FACULTY OF VETERINARY MEDICINE
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Valorisation of boar meat and analytical approaches for the fast detection of boar taint at the slaughter line

Kaat Verplanken

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AGENTSCHAP INNOVEREN & ONDERNEMEN

PROMOTERS

Prof. Dr. Ir. Lynn Vanhaecke
Faculty of Veterinary Sciences, Ghent University

Dr. Jella Wauters
Faculty of Veterinary Sciences, Ghent University

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Vion Food Group

Dr. Sara Stead
Waters Corporation

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LIST OF ABBREVIATIONS

2-MID	2-methylindole
3-β-HSD	3-β-hydroxysteroid dehydrogenase
4-VP	4-vinylpyridine
5-MID	5-methylindole
6-MOID	6-methoxyindole
ACN	acetonitrile
ADD	1,4-androstadiene-3,17-dione
AEON	androstenone (5α-androst-16-ene-3-one)
AGC	automatic gain control
AIBN	2,2'-azobis(isobutyronitrile)
AMS	ambient mass spectrometry
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionisation
API	atmospheric pressure ionization
B_{max}	maximum amount of binding sites
CAR	carboxen
CCF	central composite face-centred
CI	chemical ionization

CLT	central location test
COUP-TF1	COUP transcription factor 1
CYP2E1	cytochrome P450 2E1
DAPCI	desorption atmospheric pressure chemical ionization
DART	direct analysis in real time
DCBI	desorption corona beam ionization
DEMI	desorption electrospray/metastable-induced ionization
DESI	desorption electrospray ionization
DFD	dark firm dry
DLS	dynamic light scattering
DMP	2,2-dimethoxy-2-phenylacetophenone
DVB	divinylbenzene
e-noses	electronic noses
EC	electron capture
ECD	electron capture detector
EGDMA	ethylene glycol dimethacrylate
EI	electron impact ionization
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay

ESI	electrospray ionization
F1-Bactoferm	*Pediococcus pentosaceus* + *Staphylococcus xylosus*
F2-Bactoferm	*Lactobacillus farmicis* + *Staphylococcus carnosus* + *Staphylococcus xylosus*
FIA	fluoroimmunoassay
FID	flame ionization detector
FSH	follicle-stimulating hormone
FTIR	fourier transform infrared spectroscopy
FWHM	full width half maximum
GC	gas chromatography
GnRH	gonadotropin-releasing hormone
HCD	high-energy collision dissociation
HRMS	high resolution mass spectrometry
HS	headspace
HUT	in-house use tests
IF	imprinting factor
iKnife	intelligent knife
IND	indole (2,3-benzopyrrole)
IR	infrared

K_D	apparent dissociation constant
LC	liquid chromatography
LDA	linear discriminant analysis
LDSPI-MS	laser desorption spray post-ionisation mass spectrometry
LH	luteinizing hormone
LIAD	laser-induced acoustic desorption
LOD	limit of detection
LOQ	limit of quantification
MAA	methacrylic acid
MALDI	matrix assisted laser desorption
MC4R	melanocortin-4-receptor
MeOH	methanol
MIP	molecularly imprinted polymer
MOS	metal-oxide semiconductor
MS	mass spectrometry
MUFA	monounsaturated fatty acid
NAPH	naphthalene
NIP	non-imprinted polymer
NSAID	non-steroidal anti-inflammatory drug

OPLS-DA	orthogonal partial least-square discriminant analysis
PA	polyacrylate
PCA	principal component analysis
PdI	polydispersity index
PDMS	polydimethylsiloxane
PI	penning ionization
PT	proton transfer
PUFA	polyunsaturated fatty acid
QTL	quantitative trait loci
RADIO	radio frequency acoustic desorption and ionization
REIMS	rapid evaporative ionization mass spectrometry
RIA	radioimmunoassay
ROC	receiver operating characteristic
RP	reverse-phase
S/N	signal-to-noise
SEM	scanning electron microscopy
SERS	surface-enhanced RAMAN spectroscopy
SFA	saturated fatty acid
SIM	single ion monitoring

SK	skatole (3-methylindole)
SNP	single nucleotide polymorphisms
SPE	solid phase extraction
SPME	solid phase microextraction
SULT1A1	sulfotransferase family 1A member 1
SULT2A1	sulfotransferase family 2A member 1
T-SC-150 Bactoferm	*Lactobacillus sakei* + *Staphylococcus carnosus*
TCD	thermal conductivity detector
TOF	time-of-flight
TRIM	trimethylpropane trimethacrylate
(U)HPLC	(ultra-)high performance liquid chromatography
WLSLR	weighted least squares linear regression

CHAPTER I

GENERAL INTRODUCTION

1. PIG INDUSTRY: BACKGROUND AND LEGISLATION

With a livestock of approximately 186 million pigs each year, the pig industry is one of the major sectors in agriculture in Europe. Within Europe, the largest shareholders are Germany, Spain and France, respectively. Belgium accounts for the slaughter of 4% of the total number of pigs in Europe, which corresponds to approximately 12 million pigs and a total carcass weight of 1.13 million tonnes each year (Fig 1)[1]. Consequently, the pig industry is an economically very important sector in Belgium and especially in Flanders, which is characterized by a self-sufficiency rate of 260% and annual trade balance of €1 billion [2].

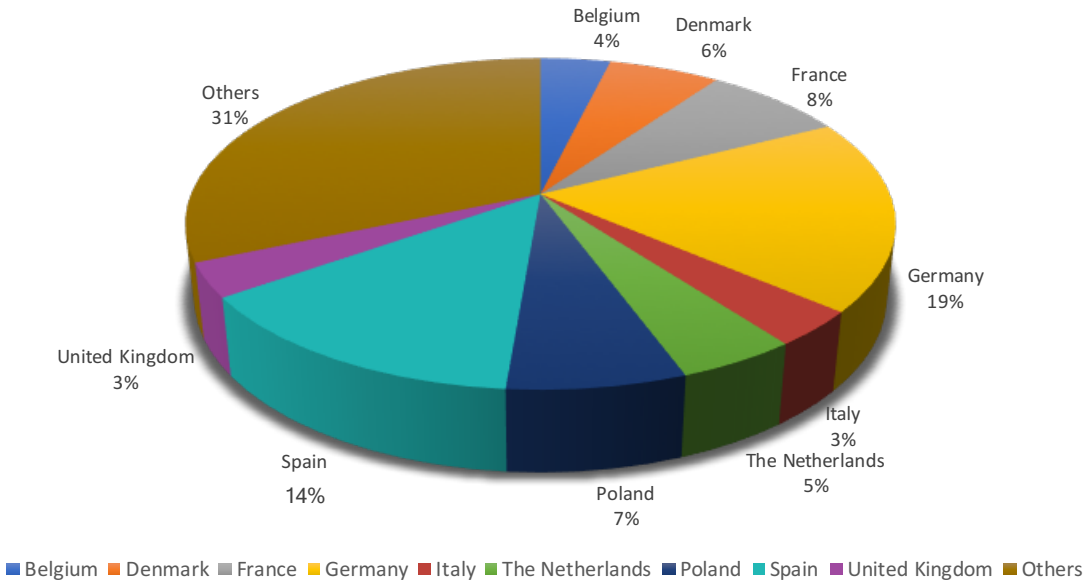

Fig 1 Percentage of pigs slaughtered in Europe in 2014.

During the past decades, increasing concerns regarding animal welfare led to several adaptations within pig husbandry to improve the life quality of pigs. As such, minimum requirements for the protection of pigs were enforced and standards for the protection of pigs as regards measures to reduce the need for tail docking were laid down [3, 4]. Despite these requirements, tail docking is still widely practiced. Apart from the above measures, important efforts in terms of the surgical castration of piglets have been imposed. Indeed, in 2001, a Commission Directive was implemented, stating that

the surgical castration of piglets must be performed by other means than the tearing of tissues [5]. Moreover, from 2008, the castration of piglets older than 7 days must be performed under sedation and prolonged analgesia and may only be executed by a veterinarian [4]. Additionally, in 2010, several European actors in the pig sector agreed to voluntarily implement alternatives for the surgical castration of pigs [6]. As a consequence, since 2012, the surgical castration of pigs should only be performed under prolonged analgesia and/or anaesthesia. In the long run, the surgical castration will phase out as the pig sector agreed on a voluntary abandonment of the latter by January 1st 2018, provided that alternatives for surgical castration are economically feasible. However, as this voluntary agreement has not been converted into European law, compliance with these engagements is questionable and still under discussion [7, 8].

Originally, the surgical castration of pigs was implemented to prevent undesired sexual and aggressive behaviour in pigs. In addition, it was also intended to prevent the occurrence of boar taint, an off-odour that is released when meat or fat of non-castrated boars is heated [9, 10]. Furthermore, castration was also a market driven choice, seen it increases the proportion of fat in carcasses, which was desired by consumers [10]. Because of the intended ban on the surgical castration, the rearing of entire male pigs has gained increased attention. However, the main problem associated with the latter is the possible re-occurrence of boar taint, which evokes negative consumer responses and can lead to significant economic losses, not only in Belgium, but also in Europe [7]. For this reason, three conditions were postulated in the European declaration that should be met in order to render entire male pigs as a sustainable alternative for surgically castrated pigs. One of these conditions involves the reduction of boar taint prevalence in entire male carcasses through pig breeding, management and feeding strategies. Second, the acceptance of products of entire male pigs by consumers, authorities and export markets should be ensured. Finally, in order to prevent tainted carcasses to reach consumers, rapid and accurate detection methods for boar taint should be implemented at the slaughter line [6]. The focus of this PhD study fits in the latter two conditions, i.e. valorisation of boar meat and the development of fast detection methods for boar taint at the slaughter line.

2. ALTERNATIVES TO SURGICAL CASTRATION

Annually, approximately 100 million pigs or 83% of the male pig population are surgically castrated in Europe [11]. Until recently, surgical castration was mostly performed without the use of anaesthesia and/or analgesia. However, contrary to what was presumed, surgical castration causes pain, even in very young piglets (< 7 days) [12]. For example, the testis and skin of the scrotum are innervated with nociceptors, which makes castration a painful and stressful process [13]. Consequently, castration causes an acute pain sensation induced by the castration procedure and chronic pain that may be noticed after castration [14]. Moreover, the pain and distress associated with the castration procedure may create behavioural changes in piglets, which are evident days after castration [12, 15].

Since research showed that castration causes pain, societal pressure to abandon the surgical castration of pigs has increased. In order to gradually abandon the surgical castration of pigs by 2018, different alternatives thereto were proposed in order to effectuate an improvement in terms of animal welfare [6]. Research revealed several alternatives for the surgical castration of pigs: (i) surgical castration of pigs with analgesia and/or anaesthesia, (ii) immunocastration, (iii) production of entire male pigs and (iv) sexing of sperm.

2.1. Surgical castration with analgesia and/or anaesthesia

One of the alternatives for surgical castration of pigs that could be implemented in the short term, is surgical castration in combination with analgesia and/or anaesthesia. The use of castration with anaesthesia, whether or not in combination with analgesia, is a common practice in Norway, Switzerland, Germany and Sweden as it is mandatory by law since 2002, 2010, 2013 and 2016, respectively [7, 16]. In The Netherlands, market-driven initiatives have led to the implementation of the use of anaesthetics and/or analgesia during castration [17]. Pig producers in France that take part in the national quality assurance program (QT) use analgesia (meloxicam) during surgical castration. In Austria and Denmark, prolonged analgesia is compulsory by law [16]. In other European countries, including Belgium, an intention agreement exists on the use of analgesia and/or anaesthesia during

surgical castration since 2012. However, up until now anaesthesia is only used in a minority of herds [7, 8].

Surgical castration in combination with analgesia involves the administration of non-steroidal anti-inflammatory drugs, e.g. meloxicam, flunixin meglumine and ketoprofen or sometimes non-opioid pyrazolone derivates such as metamizole at least 10 minutes prior to castration [12, 18, 19]. Administration of analgesia through intramuscular injection is the method of choice [16]. Monitoring of the blood cortisol levels in pigs administered with different analgesia prior to castration suggested an effective pain relief with administration of meloxicam and an effect of metamizol after 4 hours post-administration [19, 20]. Another study on the other hand, in which apart from cortisol levels, also other stress indicators such as vocalisation, physiology and behaviour of piglets were monitored, indicated no beneficial effect of meloxicam on stress and pain relief [21]. Therefore, the use of analgesia to reduce pain and stress sensation during castration is not recommended as a stand-alone therapy. Lidocaine and procaine are used most frequent as a local anaesthetic and are administered through injection in the testis or spermatic cord [13]. Recent reports indicate the effectiveness of lidocaine administration by a topical spray or gel. However, these methods are currently not in use in practice [16]. Local anaesthesia alleviates most of the acute pain caused by castration, but is not effective against post-operative pain, whereby it is often combined with analgesia [11]. Other studies failed to find evidence for effective pain relief by administration of local anaesthetics prior to castration [22, 23]. This is most likely due to the induction of stress and pain caused by intratesticular or –funicular injection of the anaesthetic, which partly nullifies the beneficial effect of the anaesthetic [12]. Apart from analgesia and/or local anaesthesia, also general anaesthesia can be applied through inhalation or although used to a lesser extent, injection [12]. Carbon dioxide seemed a promising gas for anaesthesia through inhalation, as it is safe to use, cheap and an effective alternative for gases such as isoflurane and halothane. The latter indeed pose a risk for the administrator if no gas evacuation system or adequate ventilation is provided [13, 21]. However, the use of carbon dioxide is not recommended because of its aversive impact on the animal and limited safety margins [16]. Also

general anaesthesia by injection provides satisfactory pain relief; however, recovery takes up to 3h and an increased mortality rate is observed (3-5%) in comparison to the administration of anaesthetic gases (1%) [12, 24].

In terms of animal welfare, the use of local anaesthesia (lidocaine) in combination with prolonged analgesia seems the most effective to reduce acute and chronic pain induced by surgical castration [12]. Moreover, taking into account all pros and cons, this method is preferred as it is associated with only moderate costs for drugs and veterinary services. General anaesthesia through inhalation (CO_2/O_2, isoflurane) should be avoided as it poses a potential health risk if not properly applied [16]. The administration of an analgesic is insufficient to relief acute pain distress, but can alleviate post-operative pain [21, 25]. Anaesthesia on the other hand only has an effect on acute pain sensation during castration. For this reason, the combined administration of anaesthesia and analgesia is advised [12]. However, overall, the effect on net welfare benefit remains questionable as the administration of analgesia and anaesthesia also induces stress and discomfort in piglets [16, 26, 27]. In addition, long lasting pain reducing drugs that could effectively alleviate pain during and after castration are not available for the use on piglets [16]. Moreover, on a larger-scale farmer level it is difficult to optimize the time between administration and castration, which consequently affects the degree to which the stress response to castration is reduced [12, 28].

In general, Norwegian consumers are satisfied with the practice of surgical castration in combination with the administration of analgesia and/or anaesthesia, as they consider surgical castration a necessary means to prevent the occurrence of boar taint, an off-odour that may occur in meat of non-castrated boars. Surgical castration without use of anaesthesia or analgesia is considered as unacceptable by consumers [11]. Tuyttens et al., on the other hand found no evidence for this preference as they observed no difference in consumers' attitude between surgical castration with and without usage of prolonged anaesthesia and analgesia [29]. Farmers on the other hand are more sceptical towards the use of anaesthesia and analgesia. The main reason for this is their concern about

associated additional costs and labour [30, 31]. Moreover, Belgian farmers are not convinced about the animal welfare benefits and show a negative attitude towards use of anaesthesia and analgesia as administration should be performed by a veterinarian [30, 32]. In some countries (The Netherlands, Sweden and Switserland), anaesthesia may be administered provided that they follow a specific training course [16]. However, in case of baring the additional costs over the entire sector, which are estimated on 0.19-0.39 euro per piglet, their attitude towards the use of anaesthesia and analgesia is more positive [17, 30].

Since the net benefits of analgesia and/or anaesthesia may be questioned, this alternative for the surgical castration of pigs is not considered as full-fledged in the long run. Indeed, the intentional agreement on alternatives for the surgical castration of pigs stated to gradually phase out surgical castration and eventually completely abandon the surgical castration of pigs with or without the use of anaesthesia and/or analgesia [6]. For this reason, implementation of other alternatives should be pursued.

2.2. Immunocastration

A different approach for castration is immunocastration or vaccination against the gonadotropin-releasing hormone (GnRH). GnRH is synthesized in the hypothalamus gland and induces the pituitary gland to release the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH). The latter hormones, in their turn, stimulate the gonads and consequently induce the development of the testis and synthesis of testicular hormones, such as androstenone (AEON) [33, 34]. Consequently, vaccination against GnRH inhibits the induction of LH and FSH synthesis and release, whereby the growth of the testis and thus production of testicular hormones, is suppressed (Fig 2) [14, 34, 35].

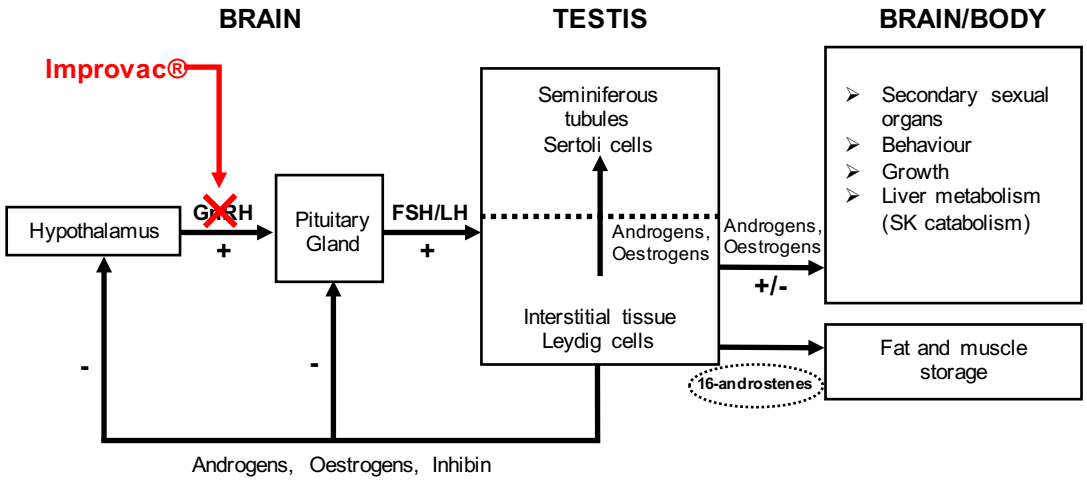

Fig 2 Overview of the principle of immunocastration through Improvac® and its influence on the hypothalamic-pituitary-gonadal axis.

