formed due to the melting step in the microwave, because these compounds were not perceivable without a melting step within the microwave. However, we do not know whether these compounds would not have been formed without using the microwave or just in such small amounts that we could not identify them via GC-O. Nevertheless, a heating step in the microwave was assumed as a realistic simulation for preparation steps of meat in households, for example, frying in a pan.

To identify key aroma compounds of each pork fat, aroma extracts were diluted stepwise 1:1 (by vol) with methanol. The original distillate and each dilution step were examined by means of GC/TOF-MS-O by three experienced panelists. In total, 15 odor active compounds were nasally identified in boar fat (Figure 1), whereas 12 and 14 odor active compounds were found in fat of female pigs and castrated pigs, respectively. With regard to boar fat, FD factors ranged from 2 to 2048 (Figure 2), whereby the highest values were found for compounds **3** (mushroom), **11** (foxy, synthetic), **10** (animal, sweet), and **13** (animal, sweet, fecal-like). With respect to fat of castrated males and females, FD factors ranged from 2 to 1024, whereby the highest values were assigned to compounds **2** (popcorn, caramel), **13**, and **14** (coconut) for fat of female pigs and to compound **3** (mushroom like), **14**, and **13** for fat of castrated male pigs (Table 1).

To achieve reliable results, the identification of odor active compounds was based on the following steps: First, mass spectra (MS-EI) of aroma active analytes were compared to spectra listed in the NIST library. Second, retention indices of aroma active analytes were determined on two GC columns of orthogonal polarity (DB-1, Carbowax) and subsequently compared to values listed in the flavornet database. Third, aroma quality, intensity, mass spectra, and retention indices of identified candidates were compared to reference compounds by means of GC-O at similar concentration levels. Due to its

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comparably high lipophilicity (K_{OW} = 400,000) identification and quantitation of androstenone was achieved by HS-SPME sampling of the aroma extracts.[15]

According to this procedure, skatole, indole, and 2-aminoacetophenone were identified as the most potent odorants in boar fat, followed by δ-decalactone and 1-octen-3-ol. As chromatographic and nasal identification of androstenone was achieved by HS-SPME sampling of the aroma extract due to the compound's high lipophilicity, a direct comparison of FD factors was not performed. In fat of castrated male pigs 1-octen-3-ol, skatole, and δ-decalactone were dominating, whereas 2,5-dimethylpyrazine, δ-decalactone, nonanal, and skatole showed the highest FD factors in fat of females.

Quantitation of Key Aroma Compounds and calculation of Odor Activity Values. With regard to androstenone, skatole, indole, and 2-aminoacetophenone, quantitation of odorants was based on SIDAs, whereas the quantitation of all other aroma compounds was realized via standard addition (Table 2). Because boar fat samples, which had already been judged as tainted by panelists, were selected, high concentrations of androstenone, skatole, and indole were expected.[16] 2,4-Heptadienal, nonanal, 1-octen-3-ol, and 2-aminoacetophenone also showed high concentrations. Somewhat lower amounts were found for indole and δ-decalactone. In fat of female pigs, highest concentrations were found for 2,5-dimethylpyrrazine, 2,4-decadienal, and δ-decalactone. In the case of fat of castrated male pigs, high amounts of 2,4-heptadienal, 1-octen-3-ol, skatole, indole, and δ-decalactone were observable.

The impact of a single odorant for the overall aroma of a certain food is based on two attributes: the possibility to interact with human odorant receptors and the extent of possible interactions with the food matrix.[17] These interactions have a direct impact on the concentration of an odorant that is present in the air above a food. Thus, it is necessary to calculate OAVs of identified aroma compounds, which is the quotient of concentration and respective odor threshold of a compound in the dominant matrix of a food. If possible, published odor thresholds of the identified compounds were used to calculate OAVs. In the case of indole, 1-dodecanol, and 2-pentadecanone, thresholds were estimated using a published protocol.[18] All odor thresholds used were determined in fatty matrices. Interestingly, many compounds were not present in concentrations above their odor thresholds, resulting in OAVs <1. In the case of methional, AEDA leads to an underestimation of its aroma contribution. As expected, androstenone, skatole, and indole share high OAVs in boar fat. Additionally, an important contribution to the overall aroma of boar fat is assigned to 2-aminoacetophenone in our experiments, resulting in an OAV of 30, which is consistent with our previous assumptions about the possible contribution of this compound to boar taint.[19,20]