The use of immunocastration, in the form of the commercially available vaccine Improvac®, is permitted in Europe since 2009 as an alternative to surgical castration. The immunisation process includes two subcutaneous injections, with a time span of minimum 4 weeks [12]. The first vaccination that induces the immune system, can be administered from an age of 10 weeks and is followed by a second vaccination mostly 4 to 6 weeks prior to slaughter. A maximum time gap of 10 weeks is advised between the first and second vaccination. The second vaccination causes neutralisation of the endogenous GnRH and thus inhibition of the testicular functions. This effect is noticeable until 22 weeks after the second vaccination [34, 36].

A major advantage of immunocastration relative to surgical castration (with or without analgesia and anaesthesia) is the better feed conversion ratio of immunocastrated pigs compared to surgically castrated pigs [37]. In comparison to entire male pigs, immunocastrates have a higher fat deposition and average body weight when slaughtered at the same age. This effect becomes apparent after administration of the second vaccination, as from then their feed intake increases and the animals become less active [37-39]. In comparison to surgically castrated pigs, immunocastrates have a significantly higher percentage of lean meat [33]. The carcass yield of immunocastrates however, is lower in comparison to surgically castrated pigs, because of its heavier gastrointestinal weight [18]. The meat thickness of immunocastrates is intermediate between that of surgically castrated pigs and

entire males. The pH and drip loss of immunocastrates is the highest in immunocastrated pigs [18]. However, these differences in meat quality are not significant and are unlikely to be perceived by consumers [38, 40-42]. The biggest advantage of immunocastration in comparison to entire male pigs is the absence of boar taint. Indeed, immunocastration effectively reduces boar taint compound levels and thus eliminates the risk on impairing consumer acceptance due to presence of the latter [36].

In contrast to the surgical castration of pigs, immunocastration effectively reduces pain and animals distress and thus provides an advantage in terms of animal welfare. For this reason, in the long run, immunocastration is seen as a viable alternative for the surgical castration of pigs [33, 43]. Moreover, immunocastrates represent less sexual and aggressive behaviour in comparison to entire males [34, 44, 45]. Consequently, less tail biting, mounting behaviour and claw injuries are observed in immunocastrates, which leads to a better general health condition in comparison to entire males [34, 43]. Overall, Norwegian and Swiss consumers are sceptical towards immunocastration as an alternative for surgical castration. Their concerns are mostly based on the fear of residues in meat and long-term health consequences for consumers [11, 46]. Also in Belgium, long-term consequences for food safety and public health were the main concern in case of immunocastration. However, overall, consumers were more in favour of immunocastration in comparison to surgical castration [47]. For farmers, the major concerns about immunocastration involve the associated costs (3.01-4.82 euro/pig) and increased labour in comparison to entire male pigs. Furthermore, also the expected lower consumer acceptance in comparison to surgically castrated pigs and safety concerns in terms of accidental self-injection contribute to farmers' reservations to produce immunocastrated pigs [13, 17, 30, 31]. Finally, although the costs associated with administration of the vaccine are compensated by the lower feed conversion ratio, farmers remained reticent about immunocastration [17, 31].

2.3. Production of entire male pigs

Apart from immunocastration, also the production of entire male pigs, whereby castration in any form is completely omitted, is considered as a viable alternative for surgical castration. Since rearing entire

males does not involve a medical intervention as for surgical castration, it is regarded as an animal friendly alternative. One of the benefits associated with the rearing of entire male pigs is the better feed conversion ratio in comparison to surgically castrated pigs. The feed conversion ratio of immunocastrated pigs on the other hand approximates that of entire male pigs or lies intermediate between surgically castrated and entire male pigs [38]. Furthermore, entire males demonstrate a lower fat deposition and thus higher meat percentage compared to castrated pigs, produced under the same circumstances [9, 24]. This translates not only in a lower percentage of dorsal and subcutaneous adipose tissue, but also in a lower percentage of intramuscular fat [38, 48]. However, in naturally low-fat pig breeds, the leanness associated with entire males can lead to a decreased succulence and tenderness of pig meat [49, 50]. Besides, the fat tissue of entire males is also characterized by a different fatty acid profile in comparison to (immuno)castrates. Indeed, entire male meat contains a higher percentage of unsaturated fatty acids, which renders it healthier [9]. Finally, entire male meat is also associated with a higher percentage of 'dark, firm and dry' (DFD) meat due to the increased aggressive behaviour when mixed in farms, during transport and in lairage at abattoirs [51].

As entire male pigs are not exposed to the pain and distress of surgical castration, the production thereof entails an improvement of the animal welfare conditions. However, at the onset of puberty, their welfare may be compromised in comparison to (immuno)castrates because of their more pronounced aggressive and sexual behaviour [12, 17, 44]. As a consequence, entire males present more skin lesions and claw and tail injuries [17]. For this reason, pig farmers are not in favour of rearing entire male pigs [30]. To overcome these behavioural issues, adjustments in terms of housing conditions, e.g. keeping entire males separated from sows and in fixed groups, may be applied [52, 53]. Furthermore, hands-on experience of farmers with rearing entire males resulted in a more positive attitude due to the improved performance and profitability as well as reduced labour demands [31].

In terms of improving animal welfare conditions, consumers have a positive attitude towards rearing entire male pigs. However, as the surgical castration of pigs was mainly intended to eliminate boar

taint, re-occurrence of the latter cannot be avoided [12, 54, 55]. Consequently, as boar taint leads to adverse consumer perception, it is the main drawback for switching to producing entire male pigs. Nevertheless, rearing entire male pigs is still considered a viable alternative for surgical castration, provided that measures are taken to prevent tainted meat to reach consumers [6]. These include investments of the industry in reduction strategies for boar taint, valorisation of tainted boar meat and fast detection methods for boar taint at the slaughter line should eliminate [51].

2.4. Sexing of sperm

Apart from the use of anaesthesia and analgesia, immunocastration or rearing entire males, research has been conducted to the sexing of sperm in order to rear only female pigs and thus preclude the occurrence of boar taint. Farmers were in favour of this alternative and agreed that sperm sexing would be the best way to avoid castration, provided that consumers would be willing to pay for the additional costs. Apart from the general positive attitude of farmers to sperm sexing, a lot of farmers also expressed a neutral response due to some degree of uncertainty on the latter practice [30]. Sex sorting of sperm is based on flow cytometric methods. However, two limitations of these methods are the low sorting efficiency and sperm viability during storage prior to artificial insemination [12, 56]. Additionally, due to the high number of sperm required for successful insemination, sperm sexing is not considered economically feasible for routine use in pig production [56, 57]. Although efforts have been undertaken to optimize the production of female litters, such as adaptations to the insemination method, no technology is available yet for commercial routine application, whereby this alternative is not considered as feasible [56]. Recently, Fast Genetics invested in an optimised sex sorting system based on detecting differences between X- and Y-bearing sperm cells. However, this method is not commercially available yet [58].

3. BOAR TAINT: A STUMBLING BLOCK FOR REARING ENTIRE MALE PIGS

3.1. What is boar taint?

Boar taint is an off-odour and -flavour that can be present in carcasses of non-castrated pigs and is perceived when meat or fat of the latter is heated. The occurrence of boar taint is mainly due to the accumulation of the (semi)-volatile compounds androstenone (AEON), skatole (SK) and to a lesser extent indole (IND) in adipose tissue (Fig 3) [59-61]. As this odour develops more strongly during puberty and is strongly related to sexual maturation of boars, it is also referred to as sex odour [62]. Boar taint is strongly present in approximately 4% of boar carcasses and moderately in 25% of boar carcasses [24]. As such, it is an important contemporary off-flavour in pig industry and should be controlled in order to prevent deterioration of pork quality of entire male pigs.

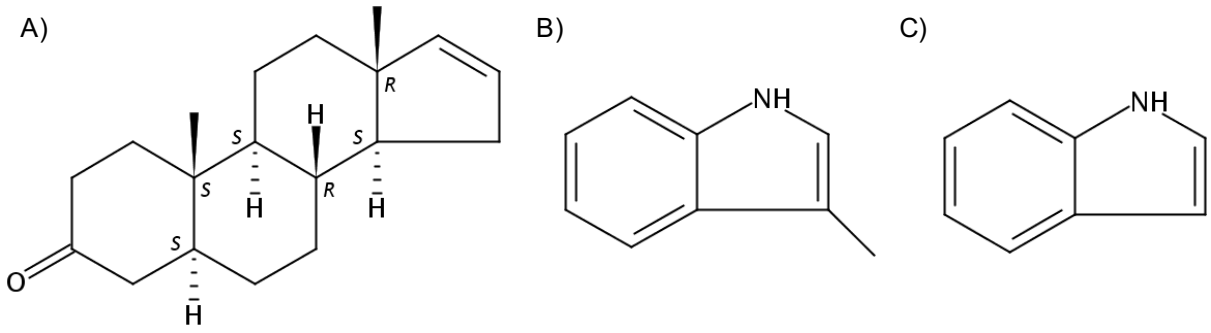

Fig 3 Overview of the boar taint compounds, with A) Androstenone, B) Skatole, C) Indole.

3.1.1. Androstenone

AEON or 5-alpha-androst-16-ene-3-one is a C19-Δ16-steroid and was first reported as a strong contributor to boar taint by Patterson in 1968 [59]. It is characterized by a urine- or sweaty-like odour. However, its perception is influenced by genetic variation in the OR7D4 human odorant receptor that is selectively expressed in the human nose epithelium [63, 64]. Indeed, these variations indicate that AEON perception also depends on gender, age and geographical location [9, 65]. Overall, women (51-89%) seem more sensitive to AEON perception compared to men (38-63%), but this was also dependent on the geographical location. Indeed, consumers in Germany, France, Spain and Sweden

were more critical towards AEON perception in comparison to Denmark, The Netherlands and The United Kingdom. Moreover, whereas AEON is mostly ascribed a urinary- or sweaty-like odour, 8% of sensitive consumers like its odour [9, 65].

AEON is synthesized in the Leydig cells of the testis and its levels are regulated by the neuroendocrine system through the hypothalamic-pituitary-gonadal axis (Fig 4). At the onset of puberty, the biosynthesis of testicular steroids and AEON increases through stimulation by GnRH and LH in the hypothalamus [10, 66]. This generally occurs in two waves, whereby a first increase in AEON levels is observed at week 14 and maximum levels are reached at sexual maturation (> 26 weeks) [10, 67]. This second wave is influenced by genotype, seasonal effects, nutrition and the social environment of pigs. Once synthesized, AEON is distributed through the blood circulation over different tissues [67]. As AEON is a lipophilic compound, its unconjugated form can accumulate in adipose tissue. The accumulated levels of AEON in adipose tissue depend on the maturity stage and genetic factors, which leads to a lot of variation [68, 69]. Moreover, the levels of AEON in fat tissue are also influenced by the degree of synthesis and metabolism in the testis and liver, respectively [70]. Apart from accumulation of AEON in adipose tissue, it is also transported to the submaxillary glands, where it binds to specific proteins and functions as a pheromone during propagation [10, 55]. Finally, AEON is also transported to the liver, where it is metabolised [10].

Biotransformation of AEON partially occurs in the testis, where it undergoes sulfaconjugation after synthesis under the influence of phase II conjugating enzymes [70]. In the liver on the other hand, biotransformation occurs in two phases. Phase I metabolisation is mediated by different dehydrogenase enzymes, which is followed by phase II conjugating enzymes such as hydroxysteroid sulfotransferases and UDP-glucuronosyltransferase [10, 71-73].

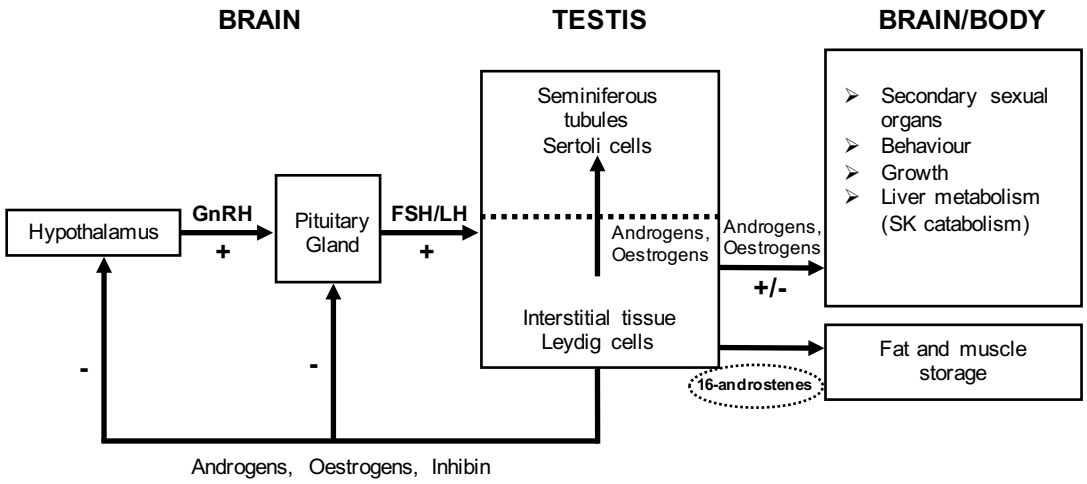

Fig 4 Overview of the hypothalamic-pituitary-gonadal axis and sexual regulations in the mature boar (+: induction, -: inhibition).

3.1.2. Skatole and indole

The contribution of SK or 3-methylindole and to a lesser extent IND was first reported in 1970 by Vold and Walstra & Maarse [60, 61]. The perception of these indolic compounds is often ascribed as faecal and can be perceived by 99% of the population [9, 67].

SK and IND are two fermentation products that are derived from the anoxic metabolism of L-tryptophan under the influence of *Clostridium spp.* and specific *Lactobacillus* strains [67, 74]. The ratio of SK and IND formation depends on the relative ratio of bacterial strains in the gut flora of pigs that compete for L-tryptophan as a substrate [67]. The formation of the latter compounds mainly depends on the availability of L-tryptophan, which mostly originates from cell debris from gut mucosa cells and to a lesser extent from feed intake. Additionally, the levels of SK and IND are also influenced by different genetic factors and the activity of enzymes involved in their metabolism [70, 75, 76]. Furthermore, SK and IND are also present in castrates, gilts, sows and other monogastric animals, although in lower amounts, suggesting that the regulation of SK and IND levels is direct- or indirectly related to the levels of testicular and thyroid hormones as the latter affect testis development [68]. Indeed, it is hypothesized that AEON inhibits the binding of the COUP transcription factor 1 (COUP-TF1) to the promotor of the CYP2E1 gene, an enzyme that is involved in the metabolism of SK and IND

in the liver. Consequently, AEON inhibits the expression of CYP2E1, which leads to a lower degree of metabolism and thus accumulation of SK and IND [70, 77]. Other hypotheses for SK and IND accumulation in entire male pigs is the formation of an adduct between SK and AEON during transport via blood circulation, and the higher cell-turnover during puberty and thus higher availability of L-tryptophan [67]. However, the mechanisms behind the accumulation of SK and IND in entire male pigs are still subject for discussion and remain to be completely unravelled.

After formation, SK and IND are partially adsorbed and transported through the blood circulation. The latter components partially accumulate in adipose tissue but are also distributed to the liver for biotransformation. In the liver, SK and IND are metabolised by CYP P4502E1 and P4502A enzymes, followed by a phase II sulfaconjugation step and excretion via urine. The remaining part of SK and IND that is not adsorbed in the gut, is excreted via faeces, but can be reabsorbed through inhalation and skin absorption [10].

3.1.3. Other boar taint contributing compounds

Up until now, boar taint perception cannot completely be explained by the presence of AEON and SK in porcine adipose tissue [54]. As such, the variation in the occurrence of boar taint and its olfactory assessment can only be explained for 35% by the presence of AEON and SK in adipose tissue [55]. Bonneau et al. on the other hand reported that the sensory perception of boar taint could be explained for 17-58% and 4-28% by the presence of AEON and SK in fat, respectively [78]. This discrepancy between the chemical analysis scores and sensory evaluation of boar taint suggests that also other, unknown compounds, may be responsible for the occurrence of boar taint [54].

In literature, several compounds that might attribute to the perception of boar taint have been highlighted. In 1967, Patterson already reported the contribution of two phenolic compounds, p-cresol and 4-ethylphenol, to boar taint. However, according to their smell, these compounds are not directly involved in the occurrence of boar taint [79]. Other compounds that might play a role in the perception of boar taint are androstenols such as 5-alpha-androst-16-en-3-alpha-ol and 5-alpha-androst-16-en-3-

beta-ol [10, 73]. Also the presence of 4-phenyl-3-buten-2-one, styrene and 1,4-dichlorobenzene and 2-aminoacetophenone as the main volatile phase I SK metabolite have been suggested as possible contributors [80-83]. Finally, aldehydes and short chain fatty acids play a dual role in the perception of boar taint as they are suggested to promote the perception of SK and AEON, or be responsible for the development of off-flavours [80].

4. OVERCOMING BOAR TAINT

Originally, the surgical castration of pigs was implemented to prevent undesired sexual and aggressive behaviour in pigs. In addition, it was also intended to prevent the occurrence of boar taint [9, 10]. Furthermore, castration was also a market driven choice, seen it increases the proportion of fat in carcasses, which was desired by consumers [10]. Because of the intended ban on the surgical castration, the rearing of entire male pigs has gained increased attention. However, the main problem associated with the latter is the re-occurrence of boar taint, which evokes negative consumer reactions and can lead to significant economic losses given the size of the pig industry, not only in Belgium, but also in Europe [7]. For this reason, the European Union postulated three conditions that should be met in order to raise entire male pigs as one of the alternatives for the surgical castration of boars. One of these conditions involves the reduction of boar taint prevalence in entire male carcasses through pig breeding, management and feeding strategies. Secondly, the acceptance of products of entire males by consumers, authorities and export markets should be ensured. Finally, in order to prevent tainted carcasses to reach consumers, rapid and accurate detection methods for boar taint should be implemented at the slaughter line [6].

4.1. Reduction strategies for boar taint in pig carcasses

One of the conditions for switching to the rearing of entire male pigs as an alternative to surgical castration, is the reduction of boar taint prevalence in pig carcasses in order to reduce the risk on rejection of entire male meat. As the boar taint levels are strongly influenced by genetics, sexual

maturity, the environment, diet and different management related factors, acting on the latter could reduce the risk on boar taint in entire male meat [84].

4.1.1. Genetic selection

Among the different strategies for boar taint reduction in pig carcasses, a lot of research has been devoted to genetic selection. Indeed, research showed that boar taint levels strongly differ between breeds, suggesting that breeding is an excellent tool for reducing the number of tainted boar carcasses [84, 85]. For example, 5-8% of boars from the Hampshire, Yorkshire and Landrace breed are identified as tainted, whereas 50% of Duroc boars are tainted [84, 86, 87]. Also, higher SK levels were found in the Large White race and Belgian Landrace stress negative, in comparison to Piétrain boars [88]. Moreover, different studies demonstrated a heritability of AEON and SK between 0.25-0.87 and 0.19-0.55, respectively [84, 89, 90].

Different strategies for genetic selection have been proposed, which consist of the identification of quantitative trait loci (QTL) or single nucleotide polymorphisms (SNPs) on responsible genes involved in AEON and SK synthesis and/or metabolism. QTLs are chromosomal regions, containing genes that affect a particular trait, e.g. boar taint. Identification of these QTLs occurs by comparing the genotype of anonymous markers located throughout the chromosome to the phenotype trait of interest. Once identified, these markers can be applied for selection during breeding [84, 91]. Different chromosomes, i.e. 3, 6, 7 and 14, have been identified to be responsible for the presence of AEON. For SK on the other hand, chromosome 6 and 14 have been demonstrated to have an influential effect. An important advantage of this strategy is that it does not require identification of genes responsible for the concerned phenotype trait [84, 89]. Another strategy for genetic selection is the identification of SNPs in candidate genes that code for key enzymes involved in metabolic pathways of the boar taint compounds. Consequently, genetic selection on these SNPs can be applied to terminal sires with a low boar taint prevalence [84, 91, 92]. In literature, different SNPs have been identified, involved in both AEON and SK synthesis and metabolism. For AEON, candidate genes responsible for the expression of

cytochrome B5, involved in the synthesis of AEON in the testis and 3-β-hydroxysteroid dehydrogenase (3-β-HSD) and Sulfotransferase Family 2A Member 1 (SULT2A1), two enzymes involved in the degradation of AEON could be identified [84]. For SK on the other hand, genes involved in the expression of enzymes responsible for SK metabolism, i.e. CYP2E1, CYP2A6, aldehyde oxidase and Sulfotransferase Family 1A Member 1 (SULT1A1) have been identified [84].

When considering these genetic selection strategies for the reduction of boar taint, the possible negative effect of genetic selection on the reproductive and growth performances of boars should be taken into account [86, 93]. Indeed, as there have been indications of unfavourable genetic associations between boar taint and female reproductive traits, selection should be based solely on genetic markers to identify pigs with low AEON levels, but otherwise normal levels of testicular hormones [84, 86, 94]. Recently, polymorphisms on the melanocortin-4 receptor (MC4R gene) have been identified to lower boar taint compound levels without compromising growth performance and carcass and meat quality in comparison to commercial boars and gilts, rendering genetic selection a feasible strategy to reduce boar taint prevalence [90, 95].

4.1.2. Slaughter at younger age and reduced weight

As the development of boar taint in entire male pigs is strongly related to sexual maturation of boars, it is hypothesized that slaughter at younger age and reduced weight decreases the chance on the occurrence of boar taint [86]. However, research showed that age is not directly correlated to sexual maturation and thus does not directly affect the AEON and SK levels, this in contrast to slaughter at reduced weight [96].

Bonneau et al. observed a strong variability in the sexual development among boars, whereby animals characterized by early sexual maturation had a higher probability for the development of high AEON levels [91]. These findings were confirmed by Chen et al., who observed significantly higher AEON levels (44% > 1000 µg kg^{-1}) in boars slaughtered at 115 kg, in comparison to slaughter at 90 kg (22% > 1000 µg kg^{-1}) [97]. No significant differences in boar taint compound levels were observed between

pigs slaughtered at 90 and 100 kg, respectively [98]. Apart from AEON levels, also reduced SK levels are observed in pigs slaughtered at lower weight, due to the influence of AEON on SK metabolism, rendering it a promising strategy for the reduction of boar taint prevalence in pig carcasses [86, 99].

4.1.3. Management strategies

Apart from genetic selection and slaughter at reduced weight, also adaptations to farm level management strategies can be applied to reduce boar taint prevalence. Among them are adaptations to the feed and different environmental factors (Fig 5) [100].

Research showed that the addition of fermentable carbohydrates such as fructooligosaccharides, raw potato starch, chicory and lupins to the diet are effective to reduce SK levels in adipose tissue of pigs [99, 101]. To explain this phenomenon, several hypotheses are proposed. First, the addition of extra dietary fibre leads to more fermentable carbohydrates in the hindgut, which in turn, leads to an increased microbial activity, whereby more L-tryptophan is incorporated in bacterial protein. Furthermore, an increase of dietary carbohydrates in the gastro-intestinal tract also leads to a decreased activity of proteolytic bacteria, leaving less L-tryptophan available for SK production [86, 99]. A second hypothesis is that extra dietary fibre causes more bulk material in the large intestine, which attracts water and leads to a dilution of SK in the gastro-intestinal tract. Consequently, there is less contact between SK and the intestinal wall, which leads to a reduced absorption of the latter and higher excretion via faeces. Additionally, dietary fibre reduces the transit time, which also contributes to a reduced absorption of SK [99]. Another hypothesis is that carbohydrates with low pre-caecal digestibility increase the butyrate production, which in turn leads to a decreased apoptosis and cell debris formation. Consequently, less L-tryptophan is available for SK production [86, 99, 102, 103]. In contrast to feeding fermentable carbohydrates, high-energy feeding leads to an acceleration of pubertal development and thus higher boar taint compound levels [91]. Moreover, a high-energy diet increases the mitotic rate of mucosa cells, which increases the L-tryptophan availability for SK production [86]. Apart from dietary supplementation of fermentable carbohydrates, also the addition

of organic acids has been evaluated. It is hypothesized that organic acids reduce the number of enterococci and lactic acid producing bacteria, which are important for the production of L-tryptophan in the colon. Consequently, dietary supplementation with organic acids could reduce SK levels in the colon; however, this effect is not observed in adipose tissue [86, 104].

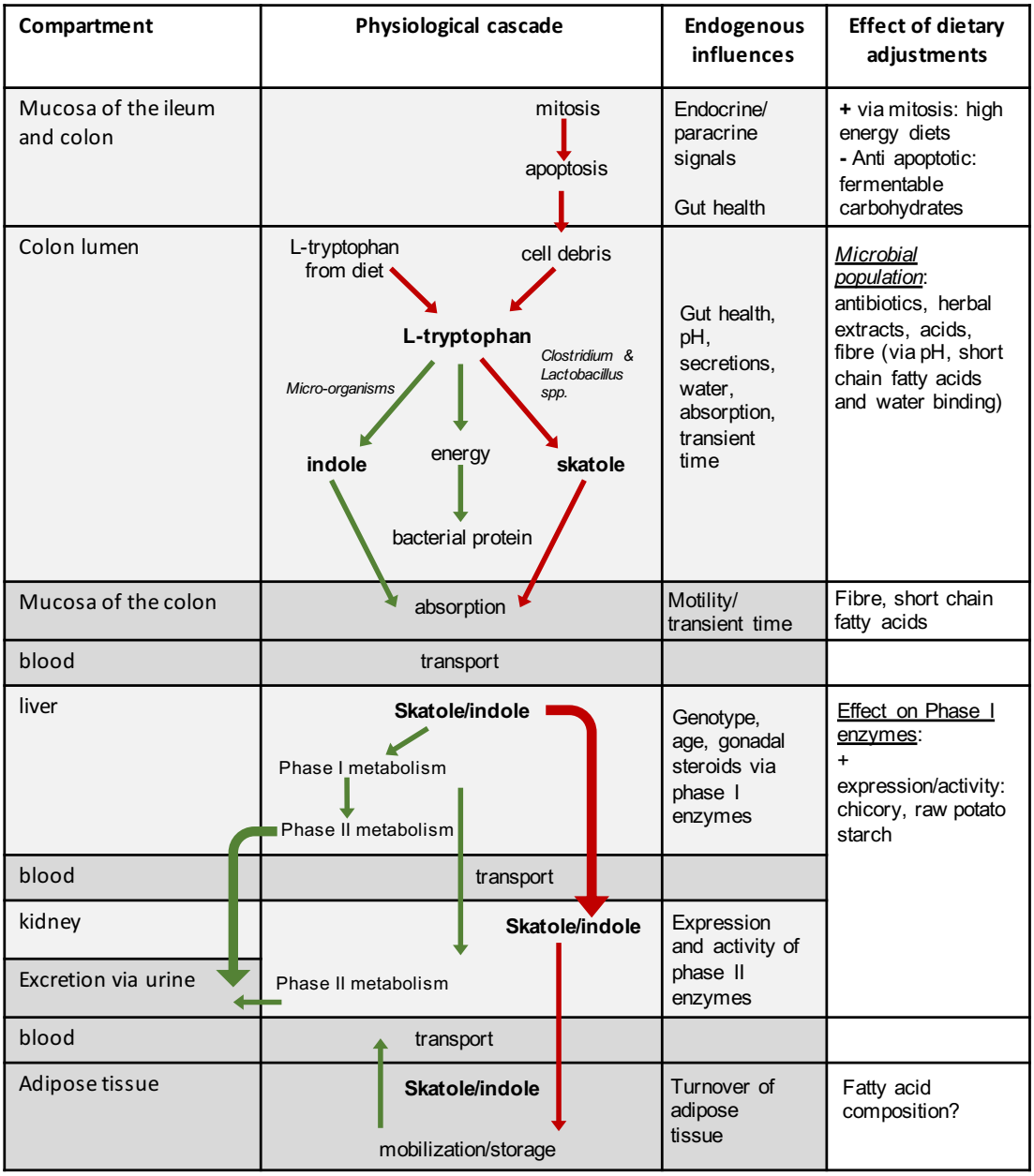

Fig 5 Cascade of physiological events influencing skatole formation, metabolism and accumulation in adipose tissue. The right column shows where distinct feeding influences exert their effects. Red arrows: steps leading to high skatole concentrations, Green arrows: skatole reducing or neutral conditions. Figure adapted from Wesoly et al. [98].

The effects of dietary supplementation of fermentable carbohydrates mainly manifest themselves on the level of SK formation, whereas AEON synthesis remains unaffected [97, 98]. Dietary supplementation with chicory root or inulin on the other hand may decrease AEON levels in adipose tissue through stimulation of liver enzymes involved in the metabolisation of AEON [105]. However, as dietary regulation mainly reduces SK levels, these should be combined with slaughter at lower weight in order to efficiently reduce boar taint prevalence in pig carcasses [98]. Moreover, the reducing effect on SK production by addition of fermentable carbohydrates is dose-dependent and should be carefully optimized prior to implementation as a farm-level intervention strategy [102, 103, 106-108]. Furthermore, Overland et al. observed that the dietary supplementation of raw potato starch only reduced SK levels in adipose tissue when adding it as a top dressing or applying liquid feeding, this in contrast to addition of the latter to the diet through pelleting [99, 109]. Finally, apart from dietary adjustments, also a 12h fasting or redraw of feed prior to slaughter has been shown effective for reducing SK levels [86, 99, 100].

Apart from adapting the diet of finishing pigs, also different environmental factors play a role in the development of boar taint. As such, strategies acting on the latter may be applied in order to reduce boar taint prevalence. First, as SK can be reabsorbed through the skin after excretion in the faeces, a clean environment reduces SK-reabsorption and thus accumulation in adipose tissue [86, 99, 110]. This effect is enhanced at high temperatures and in circumstances where the temperature of faeces and urine on pen floors may be higher [86]. In order to keep pens as clean as possible, pens with concrete or slatted floors and good ventilation are advised. Nevertheless, the effect of increasing hygiene management on SK reduction in adipose tissue appeared to be minimal and the size of this effect is not unambiguous [86, 111, 112]. Besides the cleanliness of pig pens, also other housing conditions such as group size and composition should be considered [100]. Indeed, as the development of boar taint is associated with the onset of sexual maturation, the highest AEON levels are observed in the most dominant animals. For this reason, it is important to act upon the social hierarchy and keep boars

in fixed groups until slaughter (farrow-to-finish) and separated from gilts to reduce sexual and competitive behaviour [86, 91].

4.2. Valorisation of boar meat

4.2.1. Factors influencing boar taint perception

Different studies on the acceptability of boar meat revealed that boar taint perception is influenced by a wide variety of factors, which can be subdivided in intrinsic factors related to the consumers and various extrinsic factors (Fig 6). Intrinsic factors include factors related to the consumers' profile. As such, consumers react differently to boar taint and their perception thereof is related to the gender, age, country of origin, their olfactory acuity, sensitivity and appreciation towards AEON and culinary and eating habits of consumers [113-116]. Indeed, it is widely established that women are generally more sensitive towards AEON and in addition are more critical to both odour and flavour perception of AEON and SK [113, 114, 117, 118]. Moreover, apart from gender related differences in AEON sensitivity, also differences between countries are commonly noticed. For example, the study of Blanch et al. demonstrated an overall risk of rejecting tainted meat by consumers between 14.3% and 41.0%. However, the risk was significantly lower in the United Kingdom in comparison to Spain and France, which was most likely due to the lower disliking of AEON in the United Kingdom [113]. In Norway, approximately 39% of consumers are highly sensitive towards AEON and will most likely react negatively towards meat containing high AEON levels (> 3000 µg kg^{-1}) [119]. A study of Bonneau et al. indicated that 6.5% and 3.5% of consumers were dissatisfied with pork loins of entire male pigs for its odour and flavour attributes, respectively, due to elevated boar taint compound levels. However, a lot of variation between countries was observed. In the United Kingdom, most consumers were equally satisfied with meat from entire males and gilt pork for both odour and flavour attributes. Danish and Dutch consumers on the other hand were less critical to boar flavour, but objected to the presence of boar odour. French, German, Spanish and Swedish consumers were critical towards entire male meat due to the presence of both boar taint related odour and flavour [120]. Finally, in Russia, a higher

consumer preference was demonstrated for meat with low boar taint compound levels (AEON: < 840 µg kg^{-1}, SK: < 430 µg kg^{-1}), while no differences in consumer preference were observed for Chinese consumers [121]. Apart from consumers' interindividual differences in sensitivity towards AEON, also the appreciation of AEON is a discriminating factor between consumers in boar taint perception. Although the majority of sensitive consumers dislikes AEON odour and therefore also indicate a higher dislike of boar meat, 8% of highly sensitive and 12.7% of moderately sensitive consumers may describe AEON odour as pleasant [114, 122].

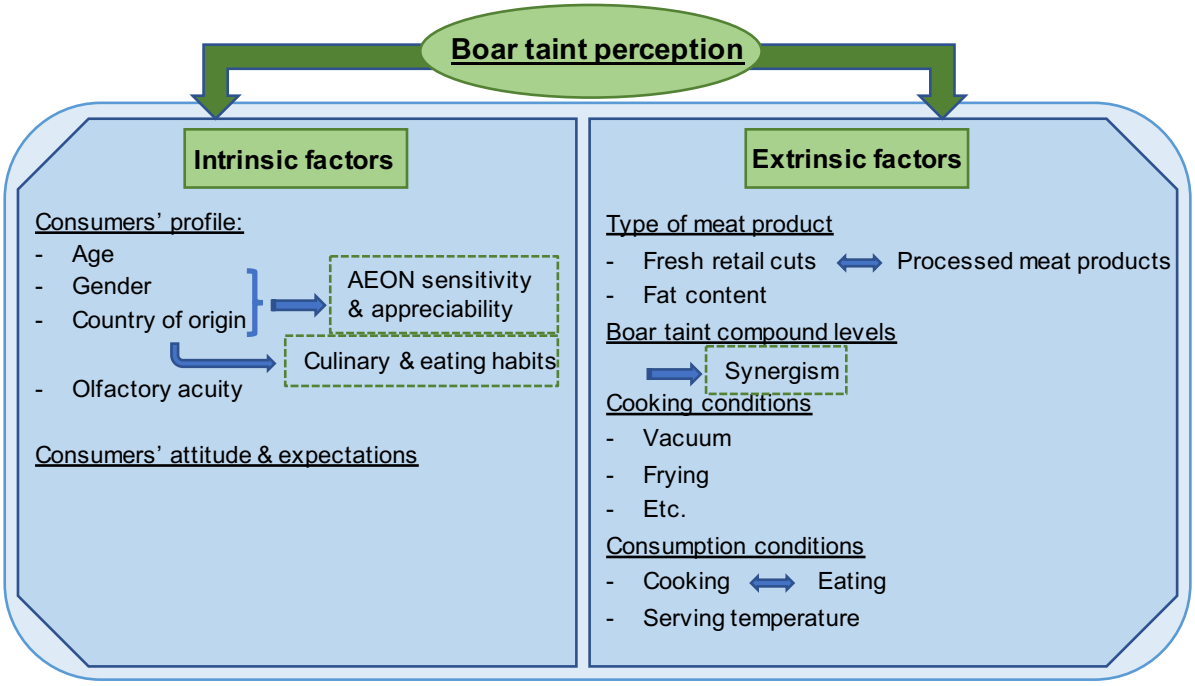

Fig 6 Schematic overview of intrinsic and extrinsic factors influencing boar taint perception.

Next, also consumers' attitude and prior information may influence the perception of boar taint. Research has shown that prior information on the boar taint issue may render more critical attitudes of consumers, nevertheless only minor differences in consumer acceptability were observed [117, 123]. Moreover, also consumers' expectations may impact the acceptability of food, which can be evoked by non-sensory cues such as labelling information given with the product. However, the label 'young boar meat' did not increase the acceptance of boar and pork loins, which may be attributed to the fact that consumers were not familiar with the term 'young boar meat', nor the issue of boar taint

in meat of entire male pigs [122]. Also in fermented sausages, no differences in acceptability between '100% pork' and '100% boar meat' sausages were observed by consumers with a background in animal husbandry [124]. Finally, as it was demonstrated that the acceptability of pork increases with an increasing frequency of cooking and eating fresh pork, also culinary and eating habits may play a role in the perception of boar taint [114, 117, 123, 125].