Comparison of Three Different Fats. The results of our experiments reveal that differences in the aroma of fat of the investigated fat types are more quantitative than qualitative in origin. Except for androstenone and 2,5-dimethylpyrazine all compounds were detectable in at least two of three samples. With regard to boar fat, 1-octen-3-ol, 2-aminoacetophenone, and methional play an important role next to the more "classical" boar taint compounds androstenone, skatole, and indole. Surprisingly, skatole, indole, 1-octen-3-ol, and methional show the highest OAVs in fat of castrated male pigs, too. However, OAVs of these odorants are lower and occur in different ratios than in boar fat. In this respect, fat of female pigs constitutes an exception, as the most relevant odorants, 2,5-dimethylpyrrazine, 2,4-decadienal, and δ-decalactone, are either not present in the other samples or are less important.

Aroma Simulation. Recombination of the overall aroma perception of boar fat was focused on descriptors of boar taint to verify if all compounds contributing to the off-flavor were correctly identified and quantitated. To that end, all aroma compounds with OAVs >1 were spiked into deodorized sunflower oil at their determined concentrations. A sensory panel familiar with the detection of boar taint identified single odor attributes describing original boar fat and an aroma reconstitution. The aroma reconstitution showed good similarities with the original boar fat (Figure 3). However, slight differences could be due to a lack of very lipophilic compounds.[21]

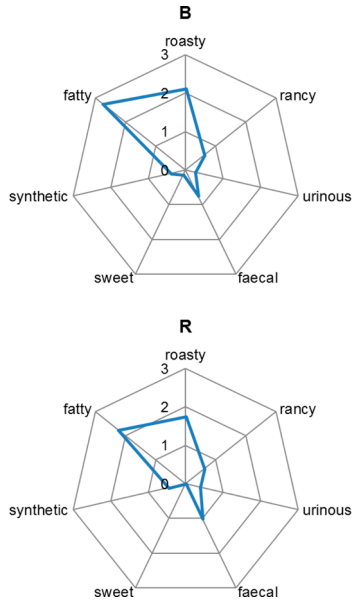

Figure 3. Aroma profile analysis of boar fat (B) and the corresponding recombinate (R). Descriptors and corresponding intensities were evaluated by six trained panelists familiar with the description of boar taint.

Possible Generation Pathways of Odorants. Androstenone, skatole, and indole are well-known key substances causing an undesirable aroma in the fat of boars.[22] Whereas androstenone is produced in the Leydig cells of the testis with the onset of puberty, skatole and indole arise from the microbiological breakdown of tryptophan in the pig's intestine. Due to their high lipophilicity these substances steadily enrich in adipose tissue after transportation via the bloodstream.[2] The absence or lower concentration of skatole and indole in female and castrated male pigs, which is reflected in lower OAVs in our experiments, is often explained by the general missing (castrated pigs) or lower concentrations (female pigs) of gonadal steroids leading to different expression levels and activities of cytochrome P450 (CYP) enzymes.[23] Additionally, the dietary composition of pigs was shown to have a strong effect on plasma and back fat levels of skatole.[24] Interestingly, our experiments revealed skatole and indole as important aroma compounds in the fat of castrated male pigs, although the occurrence of boar taint is practically avoided by the practice of surgical castration.[25] However, absolute OAVs and the ratios to other main odorants differed remarkably compared