Next to intrinsic factors influencing the perception of boar taint, the hedonic response of consumers towards boar taint can be related to various extrinsic factors such as type of meat product, boar taint compound levels and cooking conditions [126]. First, the type of meat product has a high influence on the perception of boar taint. In this context, differences in boar taint perception are observed between fresh retail cuts and processed meat products. Indeed, it has been established that the presence of boar taint is better accepted in processed meat products in comparison to fresh retail cuts, due to masking effects that can occur during processing [127-129]. Second, as the boar taint compounds accumulate in adipose tissue, it is expected that meat products with a higher fat content are more prone to impairing consumer perception [117]. Yet, this hypothesis remains inconclusive as Lunde et al. did not observe significant differences in consumer perception of marinated pork chops with a fat content of 5% and 18.9%, respectively [129]. Pauly et al. on the other hand observed a higher perception of boar odour and flavour in neck chops (14.1g fat/100g) compared to *Longissimus dorsi* chops (10.4g fat/100g), which was most likely related to the higher intramuscular fat content of neck chops [54]. Furthermore, considering the fact that SK is more volatile than AEON, it is assumed that AEON has a higher contribution to boar taint in cooked meat than SK [130]. Moreover, SK is more water soluble than AEON and is therefore expected to be more important in leaner meat cuts. However, it was observed that both AEON and SK are important in the olfactory assessment of lean muscles such as loins and tenderloins, while the importance of the two compounds relative to each other in high fat products remains to be elucidated [126]. A second extrinsic factor that influences the perception of boar taint is the boar taint compound levels and their interaction [113]. As such, different levels of AEON and SK in boar meat lead to different perceptions. Especially for SK, different

concentrations may exhibit different odour qualities, which complicates ascribing a perceived odour to a specific attribute [131]. Moreover, it has been observed that meat with low AEON (< 333 µg kg^{-1}) and SK (40-140 µg kg^{-1}) levels is well accepted by French consumers, both sensitive and anosmic for AEON. Meat with high levels of AEON (590-4300 µg kg^{-1}) and SK (60-640 µg kg^{-1}) levels on the other hand were found unacceptable by sensitive consumers, while no differences in acceptability between meat with high AEON (1260-3430 µg kg^{-1}) and low SK (< 50 µg kg^{-1}) and control were observed. Consequently, in the absence of high SK, a higher threshold for AEON was observed [125]. This suggests that there is a synergistic effect between AEON and SK in the perception of the intensity of the boar taint compounds [127, 131]. Furthermore, Annor-Frempong et al. demonstrated that the perception of boar taint also depends on the interaction of the boar taint compounds with trained assessors [132]. Indeed, it was observed that SK affected both odour and flavour perception of trained assessors, while AEON particularly affected the flavour attribute. However, this also depended on the meat cut under investigation [127, 133]. Thirdly, consumers' perception is also influenced by the cooking conditions [117]. For example, it has been shown that vacuum or sous-vide cooking prevents the development of some off-flavours but also the loss of flavour volatiles. Frying on the other hand may affect sensory perception due to the formation of aromatic compounds. Comparison of the latter cooking methods, i.e. vacuum cooking and frying, counteracted the perception of boar taint, but no differences were observed between the two methods [134]. Moreover, it is well established that boar taint is more readily perceived during cooking than eating, and that its perception increases with the serving temperature [117, 125, 126, 135]. In addition, the contribution of AEON and SK differs between these situations [125, 126]. A final factor that influences boar taint perception is the location of the test, i.e. central location test (CLT) or in-home use tests (HUT), which depends on the scope of the study [117]. The main advantage of a CLT test is that all meat samples are prepared in controlled and standardized circumstances and afterwards evaluated by consumers. In HUT tests, it is more difficult to standardize the preparation of meat samples as each consumer has its own culinary habits. However, this can be partially solved by including a recipe or protocol on how to prepare the meat samples [117, 135].

Nonetheless, this variability cannot completely be accounted for. However, HUT tests give a more representative image of boar taint perception, as the evaluation of meat occurs in a real meal context and therefore the acceptance of boar meat is not underestimated [136]. Moreover, in HUT tests, the perception of boar taint during cooking or frying is taken into account, this in contrast to CLT studies, where only the perception during consumption is taken into account [117].

4.2.2. Reduction strategies for boar taint in meat

In order to rear entire males without impairing consumer perception, over the past years, efforts have been undertaken to reduce boar taint prevalence in pig meat. Nonetheless, the risk on the occurrence of boar taint remains. For this reason, one of the requirements set by the European Union to rear entire male pigs includes the acceptance of tainted boar meat by consumers [6]. Over the past years, different routes for processing meat that is unfit for marketing due to the presence of boar taint have been evaluated. However, it remains unclear to what extent these strategies can completely mask boar taint. Hence, the question on how to deal with tainted, especially severely tainted boar meat, still stands [137].

4.2.2.1. Smoking

Several studies indicate that smoking is a feasible strategy for masking the presence of boar taint in meat products, and is most likely attributable to an interaction between SK and smoke components such as formaldehyde [9, 129, 138]. The study of Kallas et al. regarding boar taint acceptance in smoked sausages, type frankfurter, indicated that natural smoking in combination with the addition of spices could completely mask the odour attribute of boar taint, but not the boar taint related flavour [128]. Another study comparing 5 different strategies to mask boar taint indicated that smoking was the best strategy to mask AEON odour. Flavour-wise, however, only the combined use of spices (white pepper, mustard seed, paprika, nutmeg mace, coriander seed, cardamom fruit and sweet marjoram herb) and smoking was able to eliminate the perception of AEON [130]. Also Aaslyng et al. demonstrated the beneficial effect of smoking on the masking of boar taint in smoked streaky bacon and pork belly roll,

whereby no significant effect of AEON or SK on consumer liking was observed [126]. In a study of Stolzenbach et al. on the other hand, commercial smoking strategies were not able to completely eliminate the perception of boar taint, this in contrast to the use of liquid smoke in model sausages. However, the results indicated that smoking was a promising masking strategy, provided optimization of the duration of smoking cycles, penetration of smoke, type of smoke, etc. [139].

4.2.2.2. Fermenting

Different studies demonstrated that fermenting, whether or not in combination with smoking, reduces boar taint perception in meat. In particular, fermented sausages appeared as a promising product to mask boar taint as it was safe in terms of consumer acceptance to include up to 50% raw material with boar taint compound levels ranging from 950-13,140 µg kg^{-1} and 160-610 µg kg^{-1} for AEON and SK, respectively, without impairing the overall liking [137]. Also in fermented sausages type salami, containing 100% boar meat, no differences in the hedonic score given by consumers were observed [124]. This masking effect caused can be explained by the use of starter cultures for fermentation, which cause fast acidification of the meat but also aroma development. Stolzenbach et al. demonstrated that the use of T-SC-150 Bactoferm (*Lactobacillus sakei* + *Staphylococcus carnosus*), F1-Bactoferm (*Pediococcus pentosaceus* + *Staphylococcus xylosus*), F2-Bactoferm (*Lactobacillus farciminis* + *Staphylococcus carnosus* + *Staphylococcus xylosus*) could mask boar taint but was insufficient for the complete elimination of boar taint perception [139]. More recently, also the use of yeast has been proposed as an alternative to mask boar taint odour, as it is involved in different biochemical mechanisms leading to aroma development. Indeed, the oxygen-scavenging and lipolytic activities of yeast delay rancidity and catabolize fermentation products such as lactate, which decreases the pH of meat and contributes to the development of less tangy and more aromatic sausages. In this context, the use of *Debaryomyces hansenii* yeast was able to counteract boar taint perception in fermented sausages, whereby yeast inoculation caused degradation of SK. Moreover, the growth of *Debaryomyces hansenii* on the surface of the sausages also contributed to the regulation of water

release during ripening of sausages, which contributed to a reduced hardness and chewiness, which is often observed in sausages of entire male meat [140].

4.2.2.3. Dry-curing

Dry-curing is a popular processing technique, whereby meat is salted and dried during a long period. Especially in Mediterranean countries such as Spain and Italy, the production of high-quality dry-cured hams is an important sector in meat industry. Due to the occurrence of boar taint, the quality of these meat products could be compromised, especially as high weight carcasses are used for the production of dry-cured hams due to their higher percentage of intramuscular and subcutaneous fat, which contributes to the flavour profile of dry-cured hams [141]. However, high weight carcasses are also associated with a higher risk of boar taint occurrence. Different studies showed that dry-cured hams of castrates were better accepted, which was related to a higher perception of off-flavours and odours in dry-cured hams from tainted boar carcasses [127, 141, 142]. Furthermore, although a higher acceptance of boar taint was observed in dry-cured ham in comparison to cooked meat, its eating quality was negatively affected by it, especially for the boar odour attribute. Moreover, dry-cured meat of boars was associated with less aroma, taste, juiciness and tenderness [141]. In addition, it has been demonstrated that entire male meat is more prone to processing losses and salt intake, which makes dry-cured hams of the latter drier, less marbled, harder and more intensely coloured [142].

4.2.2.4. Cooking

The beneficial effect of cooking as a masking strategy for boar taint perception has been demonstrated in different meat products. For example, although masking could not be achieved to the same extent as with fermenting, boar taint perception could be reduced to some extent in boiled sausages [130, 137]. The masking effect could be explained by the reduction of AEON and SK levels during cooking; however, the mechanism behind the degradation of boar taint compound levels remains unclear [127]. Nevertheless, it is assumed that AEON makes a greater contribution to the perception of boar taint in

cooked meat as it has a lower volatility than SK [130]. However, this could not be demonstrated by Bonneau et al., who observed that AEON and SK have similar contributions to the presence of off-odours in cooked hams of entire male pigs [143]. In cooked hams, reduction of boar taint perception is hypothesized to be related to the final internal temperature of the meat. As such, less aberrant odour and flavours were observed in hams with a final internal temperature of 80 °C in comparison to 68 °C. However, it should be noted that a higher final temperature may result in a decrease in moist and tenderness, which is unfavourable for cooked ham quality [9]. Nevertheless, cooking processes increased the acceptable levels of boar taint compounds in cooked ham, which were found to be 1500 µg kg^{-1} and 750 µg kg^{-1} for AEON and SK, respectively [143]. In boiled sausages on the other hand, it was shown that cooking as a masking strategy needs more optimization to completely eliminate perceptible off-flavours prior to implementation in the industry processes [137].

4.2.2.5. Seasoning

While different masking strategies are available for processed meat products, masking boar taint perception in fresh meat cuts is less straightforward. However, the use of spices and marinades may improve the acceptability of fresh boar meat [129]. Indeed, marinating adds taste and aroma to meat and therefore has the potential to mask boar taint related off-flavours. This was demonstrated for the first time by McCauley et al., who, to some extent, observed the masking of boar taint in oven cooked pork by sweet and sour marinades. However, still significant differences in acceptance were observed between low (AEON: 250 $\pm$ 280 µg kg^{-1}; SK: 60 $\pm$ 45 µg kg^{-1}) and highly (AEON: 1100 $\pm$ 600 µg kg^{-1}; SK: 170 $\pm$ 60 µg kg^{-1}) tainted carcasses [144]. In neck chops, the use of marinades could mask the presence of SK up to levels of $\pm$ 40 µg kg^{-1}. Chops with SK > 70 $\pm$ µg kg^{-1} however, remained unmasked despite the use of strongly flavoured marinades. Nevertheless, the use of liquid smoke and oregano extracts appeared to have the most potential for masking pork chops [129]. Furthermore, also seasoning of loin chops with a garlic and parsley breaded crust could mask the perception of boar taint in case of high AEON levels (> 2020 µg kg^{-1}), as a similar overall liking between tainted and control samples was

observed by consumers [134]. Apart from fresh meat cuts, the beneficial effects of seasoning were also demonstrated in processed meat products. The addition of 0.15% of fennel herb significantly increased the acceptance of tainted boar meat in sausages, type 'Bologna'. However, in frankfurter type sausages, the latter herb only demonstrated a minor masking effect [145]. In a study of Kallas et al., the use of a mixture of spices (white pepper, paprika, mustard seed, nutmeg mace, coriander, sweet marjoram and small green cardamom) in frankfurter sausages was demonstrated promising as a boar taint masking strategy [128]. In cooked sausages on the other hand, the use of spices (white pepper, mustard seed, paprika, nutmeg mace, coriander seed, cardamom fruit, sweet marjoram herb) could only eliminate the complete perception of boar taint in combination with smoking [130]. Also in smoked streaky bacon and pork belly roll, the use of spices (salt, pepper & others) in combination with smoking proved promising for the elimination of boar taint perception [126]. In minced meat, consumer liking was strongly impaired during frying. However, serving in combination with a ready-made spaghetti Bolognese sauce indicated a masking effect of the sauce on boar odour perception, but not the flavour attribute [135].

4.2.2.6. Diluting

Finally, apart from masking strategies involving different production related processes, also diluting tainted meat has been proposed for masking boar taint in processed meat products [146]. In this context, sausages appear promising as the amount of boar taint can be controlled by blending of raw material [137]. As such, in cold consumed smoked sausages, 25% of tainted meat could be processed without the risk of impairing consumer acceptance [9, 123]. However, in the case of warm consumed sausages, a blending percentage of 6-12% was accepted in case of carcasses with AEON levels exceeding 3000 µg kg^{-1} [145]. Furthermore, as a natural boar taint prevalence of 3% severely tainted (sensory score 4, on a scale of 0 (no taint) to 4 (severely tainted)) carcasses and an observed prevalence varying from 0-14% on Belgian and Dutch farms was observed, it can be expected that blending tainted meat in commercial blend products is also economically feasible to mask boar taint [24, 100].

4.3. Analytical approaches for the fast detection of boar taint at the slaughter line

Since the rearing of entire male pigs entails the possible occurrence of boar taint, there is a need for the detection of aberrant carcasses at the slaughter line. Over the years, several attempts to develop at-line methods have been made, which can be subdivided into sensory (human nose) or analytical approaches, both characterized by several (dis)advantages. On a short-term basis, the use of the human nose methodology is regarded as a possible solution. However, analytical methods may be more desirable, depending on the requirements for at-line applications in individual slaughter houses [147].

Table 1. Performance criteria for sensory and instrumental methods for at-line sorting of boar carcasses according to the final BoarCheck report regarding the study on rapid methods for boar taint used or being developed at slaughter plants in the European Union [147].

Method parameters	Sensory panel	Instrumental (targeted)	Instrumental (untargeted)
Methods	1 method	1 method [i]	1 method [i]
Accuracy			
Sensitivity	95-100%	LOQ on carcass: AEON: 50-120 µg kg^{-1} SK: 25-50 µg kg^{-1}	β-error < 5%
Specificity	90-100%	No matrix/spectral interference	α-error < 10%
Precision	RSD ≤ 10%	RSD ≤ 10%	RSD ≤ 10%
Method capacity			
Analysis/hour	100-800 carcasses	100-800 carcasses	100-800 carcasses
Analysis speed/sample	4-40 s	4-40 s	4-40 s
Sampling time/sample	0.5-20 min	< 20 min	< 20 min
Result reporting			
Off-line method	< 30 min	< 30 min	< 30 min
On-line method	< 1 min	< 1 min	< 1 min
Costs			
Running cost/carcass	< 2.0 euro	< 2.0 euro	< 2.0 euro

[i] agreement with the assigned value of a reference standard, or with the content derived by a reference method within maximum relative uncertainty of 10%

Requirements for implementation of at-line methods include the carcass sorting criteria (sensitivity and specificity) and method performance criteria such as sample throughput, analysis time, reproducibility, accuracy, etc. (Table 1). Other issues that need to be taken into consideration are related to the individual requirements of slaughter houses, which depend on the plant size. As such,

small slaughter houses (< 2000 assays/day) might benefit from implementation of sensory methods, whereas automated instrumental methods may be more cost-effective in large-scale slaughterhouses. However, overall, the applied methods should not increase production costs substantially, whereby it is strived to cut costs down to < €2 / analysis. Moreover, the applied methods require that the expected increase in rearing entire male pigs does not impair consumer behaviour from buying pork [147].

4.3.1. Human nose methodology

Currently, pork industry suffers from a lack of analytical methods for monitoring boar taint occurrence at the slaughter line. Consequently, the use of sensory assessment of carcasses or the human nose methodology is a necessary means to detect anomalies at the slaughter line [148, 149]. Methods for the sensory assessment of boar carcasses involve a heating step for releasing the malodourous compounds from the carcass, often obtained by singeing subcutaneous fat, which is followed by sensory evaluation by one trained assessor or a trained panel [148, 149]. However, as the sensory assessment depends on the human nose, a lot of variability is observed due to variations between trained assessors, scoring systems and heating methods. Consequently, methods should be carefully standardised in order to allow reliable measurements [150]. Moreover, the sensory evaluation of boar taint requires performance assessment for validation in terms of sensitivity (β-error or false negative rate) and specificity (α-error or false positive rate), and further improvement of the methodology for successful at-line implementation [151].

4.3.1.1. Training of expert panels

Different factors affect the performance of the human nose methodology and should be harmonized, but most variation stems from variability between trained experts, stipulating the need for careful selection of experts and standardisation of the training protocol. Indeed, Annor-Frempong et al. demonstrated that the individual detection thresholds for AEON varied between 200 and 1000 µg kg^{-1} [132]. In another study, even a ten-fold variation in individual ability to perceive AEON between trained experts was observed using smell strips [115]. Also Mathur et al., observed a high variation

between panellists (18-32%), which was in line with the findings of Whittington et al., who observed significant differences between the sensory scores of individual panellists, even though they had over 15 years of experience and followed the same sensitivity selection and training test [150, 152, 153]. Moreover, even an inter-individual variation of on average 23% variation was observed, indicating the need for careful selection of panellists [116, 150].

As different studies indicate, one way to minimize these substantial differences among experts is by using multiple experts in a panel and calculating the average sensory score instead of using the assessment of only one single assessor [150, 154]. For example, Mathur et al., demonstrated that the use of 1 panellist is associated with a 25% risk of not detecting samples with low boar taint levels [150]. Moreover, in order to achieve a false negative rate (1-sensitivity) below 10% for the detection of carcasses with a sensory score of 3 (strongly tainted), one would have to use the average sensory scores of at least 9 panellists, if the reproducibility of the sensory measurements exceeds 20% [150]. In case of using the average score of 5 panellists, a sensitivity and specificity of 61-69% and 77-85% were observed, respectively, whereas the performance of individual assessors varied highly between 47-86% and 45-88% for the sensitivity and specificity, respectively [154]. However, due to limited economic resources for the detection of boar taint at the slaughter line, the use of multiple assessors significantly increases costs and is therefore doubtful for successful implementation at the slaughter line [150].

Other measures that can be taken to improve sensory performance include harmonization of the selection and training protocol for sensory experts. However, currently, guidelines for sensory training for boar taint are lacking, which leads to a lot of variability between procedures for selection and training of panellists. Although in most recent studies, similar selection and training steps were followed, also substantial differences were observed [65, 115, 116, 152, 155]. All different protocols included a selection step based on the candidates' abilities to perceive AEON. Afterwards, training was followed by identification of odd samples containing AEON and SK, ranking of samples according to

different AEON and SK concentrations and evaluation on biological samples (fat or meat samples) [65, 115, 116, 155]. In the study of Trautmann et al., an additional step involving the odour threshold determination of individual experts was included, whereby the experts were exposed to different dilution levels of AEON and SK [116]. Differences among studies mostly included the use of different carriers, different concentration levels of AEON and SK, the use of different scales for evaluation and the number of times repeating every training step, which may significantly affect the performance assessment of trained panels. Indeed, it was previously demonstrated that the type of carrier (smell strips, bottles, fat tissue, etc.) significantly influences odour and flavour perception [116, 155]. A possible explanation for these differences lies in the binding of the boar taint compounds with muscle proteins, which complicates the perception of the boar taint compounds. Moreover, also the pH and addition of salt may play a role in the olfactory perception of boar taint in biological samples [155]. Consequently, training of panellists should always include evaluation of the sample matrix of interest, e.g. fat tissue for screening at the slaughter line. Moreover, as research illustrated that the uncertainty about sensitivity and specificity of sensory assessment is influenced by the total number of evaluated samples and the prevalence of boar taint in these samples, a large sample size is required to reliably identify sensitive assessors [151]. Second, the different concentration levels of AEON and SK used during training may affect the abilities of the trained panel to perceive the latter compounds. For this reason, panellists should be selected based on their olfactory acuity and their sensitivity to AEON should be taken into account for harmonization purposes [115, 116, 151]. Moreover, it has been demonstrated that repeated exposure to boar taint, especially to AEON, may increase panellists' sensitivity. Consequently, mere exposure during repeated training sessions may increase the candidates' ability to correctly discriminate between tainted and untainted samples [156]. Finally, different evaluation scales can be applied for sensory assessment, ranging from the use of ordinal scales (visual analogue scales with anchor points or continuous scales) or the assessment on an IN/OUT or A/Not A scale to indicate whether or not the sample is deviant [148, 151]. However, the type of applied scale should be considered in accordance to the goal of the sensory assessment. Consequently,

as at the slaughter line a very high-throughput is expected, the use of a simple scoring system is preferred [135].

Currently, sensory detection methods for the screening of boar taint lack thorough validation, which may compromise the quality of sensory assessment. Accordingly, it is advised to monitor the performance of these methods in terms of sensitivity (false negative rate) and specificity (false positive rate) continuously [116, 151, 155]. However, the latter parameters are often poorly documented as a consequence of the lack of an unambiguous definition for boar taint or a gold standard giving information on the correct classification of tainted or untainted carcasses. In this regard, consumer acceptance thresholds would be the ideal gold standard for boar taint. However, due to the high variability in consumer perception between countries, product types and consumers, and the high costs for performing large-scale consumer surveys, it is hard to put this into practice. Another option is to use the sensory score of a reference expert or the average score of a trained expert panel, provided that they are sufficiently related to the consumer perception of boar taint. Finally, the most commonly applied gold standard is the use of the chemically determined levels of AEON and SK in adipose tissue and is also referred to as the so-called “safe box” approach. However, because of the lack of harmonised and standardised methods in this field, no universally recognised thresholds are available [147, 151, 157, 158]. As such, acceptance levels for AEON and SK vary between 500-3000 µg kg^{-1} and 200-250 µg kg^{-1}, respectively [9, 55, 119, 125, 159, 160]. Consequently, reported values for sensitivity and specificity vary widely among studies. This may be related to different factors such as the differences between the applied sensory protocols, or differences between methodologies applied as a gold standard for the classification of samples (Table 2) [116, 149-151, 154, 158]. For example, when applying low or more strict cut-off levels for AEON and SK, a higher proportion of samples outside the safe box was observed according to chemical analysis, and thus considered as truly positive for boar taint in comparison to the actual proportion of tainted samples by means of sensory analysis [150]. Consequently, this may be associated with a lower sensitivity and lead to an overestimation of true positive boar taint samples. Hence, in practice, a high number of carcasses will be sorted out at

the slaughter line following this approach, leading to economic losses [150, 158]. However, in the opposite case, the safe box approach does not account for possible interactions between AEON and SK in the olfactory perception of boar taint. Consequently, samples for which AEON and SK do not exceed the applied thresholds, but are considered as truly tainted according to sensory analysis, could potentially be missed as the safe box approach does not model this interaction well. To overcome this, applying a more curved approach may be more suitable as underlying model for at-line sorting of carcasses [158].

Table 2 Overview of performance characteristics (sensitivity, specificity, accuracy) of different applied sensory methods compared to chemical analysis as a reference method and based on the given threshold levels.

Reference	Sensory method	Scale	Limits of sensory scale	Gold standard	Sensitivity	Specificity	Accuracy
Meier-Dinkel et al., 2015 [154]	Microwave method	6-point scale	0: no deviation from standard fat 6: strong deviation from standard fat	Chemical analysis (ELISA) CHEM-LOW: AEON 1500 µg kg^{-1}, SK 200 µg kg^{-1} CHEM-HIGH: AEON 2000 µg kg^{-1}, SK 250 µg kg^{-1}	 64% 69%	 85% 81%	 75% 71%
Trautmann et al., 2014 [116]	Microwave method	2-point scale	deviant (= boar taint)/standard	Chemical analysis (HPLC-UV & ELISA) AEON 1500 µg kg^{-1}, SK 200 µg kg^{-1}	 53%	 63%	 61%
Mörlein et al., 2016 [158]	Microwave method	6-point scale	0: no deviation from standard fat 6: strong deviation from standard fat	Chemical analysis (SIDA-HS-SPME-GC-MS) CHEM-LOW: AEON 1500 µg kg^{-1}, SK 200 µg kg^{-1} CHEM-HIGH: AEON 2000 µg kg^{-1}, SK 250 µg kg^{-1}	 85% 86%	 69% 70%	 72% 73%
Mathur et al., 2012 [150]	Hot iron method	5-point scale	0: no boar taint 4: strong boar taint	Chemical analysis (immunoassay & LC-MS) AEON: 1000 µg kg^{-1}, SK 250 µg kg^{-1}	 16%	 97%	 57%
Trautmann et al., 2016 [149]	Microwave method	6-point scale	0: no deviation from standard fat 6: strong deviation from standard fat	Chemical analysis (SIDA-HS-SPME-GC-MS) CHEM-LOW: AEON 1500 µg kg^{-1}, SK 200 µg kg^{-1} CHEM-HIGH: AEON 2000 µg kg^{-1}, SK 250 µg kg^{-1}	 88% 94%	 68% 63%	 78% 79%
	Hot water method	6-point scale	0: no deviation from standard fat 6: strong deviation from standard fat	Chemical analysis (SIDA-HS-SPME-GC-MS) CHEM-LOW: AEON 1500 µg kg^{-1}, SK 200 µg kg^{-1} CHEM-HIGH: AEON 2000 µg kg^{-1}, SK 250 µg kg^{-1}	 68% 72%	 91% 85%	 80% 79%
	Hot iron method	6-point scale	0: no deviation from standard fat 6: strong deviation from standard fat	Chemical analysis (SIDA-HS-SPME-GC-MS) CHEM-LOW: AEON 1500 µg kg^{-1}, SK 200 µg kg^{-1} CHEM-HIGH: AEON 2000 µg kg^{-1}, SK 250 µg kg^{-1}	 88% 87%	 100% 81%	 94% 84%

4.3.1.2. Heating method

In order to sufficiently perceive the boar taint compounds during sensory assessment, subcutaneous fat is heated, volatizing the boar taint compounds. This can be achieved by different methods, e.g. melting, microwave heating, singeing (hot iron or soldering iron) or boiling (hot water method) [148, 149, 153, 161]. Comparison of different heating methods showed that both the microwave, singeing with a hotwire and boiling method were suitable for the detection of SK. However, low correlations were observed between the sensory scores and AEON levels with the hotwire and boiling method (Table 3). Consequently, the microwave may be preferred for heating samples due to better agreement between sensory and chemical analysis on the one hand its simplicity and high-throughput [153]. Another study comparing the microwave, soldering iron and pyropen indicated that the sensory score was significantly affected by the type of heating method. Correlations between the sensory score (average of 4 trained assessors) and the levels of boar taint compounds as assessed by ultra-high performance liquid chromatography hyphenated to high resolution mass spectrometry (UHPLC-HRMS) were highest for the soldering iron and pyropen (Table 3). However, the study demonstrated that all heating methods seem suitable and that the choice of method mostly depends on the habits of the trained assessors [148]. However, when taking into account practical at-line considerations, the use of the pyropen may be preferred because it is easy to handle (wireless) and makes no contact with the subcutaneous fat [148]. Indeed, use of the microwave or boiling method requires sampling and weighing, which is not feasible considering the high speed of the slaughter line as additional manual actions are required [149]. Moreover, in comparison to other gas burners (flame temperature 1300 °C) that were originally used to singe fat, the pyropen operates at lower temperatures (350-500°C), decreasing the risk of burning fat tissue and eliminating the release of burnt odours which could mask the perception of boar taint [148, 161]. However, the release of gas may also, although to a limited extent, hamper boar taint perception [148]. In contrast to previous studies, Trautmann et al. demonstrated that the choice of heating method significantly affected the probability of a deviant rating. As such, comparison of three heating methods (microwave, hot iron and hot water) revealed

that the best performance in terms of sensitivity and specificity was obtained with the hot iron method, in case the levels of AEON (cut-off 1500 µg kg^{-1}) and SK (cut-off 200 µg kg^{-1}) were set as the true condition of the test outcome [149].

Table 3 Pearson (Whittington et al., 2011) and Kendall's (Bekaert et al., 2013) correlation coefficients between odour scores and boar taint compound levels obtained for different heating methods to heat fat samples.

	Whittington et al., 2011 [153]		Bekaert et al., 2013 [148]	
	Androstenone	**Skatole**	**Androstenone**	**Skatole**
Microwave	0.25	0.48	0.35	0.26
Hotwire	0.06	0.46	-	-
Melting	0.16	0.34	-	-
Boiling (75°C)	0.04	0.44	-	-
Boiling (25°C)	0.03	0.32	-	-
Soldering iron	-	-	0.37	0.41
Pyropen	-	-	0.34	0.36
Pyropen with plate	-	-	0.35	0.16

In addition to the choice of heating methods, also other factors related to the heating method such as temperature, duration of heating and sample type or part of the carcass should be standardised for reliable at-line detection. For example, the time needed for scoring decreases with increasing temperatures; however, this reaches an upper limit as too high temperatures hamper boar taint perception due to the release of burnt odours [148, 153, 161]. Similarly, singeing fat twice at the same place significantly lowers the sensory score of the second assessment, although this was limited to blank samples [148]. Moreover, it has been demonstrated that cleaning the soldering iron in between two samples significantly affected the sensory score. In general, samples were scored higher in case the soldering iron was not cleaned, which could be related to cross-contamination of samples from fat residues present on the soldering iron [148]. These concerns further address the need for harmonisation and the development of standardized procedures for sensory assessment of carcasses for reliable detection of boar taint at the slaughter line.

4.3.2. Analytical methods

Over the past decades, numerous methods for the determination of boar taint compounds have been developed, ranging from immunological to specialized chromatography and MS based protocols [157].

However, these methods involve complicated and labour-intensive sample pre-treatment procedures, making them unsuitable for at-line implementation. Recently, more research has been devoted to the development of rapid detection methods for boar taint, that could potentially be used for at-line detection. These include different approaches such as the use of spectrophotometry, sensor technologies, development of immunoassays, spectroscopy and MS-based techniques. However, feasibility studies on the use of these methods remain limited and most methods have not been properly validated for at-line use [157].

4.3.2.1. Spectrophotometry

Spectrophotometric methods are based on the interaction between molecules in solution and radiation of wavelengths between 200 and 800 nm, in the ultraviolet-visible (UV-VIS) spectral region. In case radiation is absorbed by a molecule, its energy state changes, whereby the molecule transitions to an excited state. According the Lambert-Beer law, the amount of absorbance or extinction is directly proportional to the concentration of absorbing molecules in the solution and the applied wavelength. Consequently, spectrophotometric methods can be used to quantitatively determine the concentration of absorbing species [162, 163].

Mortensen & Sorensen developed a colorimetric method for off-line boar taint detection in slaughterhouses in 1983, which is currently applied in different slaughterhouses in Denmark [164]. This method allows to analyse up to 200 samples per hour, including 20 quality control samples, and the analysis time per sample takes 15 to 20 minutes. Analysis involves the physical removal of a back fat sample from the carcass at the beginning of the slaughter line, which is subsequently analysed by an automated robot. As such, results on individual samples are available when the carcass leaves the chilling tunnel at the end of the slaughter line [164, 165]. However, taking into account the current rates at which pigs are slaughtered in large scale abattoirs (up to 800-1200 per hour), this method would be insufficient for implementation in terms of analysis speed. Screening of the samples is based on a colorimetric reaction between the indolic compounds and the colorimetric reagent

dimethylaminobenzaldehyde (Ehrlich's reagent), which is added to an extract of the fat sample. However, as this reagent is not specific, the method does not allow to discriminate between the indolic compounds SK and IND. Consequently, the total concentration of indolic compounds is expressed in SK equivalents, as fitted by a linear model including 75.9% (a) of SK and 44.2% (b) of IND as determined by HPLC analysis, and a constant of 0.027 (c).

$$a\ SK_{HPLC} + b\ IND_{HPLC} + c = SK_{equivalents\ colourimetric\ method}$$

By means of the obtained regression curve, it is possible to predict the SK equivalent from the levels of SK and IND as obtained by HPLC analysis, but not the other way around. However, by applying a multi component algorithm from the obtained HPLC results and spectra of the spectrophotometric method, it is possible to estimate whether or not a sample is tainted [166]. As such, although the method lacks specificity, the results obtained by this colorimetric method correlated well with the sensory evaluation of boar taint (r = 0.8) [157]. Another drawback of this method is that it is not suitable for the detection of AEON. Consequently, sorting of carcasses is solely based on the presence of indolic compounds, which may lead to an underestimation of tainted carcasses as AEON is not taken into account and the presence of SK only explains 4-28% of the sensory perception of boar taint [78]. However, other studies indicated that in case a sorting criterion of 200 µg kg^{-1} SK equivalents was applied, a false negative rate of only 0.8 % was observed [165].

Other colorimetric methods for the detection of SK in adipose tissue involve the reaction between SK and dimethylaminobenzaldehyde as a colouring reagent. However, this method was developed for in-laboratory use and also an overestimation of SK was observed as the used colouring reagent also interacted with other indolic compounds [120, 167]. Also for the determination of AEON in the salivary gland and adipose tissue, a colorimetric method has been developed, based on the reaction of 16-androstene steroids and resorcaldehyde [168]. However, this method showed interference with cholesterol in fat samples, as the latter compound also interacts with resorcaldehyde [167]. To

overcome this, separation between cholesterol and the 16-androstene steroids was required, impairing the applicability of the method at the slaughter line [169].

4.3.2.2. Other spectroscopic techniques

Apart from spectrophotometry, also the feasibility of other spectroscopic techniques including RAMAN and Fourier transform infrared spectroscopy (FTIR) spectroscopy has been evaluated for the fast detection of boar taint [170, 171]. As research has shown that the boar taint compounds have distinguishable infrared (IR) spectra in the gas phase, the use of spectroscopic techniques could allow direct detection of the boar taint compounds in the vapour. However, gas sampling is the main critical point and requires a standardised method [170]. Recently, Sorensen et al. developed a targeted method for the quantification of AEON and SK by surface-enhanced RAMAN scattering (SERS) and multivariate data analysis [171]. Melted fat samples were extracted with methanol, followed by spectral analysis of the extract. Although the multivariate models were optimized, high prediction errors were observed of 1460 µg kg^{-1} and 173 µg kg^{-1} for AEON and SK, respectively, which may be due to an incomplete extraction [171]. An untargeted profiling approach of fat samples with a portable RAMAN device on the other hand showed better performance accuracy (81%). With this method, RAMAN spectra of both inner and outer layers of subcutaneous fat of tainted and untainted boars were determined. Discrimination between the latter two groups of samples was based on varying levels of fatty acid saturation in the fat tissue, which was most prominent in the inner fat tissue layer [172]. Consequently, although further development and optimization is required, the use of this untargeted RAMAN approach was promising for the fast classification of boar taint.

4.3.2.3. Sensor technology

During the past decades, advances in the development of sensor devices have allowed their application in many technological fields, including various food applications [173]. For example, different sensor devices have been developed that provide a fast, simple, non-expensive and non-destructive

assessment of food quality control [174, 175]. Also in the field of rapid boar taint detection, efforts have been undertaken to develop sensors that could potentially be applied at the slaughter line for the screening of boar carcasses. These sensor devices include electronic noses (e-noses), gas phase sensors, amperometric sensors, thickness shear mode resonators and parasitic biosensors [176-181].

The operating mechanism behind gas sensor devices is based on physical or chemical adsorption and desorption, optical adsorption or chemical reactions of a compound in the gas phase or at the surface of the gas phase and the sensor. As a consequence, these interactions cause physical changes in the sensor, which can subsequently be detected [9]. Bourrounet et al., developed a multi-gas sensor device for the detection of boar taint. The sensor system consisted of 5 commercial metal-oxide semiconductor (MOS) sensors, which represented maximal responses for the detection of volatiles in adipose tissue. Measurement of the volatile fraction of melted adipose tissue revealed a correct classification rate of 84.2% of the samples, which were classified according to their AEON content (< 700 µg kg^{-1} and > 1700 µg kg^{-1}) as determined by an enzyme-linked immunosorbent assay (ELISA) [176, 182]. However, the study did not investigate the classification according to the SK levels present in carcasses or both AEON and SK. Moreover, the results were preliminary and lacked thorough validation.

Another sensor developed for the detection of AEON in gas phase involves the use of thickness shear mode resonator sensors, which showed comparable sensitivity to the human olfaction of AEON. However, experimental minimum errors of 320 and 480 µg kg^{-1} were observed, limiting the potential of this sensor in terms of robustness for at-line implementation. Moreover, sample preparation consisted of heating fat samples for 30 min, after which the headspace (HS) was extracted and guided to the sensor chamber, which does not meet the requirements for at-line implementation [177].

Also the use of electronic noses has been proposed as a potential technique for the fast detection of boar taint. The first study of the development of a sensor for the detection of agricultural malodourous compounds dates from 1996. The latter sensor consisted of 20 conducting polymers and showed a

signal proportional to the concentration of volatiles in pig slurry, including SK and IND. However, the sensor was not specific for the latter components as interference was observed with other volatiles such as short chain fatty acids and phenolic compounds present in pig slurry [183]. In 2006, Vestergaard et al. developed an electronic nose based on ion mobility spectrometry for the detection of boar taint in fat samples with varying concentrations of AEON (90-880 µg kg^{-1}) and SK (10-260 µg kg^{-1}) levels. The applied sensor demonstrated good repeatability (86.7%) and a high and moderate correlation between the e-nose data and boar taint compound levels were observed for AEON (r = -0.948) and SK (r = -0.629), respectively. In contrast to the chemically determined boar taint compound levels, the observed correlation with the sensory evaluation was lower (r = 0.409) [180]. In the study of Annor-Frempong et al. on the other hand, a higher correlation was observed between the e-nose, consisting of 12 conducting polymers, responses and sensory evaluation by a trained panel. However, also some limitations in terms of sampling time and specificity of the electronic noses were observed, which should be overcome prior to at-line implementation of these systems [178, 184].

Finally, apart from chemical sensors, also the potential of biosensors based on parasitic wasps has been investigated for detecting tainted samples. As these parasitoid species present good learning abilities, high sensitivity, flexibility and reliability in exhibiting conditioned behavioural responses, their use in biosensors is evident [181]. The application of these types of sensor for boar taint detection demonstrated that the wasps could be successfully trained for the detection of the indolic compounds, separately or all boar taint compounds presented in a 1:1:1 mixture. However, the conditioned response towards the exposure to AEON separately was moderate and needs further optimization [181]. However, another study showed that the wasps were able to discriminate between low, medium and high concentrations of both AEON, SK and IND in boar fat after associative learning of the wasps through positive and negative feeding experiences [179]. However, further studies are needed to determine the minimum detection thresholds of the wasps and their performance in an industrial setting [157, 179, 181].