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to boar fat. A synergistic effect of aroma components resulting in new odor perceptions deviant from the simple sum of individual aroma contributions is well-known and could serve as an explanation here.[26] 2-Aminoacetophenone was reported to be one of seven major porcine hepatic phase I metabolites of skatole.[27] This compound was already shown to be the perpetrator of an off-flavor in white wines, which led us to the idea that it could be involved in the origin of boar taint, too.[28−30] In this context we determined high formation rates for 2-aminoacetophenone in boars of Pietrain × Baden-Württemberg hybrid type in in vitro experiments with liver microsomes.[20] These formation rates could serve as an explanation for back fat levels of 2-aminoacetophenone above a determined sensory threshold of 150 ng/g_{fat} that were found recently.[19] In the light of these findings, the high FD factor and OAV of 2-aminoacetophenone in Pietrain × Baden-Württemberg hybrid type boars emphasize the possible involvement of this compound in the origin of boar taint. Other compounds previously reported to be associated with boar taint, such as *p*-cresol, 3-*α*-androstenol, 3-*β*-androstenol, and 4-phenyl-3-buten-2-one, were not detected in this study.[8,9,16] Methional was already reported as a degradation product of methionine via Strecker reaction in meat and meat products, whereas 1-octen-3-ol was identified as a product of the oxidation of fatty acids.[31,32] As the formation of *δ*-lactones from hydroxy fatty acids has already been described in milk fat, it seems reasonable to assume that similar pathways can occur in porcine fat, especially during the heating process.[33] In samples derived from female pigs, calculation of OAVs revealed important aroma contributions of 2,5-dimethylpyrazine, 2,4-heptadienal, and 2,4-decadienal besides *δ*-decalactone. Among these compounds 2,5-dimethylpyrazine, the odor of which is often described as roasty or earthy, has already been identified as a product of Maillard reactions in roasted lamb and beef meat.[34,35] The formation of 2,4-decadienal as a degradation product of methyl 9-hydroperoxy-(*E*,*Z*)-10,12-octadecadienoate (9-HPOD) has been demonstrated in thermally treated fats. In this context 2,4-decadienal occurred as an intermediate in reaction pathways leading to the potent aroma compound *trans*-4,5-epoxy-(*E*)-2-decenal (ED), a compound that was identified as a key odorant in the warmed-over flavor in beef or the fishy off-flavor of butter fat.[36] However, no ED could be found in our experiments. Elmore et al. found elevated levels of 2,4-heptadienal in cooked beef and lamb muscle from animals fed dietary supplements high in *n*-3 fatty acids and identified this compound as an oxidation product of these substrates.[37,38]

In summary, 15 aroma active compounds in boar fat of type Pietrain × Baden-Württemberg hybrids were identified and quantitated, leading to a successful reconstitution of the off-flavor profile. Our experiments revealed that the enhanced formation of most aroma contributors was due to the heating procedure in the microwave. Qualitative and quantitative differences between the fat of female pigs and castrated male pigs were also elucidated by means of AEDA and the calculation of OAVs. However, an important aspect that has to be taken into account are addition and suppression effects of odorant mixtures, which have previously been demonstrated in binary systems.[39,40] Recently, a trained sensory panel demonstrated such effects between the boar taint perpetrators androstenone and skatole.[41] Such findings emphasize the need of broad consumer studies in which such effects can be investigated.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b04747.

Table S1: Concentrations of the most important aroma compounds in porcine fat (PDF)

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Notes

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3 Closing remarks and outlook

In the presented thesis hepatic phase-I-metabolites of skatole were identified as putative contributors to the off-flavour boar taint for the first time. Special focus was put on 2-aminoacetophenone, a compound that has extensively been investigated in the context of aroma research. Two questions served as basis for the conducted studies: First, is 2-aminoacetophenone a possible contributor to boar taint? Second, what are the reaction pathways leading to the compound's formation in pigs?

The presented publications dealing with the quantification of 2-aminoacetophenone in boar fat, the determination of odour thresholds and the elucidation of the overall aroma composition of boar fat were addressed to the first question. In this context, 2-aminoacetophenone concentrations above the nasally perceivable threshold were determined in boars of Pietrain x Baden-Württemberg hybrid type by the adaption of a recently developed SIDA-HS-SPME-GC/MS methodology. Furthermore, a decisive contribution of the compound to the overall aroma of boar fat was demonstrated by the application of AEDA to fat of boars, female and castrated pigs.

The second opening question is addressed by the publications regarding the product profiles of *in vitro* incubations using porcine liver microsomes. To the best of the author's knowledge, such incubation experiments were never before investigated with respect to their importance for aroma development using HS-SPME sampling. The presented results elucidate the reaction pathways leading to 2-aminoacetophenone formation in pigs and provide evidence for the involvement of specific CYP 450 isoforms.

Nevertheless, boar fat with and without addition of 2-aminoacetophenone has to be evaluated by trained panellists in large scale experiments to verify its boar taint enhancing effect. Additionally, such results, which are produced under standardized conditions by trained assessors, must be confirmed in broad consumer studies to finally substantiate the influence of 2-aminoacetophenone on the liking or disliking of boar meat.

The presented results regarding the biogenesis of 2-aminoacetophenone in pigs are of particular interest for boar fattening issues. Only if all reaction pathways, involved enzymes and interfering factors are identified and understood, efforts can be made to reduce 2-aminoacetophenone levels by means of breeding, raising and feeding. To that end, it is very important to expand future studies to other breeds in order to understand breed related differences in the hepatic metabolism of pigs. Furthermore, individual CYP 450 isoforms

involved in the generation of 2-aminoacetophenone in pigs have to be identified by the application of individually expressed CYP 450 in incubation experiments. This practice would also allow to draw further conclusions by the investigation of kinetic isotope effects.

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