4.3.2.4. Immunoassays

Immunological methods are based on the interaction between an analyte (antigen) and its complementary recognition element, in which the concentration of the analyte can be determined by measurement of labelled units, e.g. radioactive isotopes, enzymes or fluorescent components. Depending on the design of the immunoassay, either the analyte can react with the labelled recognition element, or it can compete with labelled antigens [157]. Most immunological methods incorporate antibodies as recognition elements. However, recently, also molecularly imprinted polymers (MIPs) have surfaced as artificial recognition elements and are more and more applied in immunoassays.

Antibodies

Different immunological methods have been developed for the detection of AEON, including radioimmunoassays (RIA), enzyme immunoassays (EIA) and fluoroimmunoassays (FIA). Different RIAs were developed for AEON and all delivered sensitive and moderately accurate to accurate results [185-188]. However, RIAs imply the use of radioisotopes of which the use is restricted to specialized laboratories due to safety precautions. In this regard, the use of EIAs, ELISA and FIAs has gained an increased interest.

One of the main bottlenecks in the development of immunoassays against AEON is their cross-reactivity with other 16-androstene steroids, which impairs selectivity of the measurements. As such, Claus et al., reported high cross-reactivities against 5α-androst-16-en-3α-ol (8%), 5α-androst-16-en-3β-ol (12%) and 4,16-androstadien-3-one (80%) [189]. In the study of Andresen et al., even 100% cross-reactivity was observed with 4,16-androstadien-3-one [185]. In the study of Squires and Lundstrom on the other hand, significantly higher selectivity was demonstrated, as cross-reactivities between 0.2 and 2.6% were observed for 5α-androsten-17β-ol-3-one, 5α-androstan-3-ol-17-one and 4-androsten-3,17-dione [76]. Also the time-resolved FIA for AEON developed by Tuomola et al., the cross-reactivity did

not exceed 10% for 5-androst-16-en-3-ol [190]. However, although most immunoassays for AEON demonstrated high cross-reactivity with other 16-androstene steroids, it was suggested that this only minorly affected the performance of the assays as the latter compounds are only present in adipose tissue in very low concentrations [157].

In contrast to AEON, only limited attempts have been made to develop immunoassays for the detection of SK, which is most likely due to its small size and low molecular weight. Consequently, SK lacks antigenic properties and is considered as a poor epitope [157]. Currently, only 2 immunoassays are available for the detection of SK [191, 192]. Aguilar-Caballos et al. developed a stopped-flow FIA for SK based on the use of europium(III) as a label and kinetic measurements. However, only limited data of the performance of the assay were available [191]. Leivo et al. recently described the development of an ELISA assay based on recombinant antibodies for SK derived from a synthetic antibody library. Although screening of this library was promising in terms of developing a specific antibody for SK, no experimental results were available and development and optimization of methods incorporating this antibody remains to be performed [192].

Although immunoassays deliver reasonably quick results and are widely applied for primary screening of samples, the analysis of adipose tissue still requires sample pre-treatment, which delays the analysis time and hampers high-throughput detection [157]. In contrast to adipose tissue, such assays can be directly applied on serum or saliva; however, from a practical point of view it is difficult to obtain the latter sample matrices post-mortem at the slaughter line [190, 193, 194].

Molecularly imprinted polymers (MIPs)

Molecular imprinting has surfaced as an emerging technique for the production of polymeric materials containing binding sites of predetermined selectivity. Due to their superior stability, robustness and higher resistance to extreme environmental and chemical conditions in comparison to antibodies, they have gained an increased interest over the past decades [195, 196]. In this context, MIPs are widely

applied as artificial recognition elements for different applications including their use in filter systems, sample clean-up procedures to their implementation in sensors and screening assays [197, 198].

MIPs are created by formation of a pre-polymerization complex through interaction between a template functional monomer. Afterwards, the pre-polymerization complex is stabilized by addition of a cross-linking polymer. During this process, unique three-dimensional cavities complementary in size and shape to the template are formed, which are characterized by a high specificity towards the template. After removal of the template molecule, these cavities become available for re-binding of the analyte of interest [199] (Fig 7).

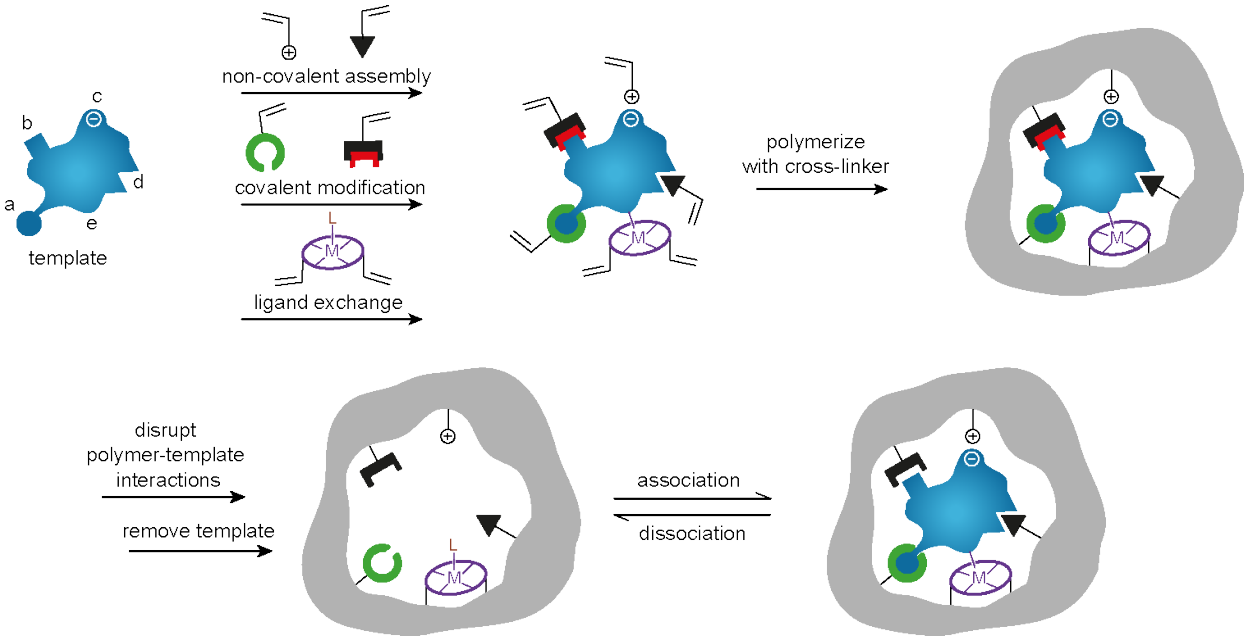

Fig 7 Schematic overview of the molecular imprinting process.

Until now, the molecular imprinting technique has not been applied for boar taint. However, a lot of research has been conducted to the development of MIPs against steroidal compounds including various androgenic steroids [200-204] and androstane steroids [205, 206]. However, most MIPs demonstrated a high degree of cross-reactivity and were applied as group-selective recognition elements. For SK, magnetic MIP particles have been developed for selective adsorption of SK or 3-methylindole from oil. These MIPs demonstrated a certain degree of selectivity towards SK in

comparison to structure analogues. However, they also represented low binding capacity, which makes them unsuitable for implementation in fast screening assays for boar taint [207].

4.3.2.5. Chromatography hyphenated to mass spectrometry

Routinely used techniques for the analysis of complex samples or sample matrices are based on chromatography coupled to MS. Chromatography is performed to separate compound mixtures, after which individual compounds are detected by a flame ionisation detector (FID), thermal conductivity detector (TCD), electron capture detector (ECD) or mass spectrometer (MS). In the context of this PhD thesis, only the use of mass spectrometry (MS) will be discussed.

Liquid and gas chromatography

Chromatography is a physical separation technique, whereby compounds selectively distribute between a mobile and stationary phase. Based on the physical state of the mobile phase, a differentiation between gas and liquid chromatography can be made.

Liquid chromatography (LC) can be subdivided into different types, whereof reverse-phase (RP) is the most commonly applied. RP makes use of a polar mobile phase and apolar stationary phase, consisting of alkyl groups attached to the surface of silica particles of approximately 5 µm diameter. Reducing the size of these stationary particles sub 2 µm has led to the development of UHPLC. This progress enabled more efficient separation and faster analysis of compound mixtures. Moreover, a better resolution, increased sensitivity and reduction of matrix effects were obtained [208]. Over the years, (U)HPLC has been widely for the detection of the boar taint compounds. Garcia-Regueiro et al. were the first to describe a normal phase HPLC method coupled to UV for the detection of SK [209]. From then onwards, mostly reversed phase chromatography was applied for the detection of the indolic compounds [210-215]. In 1994, Hansen-Møller reported the first method for the simultaneous detection of the three known boar taint compounds by means of HPLC coupled to fluorescence detection. Afterwards,

several other HPLC methods have been developed for this purpose [216-218]. Nevertheless, the use of (U)HPLC remains limited to in-laboratory use.

Gas chromatography (GC) is a common type of chromatography applied in analytical chemistry for the separation of (semi)-volatile compounds according to their volatility and polarity. Originally, GC was only used in a laboratory environment. Patterson was the first to detect AEON by GC-FID and olfactory evaluation [59]. In the following decades, several other methods were developed for the detection of AEON [83, 219-221]. Although AEON is volatile and can be detected without derivatization, the latter step often precedes GC analysis to render AEON more volatile and improve reproducibility of the method [188, 219]. For example, De Brabander et al. applied derivatisation by silylation with the Florox reagent (*o*-pentafluorobenzyl)hydroxylamine hydrochloride for the derivatization of AEON [219]. Also for SK and IND, different GC based methods have been developed [60, 222-224]. Recently, also a method for the simultaneous detection of the three boar taint compounds was developed by Fischer et al. [220, 225]. The method consisted of a fast extraction step of melted fat tissue with methanol followed by a freezing and evaporation step for enrichment of the analytes. Afterwards, the boar taint compounds were measured by applying stable isotope dilution analysis and headspace solid phase microextraction (HS-SPME) coupled to GC-MS [220].

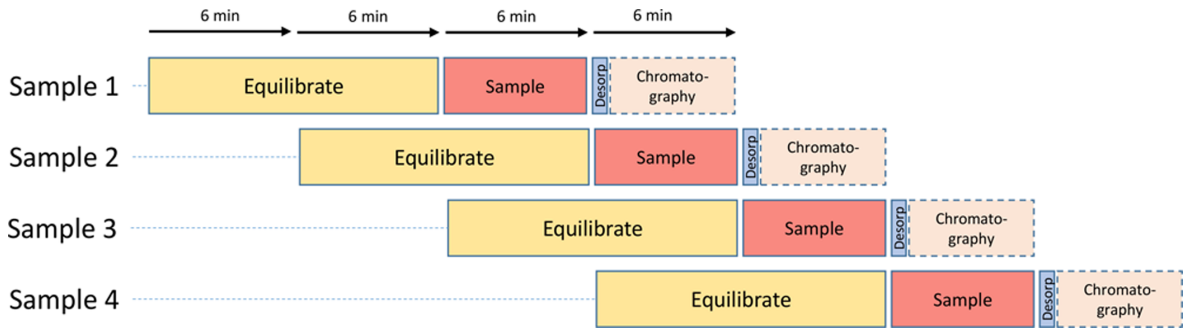

Fig 8 Sequence diagram of the high-throughput GC-MS protocol if sequenced by a multipurpose sampler robot as developed by Sorensen et al. [226].

The existing laboratory GC-MS based methods are limited from application at the slaughter line due to their extensive sample pre-treatment procedures and long chromatographic runs, compromising

their need for high-throughput detection of boar taint. However, advances in terms of in miniaturization of mass analysers, e.g. ion trap, and fast GC have led to the development of portable GC-MS instruments, which can be applied for real-time and field analysis [226]. The first portable GC instruments were developed in 1962 for space research, but further miniaturization of these instruments occurred in the late 1970s. Also in environmental field analysis, the use of portable GC instruments has gained considerable interest, especially in cases where rapid and accurate determination of analyte concentration levels is required [226]. The speed of analysis has significantly increased by the development of open tubular fused silica columns with low thermal mass properties. Consequently, these columns can be rapidly heated and cooled down, which decreases run-to-run times to less than 5 minutes. Moreover, most recent portable GC instruments use insulated wires in close proximity to the column in order to heat the latter, which eliminates the need to heat and cool down the large conventional column ovens [227]. The use of fast GC-MS for the detection of boar taint was reported by Sorensen et al., who developed a high-throughput GC-MS protocol with a possibility for automation. This method provides a first quantitative result within 24 min, followed by results from sequential carcasses every 6 minutes (Fig 8). However, although this method was significantly faster in comparison to conventional GC-MS methods, which take up-to 24 min per chromatographic run, the method still required sample collection, hampering its at-line application [228, 229]. However, due to the accurate quantification results that can be obtained by GC-MS analysis, it is still a promising technique for developing high-throughput detection methods. Moreover, the use of portable GC-MS, which has yet to be applied for boar taint, could even further increase analysis speed.

Mass spectrometry (MS)

Following chromatographic separation, the individual compounds can be detected by MS. During the past decades, the use of MS has taken the upper hand from other detection techniques, due to its superior selectivity. The combination of GC with mass spectral detection is therefore considered as very powerful for quantitative as well as qualitative analysis. The MS principle consists of generating

charged molecules or fragments thereof and sorting and detecting the intensity of the latter ions according to their mass-to-charge (*m/z*) ratio. In order to do so, the MS source is made up out of 3 essential parts, including the ionisation source, mass analyser and detector [230].

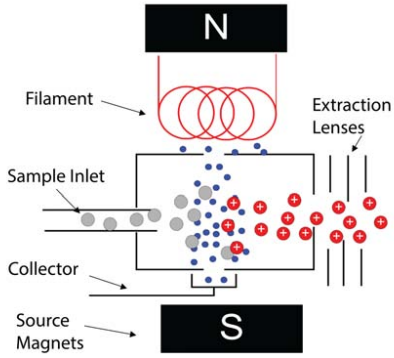

Fig 9 Schematic overview of the working principle of electron impact ionisation (EI).

Different types of ionisation techniques can be applied for the generation of ions, including electron impact ionisation (EI), chemical ionisation (CI) techniques such as atmospheric pressure ionisation (API) and electrospray ionisation (ESI), and finally matrix assisted laser desorption (MALDI). The use of API and ESI is mostly applied in combination with LC, whilst EI is often coupled to GC. With the latter ionisation technique, the sample is bombarded with accelerated electrons that cause the molecules present in the sample to lose valence electrons. Consequently, positive radical ions are formed. Moreover, EI involves a high-energy process, which involves fragmentation of the ions [220, 221, 231] (Fig 9). After ionisation, the ions are guided to the mass filter, which can consist of a quadrupole, Time-of-flight (TOF), Orbitrap or ion trap mass spectrometer.

Traditionally, for the targeted detection of compounds by GC-MS, single-quadrupole mass spectrometers were used [228]. A quadrupole consists of four parallel hyperbolic rods or poles, which are equally spaced around a central axis (Fig 10A). Under the influence of a direct current and radio frequency applied on the quadrupole rods, which is directly related to the m/z value of the ions, the ions describe a wave motion and are introduced along the axis of the poles. Afterwards, the ions are detected at a specific applied voltage [232, 233]. In a demand for more sensitive analysis, these

quadrupoles can be operated in single ion monitoring (SIM) mode, which allows to screen for one or a few single ions. However, SIM mode does not allow to obtain full scan mass spectra and thus sacrifices selectivity and compound identification [228]. To overcome this, the use of triple-quadrupoles was introduced, which consist of 3 quadrupoles placed in series, and allow multiple reaction monitoring. In this way, the second quadrupole can act as a cell for collision-induced dissociation of ions selected by the first quadrupole. Afterwards, the fragment ions can be detected by the third quadrupole (Fig 10B) [232, 233].

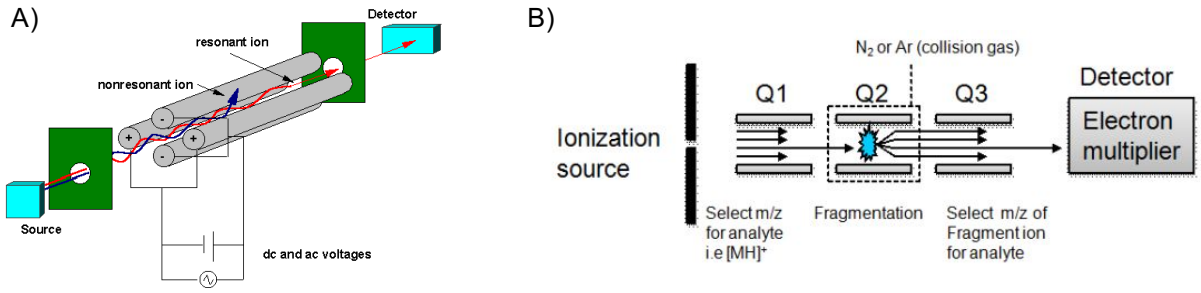

Fig 10 Schematic representation of a A) single quadrupole and B) triple quadrupole.

For screening purposes, the development of HRMS has allowed for more accurate untargeted screening of multiple compounds at the same time. In this context, the ion trap technology, among which the Orbitrap is classified, has led to progress in terms of identification of unknown compounds (Fig 11) [234, 235].

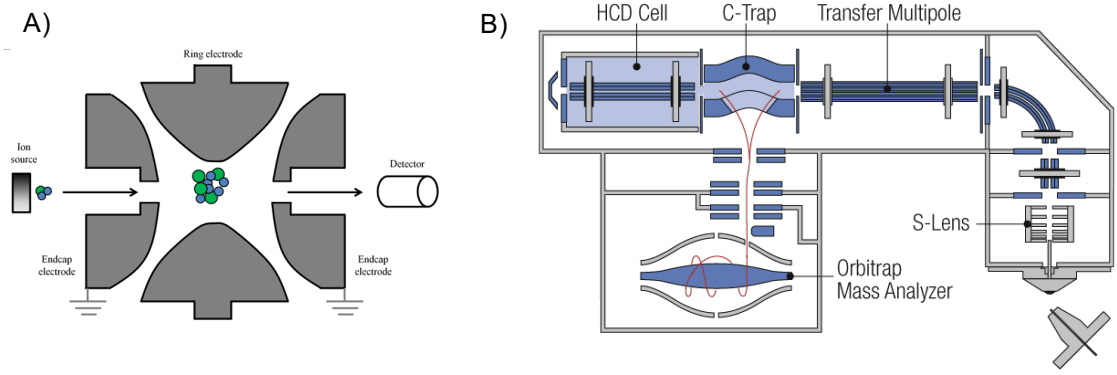

Fig 11 Schematic representation of an A) ion trap and B) Orbitrap.

Whilst ion traps are widely combined with GC, traditionally, the Orbitrap MS was limited to its use in combination with LC. However, due to recent developments in this field, its use has now been extended to the combination with GC. In an Orbitrap MS, ions are electrostatically trapped around a central electrode. Under the influence of a high voltage, these ions cycle around the central electrode and describe elliptical trajectories, which are proportional to the *m/z* value of the ions [236]. However, HRMS compromises sensitivity to gain structural information for compound identification [228].

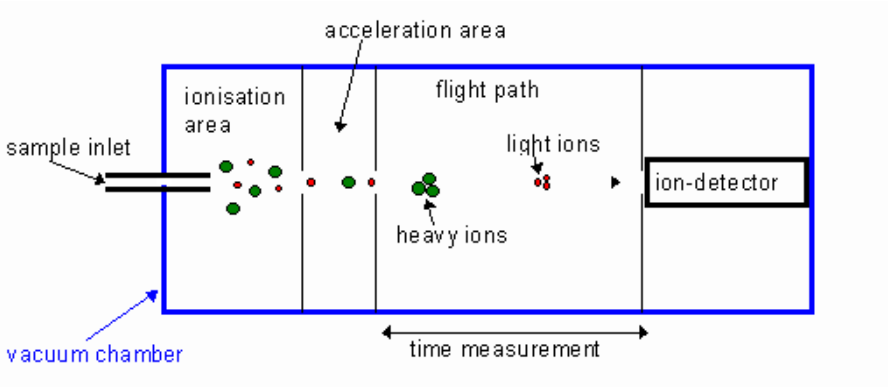

Fig 12 Schematic representation of a Time-of-Flight mass spectrometer.

In the pursuit of quantification of targeted compounds, the value of high mass resolution screening is limited to the reduction of sample matrix effects rather than structural elucidation of compounds. In addition, very high sensitivity is desired for quantification purposes. For this reason, the use of high-definition screening by means of TOF was proposed, as it delivers the benefit of matrix exclusion without compromising sensitivity (Fig 12) [228]. A TOF mass spectrometer determines the drift time of ions through the mass spectrometer. The ions are accelerated through an applied electric field so that they represent equal kinetic energies before entering the flight tube. As the kinetic energy is related to the *m/z* value of the ions, smaller ions will have a greater velocity and thus a shorter flight or transit time. As such, the measured flight time of the ions can be converted to the *m/z* value. In contrast to the quadrupole, the TOF MS is a non-scanning mass filter and is therefore well-suited for targeted as well as untargeted analysis. Moreover, the ability of the TOF MS to acquire full scan mass

range spectra without sacrificing sensitivity makes it an excellent tool for the qualitative and quantitative determination of complex samples [237].

4.3.2.6. Ambient mass spectrometry

Originally, mass spectrometric analysis was preceded by excessive sample clean-up and chromatographic separation. These time-consuming actions could be avoided with the advent of ambient mass spectrometry (AMS). As ions are formed under ambient conditions, outside the vacuum system of the mass spectrometer, AMS has the ability to register mass spectra of samples in their native environment [237]. As such, AMS allows surface sampling and real time analysis, requiring little or no sample preparation [238]. AMS has emerged during the past decade and has been widely applied in environmental, pharmaceutical and food analysis. Moreover, it is an especially useful technique in cases that require fast but accurate screening, as it generates results that are comparable to conventional applied techniques including LC and GC-MS [239, 240].

Typically, AMS techniques can be classified in different categories based on their ionisation mechanism, including spray and solid-liquid extraction-based techniques, plasma based techniques, chemical sputtering/ionisation, laser desorption/ablation, multimode techniques, acoustic desorption methods, and other techniques [241]. Differentiations within the latter ionisation techniques has led to the development of over 80 ambient ionisation techniques, yet only a few are commercially available [242].

Spray and solid-liquid extraction-based techniques

In spray desorption methods and solid-liquid extraction-based techniques, primary nebulized droplets are sprayed over the sample, whereby a liquid microlayer is formed on the sample surface. Consequently, analytes from the sample are extracted to the liquid microlayer, after which the analyte containing droplets (secondary droplets) are released from the liquid layer by pneumatically accelerated droplets (primary droplets). Finally, ions are generated from the analytes from the charged

secondary droplets by soft electrospray ionisation prior to mass spectrometric detection (Fig 13) [241-243].

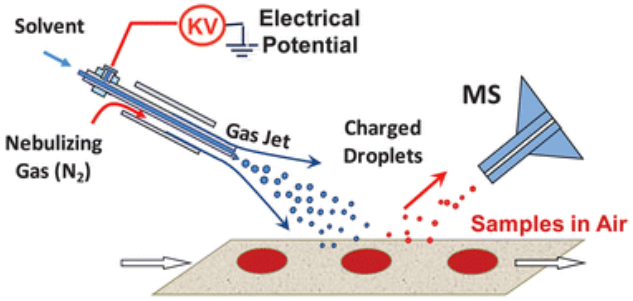

Fig 13 Illustration of desorption electrospray ionisation (DESI) [244].

The most commonly applied technique in this category is desorption electrospray ionisation (DESI), which is widely regarded as the first AMS technique created by Takats et al. and can be applied for the analysis of solid samples[245]. Variations on DESI have allowed the analysis of liquid samples through a continuous flow through a silica capillary, which is referred to as liquid-DESI [242].

Plasma based techniques

Plasma based methods rely on a direct current or radiofrequency electrical discharges between electrodes in contact with a flowing discharge gas such as helium or nitrogen. As such, the electrical discharges generate a stream of ionised molecules, radicals and excited-state neutrals. These plasma species are then directed towards the sample, with optional secondary heating of the gas stream to enhance desorption of analytes from the sample [241, 243, 246]. Afterwards, analytes can be ionised through different mechanisms including penning ionisation (PI), proton transfer (PT), electron capture (EC) and charge exchange (Fig 14). The main advantages of these plasma based techniques are the simple instrumentation, rugged construction, no need for solvents and the formation of singly charged ions, which can be more easily identified in comparison to spray techniques. Indeed, in spray techniques, multiple charged ions or adducts can be generated, making identification more difficult. Yet, ionisation in plasma based methods occurs at higher energy, leading to more ion fragmentation [240, 246].

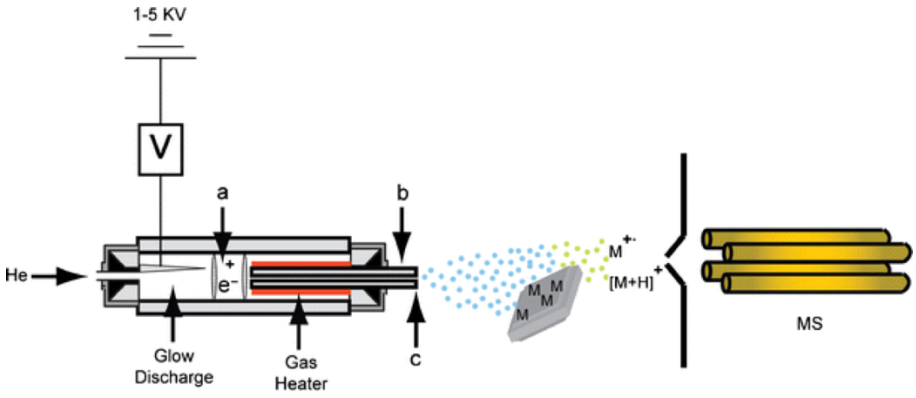

Fig 14 Illustration of direct analysis in real time (DART) [239].

The first plasma based ionisation source was developed by Cody et al. in 2005, and is referred to as direct analysis in real time (DART). Because of its ease of use, versatility and commercial availability, DART has been widely applied as a quick first screenings tool prior to confirmatory analysis by LC- or GC-MS [241].

Chemical sputtering/ionisation techniques

Chemical sputtering/ionisation techniques include desorption atmospheric pressure chemical ionisation (DAPCI) and desorption corona beam ionisation (DCBI). These approaches are somewhat related to plasma and spray-based approaches. The difference, however, lies in the fact that chemical sputtering is applied, whereby excited species bombard the sample and release analytes through a transfer of chemical energy [240-242].

Laser desorption/ablation

The main drawback of conventional laser desorption/ablation techniques such as MALDI, is the low ionisation efficiency. Particularly in biological samples, it is difficult to generate charged ions, similar to those obtained with ESI. Moreover, the demand for high vacuum conditions with MALDI hampers its application for field analysis. Hyphenating laser desorption/ablation to ESI or plasma secondary ionisation led to the development of a new subgroup of AMS approaches [242, 247].

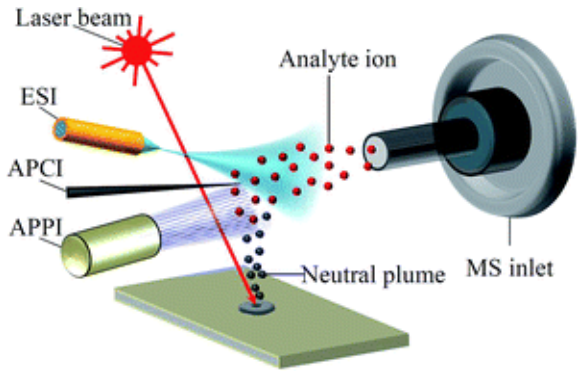

Fig 15 Illustration of laser desorption/ablation ambient mass spectrometry techniques [247].

These new techniques apply a high energy through UV or IR laser, which generates a loss of neutrals from the sample surface. Afterwards, this plume of neutrals is ionised by either electrospray ionisation or a plasma source (Fig 15). In these hybrid approaches, the desorption and ionisation step are separated. Therefore, these techniques are often referred to as "two-step" techniques. The major advantages of these approaches include the production of highly charges ions, which allows analysis of complex biological samples. Moreover, due to the small size of the laser spot, minimal amounts of samples can be analysed [247].

Multimode techniques

Classical ionisation techniques mostly apply electrospray or atmospheric pressure chemical ionisation. Recent trends in analytical chemistry have led to the development of multimode techniques, referred to as desorption electrospray/metastable-induced ionisation (DEMI), which combines both ESI and APCI-like ionisation techniques in one source. As such, the strengths of both DESI and DART techniques can be combined. Consequently, both thermally labile, non-volatile compounds with a higher molecular weight may be analysed with DESI, whereas thermally stable and lower molecular weight (< 1000 Da) compounds can be detected with DART [241, 243].

Acoustic desorption

Acoustic desorption methods can be subdivided into laser-induced acoustic desorption (LIAD) and radio frequency acoustic desorption and ionisation (RADIO). With LIAD, a high frequency laser

irradiates the backside of a thin metal foil on which the sample is located. The acoustic waves created by the laser through the metal foil causes desorption of non-volatile and thermally labile compounds from the sample matrix. Similarly, with RADIO, the energy transfer is accomplished by applying a radio frequency to a piezoelectric material [241].

Others

Finally, some other AMS approaches can be listed, which cannot be categorized in the aforementioned groups. Among them is rapid evaporative ionisation mass spectrometry (REIMS). REIMS applies a high frequency electric current by means of an electrosurgical knife, which is referred to as the intelligent knife and branded as "iKnife". This electrosurgical technique utilizes Joule heating to cut and cauterize sample tissue. The applied heat causes the production or aerosols which are guided through tubings to the MS instrument for analysis (Fig 16). The ionisation process is hypothesized to proceed through either gas phase ionisation by proton transfer and/or thermal evaporation of ionic molecules in solution due to the high water content of biological tissues [241, 242].

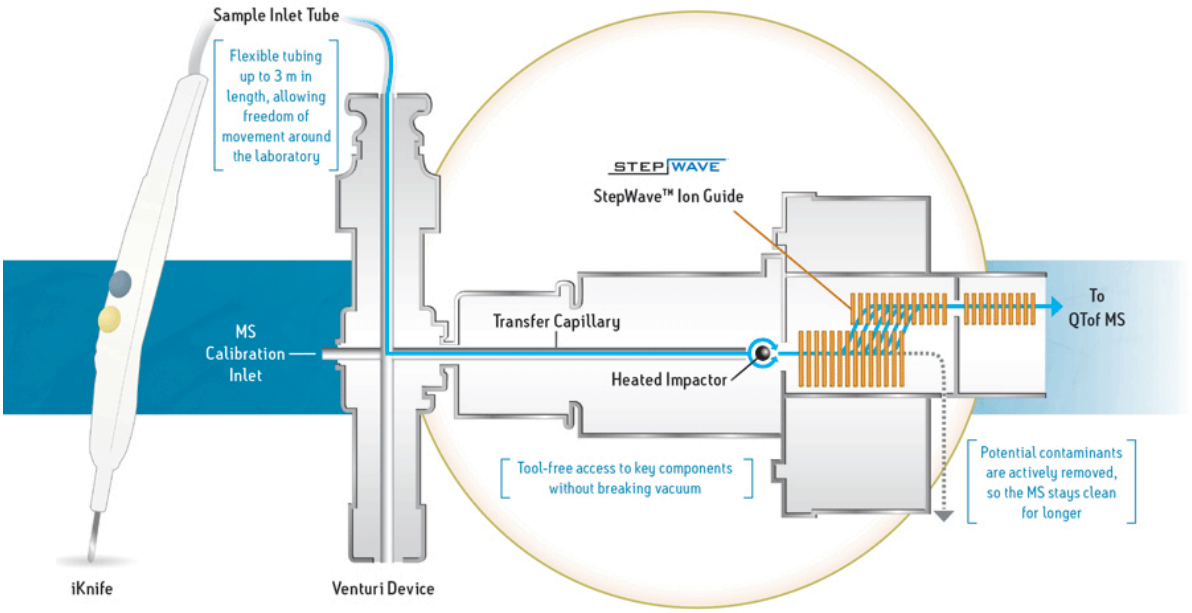

Fig 16 Illustration of the intelligent knife (iKnife) coupled to rapid evaporative ionisation mass spectrometry (REIMS) [248].

Mass spectral fingerprinting combined with chemometric data analysis can be used as a real-time monitoring platform for tissue differentiation. Tissue profiling has led to differentiation of over 60 different tissue types, based on variations in lipid profiles (phospholipids, fatty acids). Moreover, *in-vivo* studies on rats fed diets with differing fatty acid content indicated that tissue identification was not influenced by dietary intake of fatty acids. These promising results demonstrate the potential for building databases for specific tissue markers [241, 249, 250]. Since already promising results were achieved with REIMS for tissue characterization, this technique has emerged to other applications. As such, REIMS has been successfully applied for identification of bacterial colonies at species level [249-252]. Moreover, REIMS has also found different applications in food analysis, particularly for the detection of meat and fish fraud [253, 254].

5. CONCEPTUAL FRAMEWORK OF THIS STUDY

In view of improving animal welfare, a gradual phase out of the surgical castration of pigs is anticipated. Consequently, in the long run, an increase of immunocastrates and entire male pigs is to be expected. As raising entire male pigs is associated with a strong and moderate prevalence of boar taint in 4% and 25% of pig carcasses, respectively, an impairment of consumer acceptance is presumed as well. In order to prevent this, different conditions should be met as laid down in the European declaration for alternatives to the surgical castration of pigs [6]: i) the development of strategies for the reduction of boar taint prevalence in pig carcasses, ii) the valorisation of tainted boar meat and iii) the development of fast and reliable detection methods for boar taint at the slaughter line.

Over the past years, international research on boar taint mainly focussed on the primary and distribution phase in pig sector, namely, identification of reducing strategies for boar taint in pig carcasses (primary phase) as well as assessing consumer responses to boar meat (distribution phase) (Fig 17).

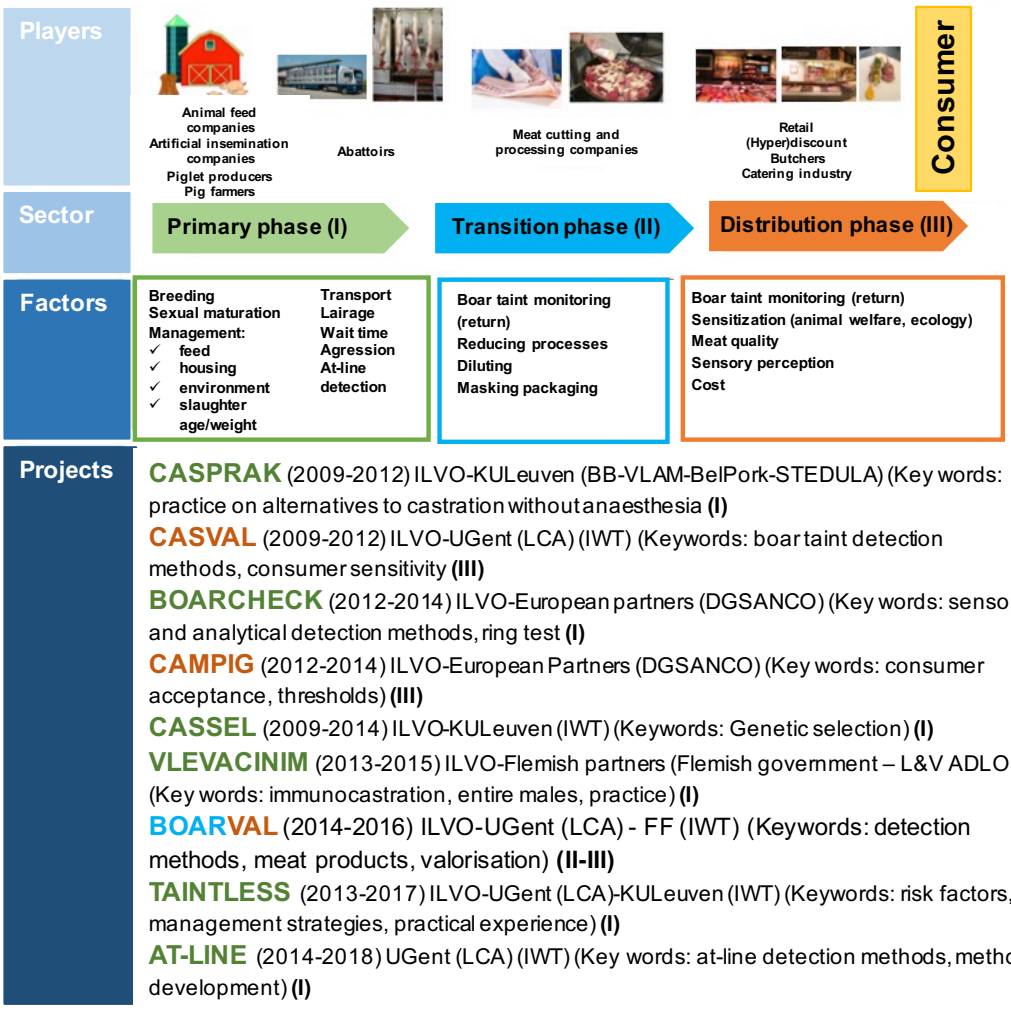

Fig 17 Overview of the pig sector, risk factors for boar taint and overview of past and on-going research in Flanders.

In Flanders, the BOARVAL project, of which the results were reported in this PhD work (Part I), was the first project to focus on this transitional phase by evaluating the influence of different processing procedures on the sensory perception of boar taint in meat. Currently, also a fast and reliable detection method for boar taint at the slaughter line is still lacking, which is indispensable for sorting and guiding tainted carcasses to alternative processing circuits in the transition phase. In view of at-line implementation of such methods, the aim of Part II of this PhD study was the development of fast and reliable detection methods for boar taint. Both part I and II of this study were characterised by specific goals, which are listed below and depicted in Fig 18.

PART I: Valorisation of tainted boar meat

- To develop and validate an analytical method for the accurate and specific determination of the boar taint compounds in different pork products (**Chapter II**).
- To assess the sensory acceptance of different boar meat products by a trained panel (**Chapter III**).
    - Evaluate differences in sensory perception between different boar categories
    - Evaluate differences in sensory perception between meat products
- To assess the sensory acceptance of different boar meat products by a trained panel and consumers (**Chapter IV**).
    - Estimate rejection thresholds for the boar taint compounds in meat
    - Evaluate the effect of diluting tainted meat on the sensory perception of boar taint
    - Confirm results by consumer panel

PART II: Development of fast and reliable detection methods for boar taint at the slaughter line

- To develop and validate a rapid analytical method for the accurate and specific determination of the boar taint compounds by means of solid phase microextraction and portable gas chromatography coupled to mass spectrometry (**Chapter V**).
- To develop and validate a molecularly imprinted polymer screening assay for the detection of skatole and indole in porcine neck fat (**Chapter VI**).
- To evaluate the feasibility of rapid evaporative ionisation mass spectrometry as an untargeted ambient mass spectrometric strategy for the high-throughput screening of boar taint (**Chapter VII**).

In **Chapter VIII**, the main research findings of this study were summarized and discussed. Finally, also the future perspectives resulting from this study were addressed.

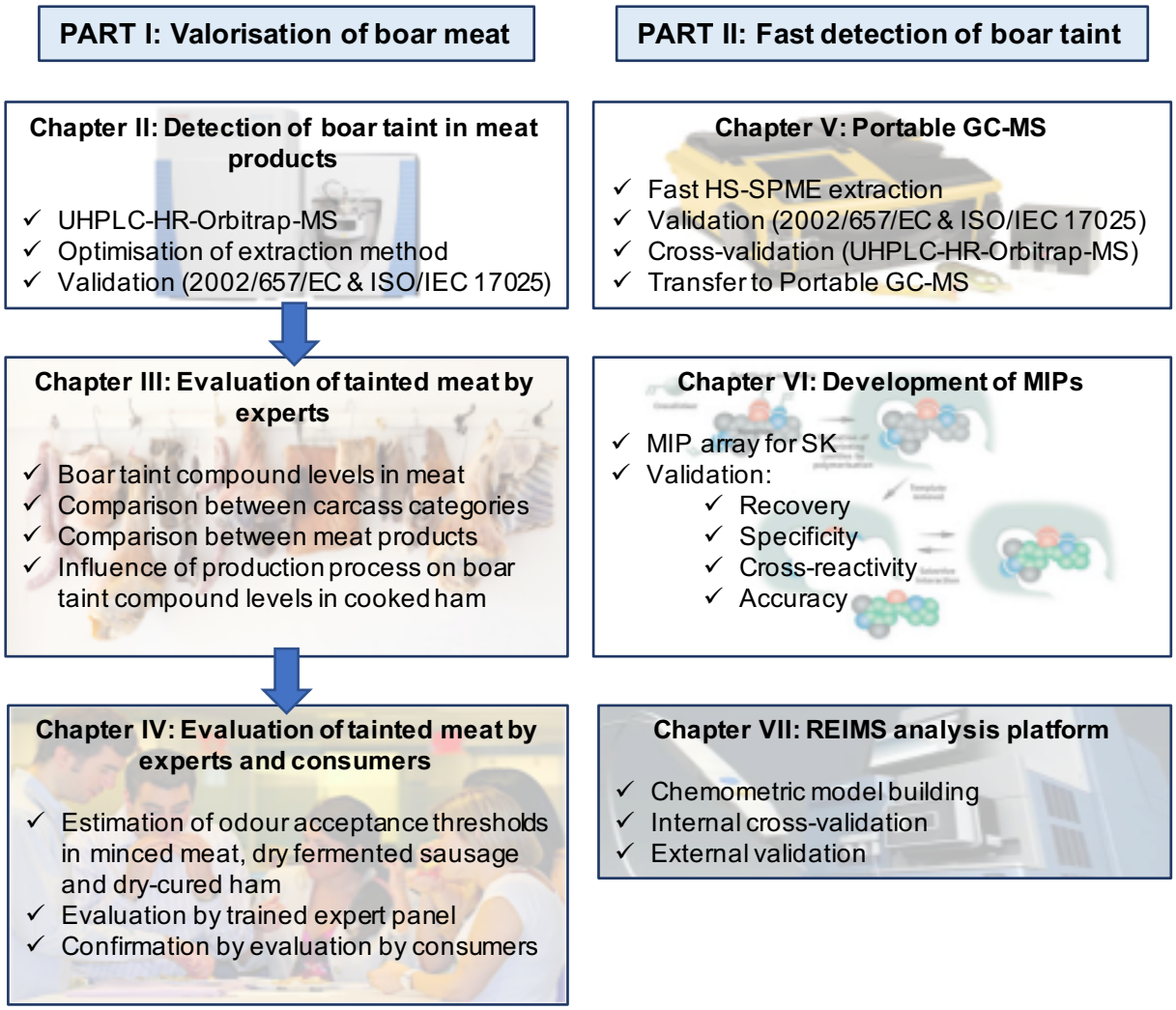

Fig 18 Schematic overview of the main research goals of this PhD study.

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PART I

VALORISATION OF TAINTED BOAR MEAT

CHAPTER II

DEVELOPMENT AND VALIDATION OF A UHPLC-HR-ORBITRAP-MS METHOD FOR THE SIMULTANEOUS DETERMINATION OF ANDROSTENONE, SKATOLE AND INDOLE IN PORCINE MEAT AND MEAT PRODUCTS

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ABSTRACT

Boar taint is an off-odour that entails negative consumer reactions. In this study two extraction and UHPLC-HRMS analysis methods, valuable for evaluation of consumer acceptance towards boar meat, were developed for quantification of IND, SK, and AEON in different meat products. Sample pre-treatment consisted of extraction with methanol and a homogenizing step (cooked ham, minced meat, tenderloin, bacon, cutlets, blade loin, dry-cured ham) or a melting step (dry fermented sausage and liver paste). Both methods were validated according to CD 2002/657/EC and ISO17025 guidelines. Good performance characteristics were obtained. Good linearity ($R^2 \geq 0.99$) and no lack of fit was observed (95% confidence interval; F-test, $p > 0.05$). Also good recovery (89% - 110%) and satisfactory precision: repeatability (RSD ≤ 14.9%) and within-laboratory reproducibility (RSD ≤ 17.2%) were obtained. Analysis of cooked ham and dry fermented sausage samples proved the applicability of both methods for routine analysis.

1. INTRODUCTION

The surgical castration of male pigs has been widely practiced for centuries. Two reasons for this practice are the prevention of undesirable behaviour and a higher percentage of fat deposition. However, the main reason for the surgical castration of pigs is the prevention of boar taint, i.e. an off-odour that can be released by heating the meat or fat of non-castrated boars [1]. The main compounds contributing to this taint are 5α-androst-16-ene-3-one (AEON) [2], 3-methylindole (SK) [3], and to a lesser extent indole (IND) [4]. AEON is a pheromone produced in the Leydig cells of the testes and contributes to boar taint by having a sweaty- or urine-like odour, whereas SK and IND are two fermentation products derived from the biological degradation of L-tryptophan by intestinal bacteria and are described as having a faecal-like odour [5-8].

Since research showed that surgical castration causes pain even in very young animals, societal pressure against the surgical castration of pigs has risen [9]. Hence, Norway, The Netherlands and Germany were the first to voluntarily implement surgical castration with anaesthesia and/or analgesia. Subsequently, in 2010 the European declaration on alternatives to the surgical castration of pigs was signed, in which participating member states engage to no longer perform surgical castration of pigs without anaesthesia and/or analgesia and in the long run to ban surgical castration of pigs by January 2018 [10]. Alternatives to surgical castration are gender selection, immunocastration and the production of entire males. The latter alternative is considered as valid in terms of animal welfare and is associated with a lower feed conversion ratio, faster growth and more lean meat production [11, 12]. However, the main problem remains the possible production of boar taint, which could entail negative consumer reactions.

In light of the impending ban on the surgical castration of pigs, it is important to valorise meat from entire males and to assess its impact on consumer acceptance. Since general agreement on acceptable levels of boar taint compounds is lacking [13-16], determination of odour thresholds for these compounds in different meat cuts and products could increase the understanding of consumer

acceptance or restraint of meat from entire male pigs. Accordingly, the determination of thresholds necessitates analytical methods for the quantification of the boar taint compounds in different meat matrices.

Over the years several methods for the detection of individual boar taint compounds have been developed [17-21]. Hansen-Møller was the first to describe a method for the simultaneous detection of the three known boar taint compounds by means of HPLC coupled to fluorescence detection [22]. Also HS-SPME coupled to GC-MS, UHPLC coupled to linear ion trap MS or HRMS by means of an Orbitrap mass analyser have been proposed for the quantification of AEON, SK and IND simultaneously [23-26]. In spite of the above mentioned methods that focus on adipose tissue, serum and plasma as sample matrices, only one previous report on the simultaneous quantification of boar taint compounds in fermented sausages is available [27].

Therefore, the aim of this study was to develop accurate, robust and fast extraction and UHPLC-HRMS analysis methods for the simultaneous quantification of AEON, SK and IND in different meat products (cooked ham, minced meat, tenderloin, bacon, cutlets, blade loin, dry fermented sausage, dry-cured ham and liver paste), which would be highly valuable for the evaluation of consumer acceptance towards boar taint affected meat and fixing thresholds for boar taint related compounds. In total, 2 extraction methods were developed and optimized for the more lean meat products and fatty products, respectively. Finally, these methods were validated according to the guidelines of CD 2002/657/EC and ISO 17025 [28, 29]. A full validation was performed for cooked ham and dry-fermented sausage. For the other meat products, a shortened validation protocol was followed.

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

The reference standards IND (2,3-benzopyrrole), SK (3-methylindole), and AEON (5α-androst-16-ene-3-one) and the internal standards 2-methylindole (2-MID) and androstadienedione (1,4-androstadiene-3,17-dione, ADD) were obtained from Sigma Aldrich (St. Louis, MO, USA). For each compound a stock solution was prepared in methanol at a concentration of 2 mg ml^{-1}. Working solutions were made for each compound in methanol in a range of 1-500 ng μl^{-1}. Solutions were stored in dark glass bottles at -20 °C. Reagents were of analytical grade when used for extraction purposes and of MS-grade for UHPLC-MS applications. They were obtained from VWR International (Merck, Darmstadt, Germany) and Fischer Scientific (Leichestershire, VS), respectively. Solid phase extraction (SPE) columns were purchased from Waters Corporation (Milford, US).

2.2. Samples

The different meat products (cooked ham, minced meat, tenderloin, bacon, cutlets, blade loin, dry fermented sausage, dry-cured ham and liver paste) were purchased at the local supermarket. After arrival in the lab, cooked ham, minced meat, tenderloin, bacon, cutlets, blade loin and dry-cured ham were each separately blended and stored in plastic bags at -20 °C until analysis. Dry fermented sausage and liver paste were directly stored at -20 °C after arrival in the lab. Processed meat products only consisted of porcine ingredients (Dry fermented sausage: porcine meat, fat, and hemoglobin; liver paste: porcine meat 12%, fat, and liver 34%).

2.3. Sample extraction and clean-up

Two different protocols for extraction and clean-up of the samples were optimized. The meat products were subdivided into two categories in accordance with their fat percentage. Cooked ham (2.5% fat), tenderloin (2.3% fat), cutlets (9.9% fat), dry-cured ham (12% fat) and blade loin (15% fat) were subjected to a protocol including direct extraction of the analytes with methanol and a homogenizing

step. Meat products with a higher fat content, among which dry fermented sausage (30% fat) and liver paste (30% fat) were extracted with a second protocol that included a melting step to release the fat fraction. Despite their high fat content, extraction of minced meat (24.6% fat) and bacon (36.2% fat) proved better with the first protocol.

2.3.1. Method 1: extraction with homogenising step

Cooked ham, minced meat, tenderloin, bacon, cutlets, dry-cured ham and blade loin were extracted using this method. Two grams of blended meat were fortified with a mixture of internal standards (2-MID: 500 µg kg^{-1} and ADD: 1000 µg kg^{-1}). To ensure a homogeneous distribution of the compounds in the sample matrix, the samples were mixed and vortexed, and subsequently rested for 5 minutes at room temperature. Methanol (5 ml) was added and each sample was vortexed thoroughly for two minutes. Further homogenization was carried out using an Ultra-Turrax (IKA® T18 Digital) and the samples were centrifuged at 13,300 x g for 10 min. Next, the supernatant was transferred into 15 ml tubes, which were frozen (-20 °C) for 60 min to clarify the supernatant. Afterwards the 15 ml tubes were again centrifuged at 12,300 x g for 5 min and 2 ml of the extract was diluted with 38 ml water prior to solid phase extraction (Oasis HLB 3 cm3 (60 mg), Waters). The cartridge was conditioned with 2 ml of 100% methanol and equilibrated with 2 ml of 5% methanol. After loading the sample, the cartridge was washed with 2 ml of 20% methanol and eluted with 1 ml of 100% methanol. Of the obtained extract, 100 µl was diluted with 100 µl of 0.05% formic acid into an LC-MS vial prior to HPLC analysis.

2.3.2. Method 2: extraction with melting step

Dry fermented sausage and liver paste were extracted using this method. However, small differences in sample size, microwave conditions and centrifugation steps between the two meat matrices were implemented. Five grams of dry fermented sausage were sliced into small pieces whereas of the liver paste, 8 grams were weighed. Both samples were fortified with a mixture of internal standards (2-MID: 500 µg kg^{-1} and ADD: 1000 µg kg^{-1}) and each sample was thoroughly mixed and vortexed. Afterwards,

the samples were rested for 5 minutes at room temperature to allow distribution of the compounds within the sample matrix. Next, the samples were melted in the microwave oven (dry fermented sausage: 3 min at 200 W; liver paste: 3 min at 100 W). Afterwards the melted fraction of liver paste was centrifuged at 17,000 x g for 1 min at room temperature to separate the fat fraction of the supernatant. From both dry fermented sausage and liver paste samples an aliquot of 150 µl of melted fat was taken and mixed with 750 µl methanol. The eppendorfs were transferred to an ultrasonic bath (Elma® Transsonic Digital) for 10 min at 32 °C (power 9) in order to enhance extraction. Afterwards the eppendorfs were frozen (-20 °C) for 15 min and centrifuged at 17,000 x g for 5 min at 4 °C. Of the extract, 500 µl was diluted with 9500 µl water prior to solid phase extraction as described under 2.3.1.

2.4. Instrumentation

The UHPLC system consisted of a Thermo Fisher (Thermo Fisher Scientific, San José, CA, USA) Accela UHPLC pumping system coupled to an Accela Autosampler and Degasser. Chromatographic separation was achieved using reversed phase chromatography with gradient elution. Separation of the compounds was carried out on a Hypersil Gold column (1.9 µm, 50 mm x 2.1 mm ID) (Thermo Fisher Scientific, San José, CA, USA). The mobile phase consisted of a mixture of methanol and 0.05% formic acid, pumped at a flow rate of 0.3 ml min^{-1}. Optimized separation of the compounds was obtained using a linear gradient.

Mass spectrometric analysis was carried out using an Exactive™ benchtop mass spectrometer (Thermo Fisher Scientific, San José, CA, USA) fitted with an atmospheric-pressure chemical ionisation source (APCI) operated in the positive ion mode. The optimal ionisation source working parameters can be consulted in Bekaert et al. [216]. A scan range of *m/z* 100 – 500 was chosen and the resolution was set at 50,000 full width half maximum (FWHM) at 2 Hz (2 scans per second). The automatic gain control (AGC) target was set at high dynamic range (3 x 10^6) and the maximum injection time was 500 ms. Initial instrument calibration was carried out by infusing calibration mixtures for the positive and negative ion modes (Thermo Fisher Scientific, San José, CA, USA). The positive calibration mixture

included caffeine, Met-Arg-Phe-Ala acetate salt (MRFA) and Ultramark® 1621. These compounds were dissolved in a mixture of acetonitrile, water and methanol, and both mixtures were infused using a Chemyx Fusion 100 syringe pump (Thermo Fisher Scientific, San José, CA, USA). The option "all-ion fragmentation" using the High-Energy Collision Dissociation (HCD) cell was turned off. The fore vacuum, high vacuum and ultrahigh vacuum were maintained at approximately 2 mbar, from 10^{-5} and below 8 x 10^{-10} mbar, respectively. Instrument control and data processing were carried out by Xcalibur 2.0.7 SP1 software (Thermo Fisher Scientific, San José, CA, USA).

2.5. Quality assurance

Prior to sample analysis, a standard mixture (1 ng on column) of the target compounds was injected to evaluate the operational conditions of the chromatographic devices. Analytes were identified based on their relative retention time, i.e. the ratio of the retention time of the analyte relative to that of the internal standard, and their accurate mass, as can be consulted in Bekaert et al. [23]. Moreover, for each target compound, the C_{12} and C_{13} isotope were analysed, which counts as 2 diagnostic ions per compound. Consequently, as HRMS mass spectrometric detection was applied, a total of 4 identification points was obtained for each target compound. This exceeds the 3 required identification points for mass spectrometric detection. For quantification purposes, matrix-matched calibration curves were prepared by spiking the different meat samples with a standard mixture of IND, SK, and AEON obtaining fourteen concentrations (0, 10, 25, 50, 75, 100, 250, 500, 750, 1000, 2000, 3000, 4000 and 5000 µg kg^{-1}). The internal standards 2-MID and ADD were added at a concentration of 500 µg kg^{-1} and 1000 µg kg^{-1}, respectively.

2.6. Method validation

Both optimized methods were validated according to the criteria of the European Commission (2002/657/EC) and/or ISO/IEC 17025 guidelines. Specificity, selectivity, linearity, trueness and precision were assessed according to the 2002/657/CD guideline. As there are no maximum residue limits (MRLs) available for the boar taint compounds, sensitivity of the method was assessed through

the limits of detection (LOD) and quantification (LOQ) in accordance to the ISO/IEC 17025 guidelines. For cooked ham and dry fermented sausage, a complete validation was carried out. For the other meat products linearity, recovery and repeatability were assessed.

In order to check the specificity and selectivity of both methods, blank as well as fortified samples were analysed and the boar taint compounds were identified taking into account their relative retention time and accurate mass. Quantification was based on 14-point matrix matched calibration curves. Linearity of the curves was verified through determination coefficients (R^2) and lack of fit. To this end, a univariate linear regression model was built (SPSS version 22.0; IBM, USA) in which the calibration concentrations were set as independent variables and the respective area ratios were set as dependent variables. To evaluate both trueness and precision of the methods, repeatability and within-laboratory reproducibility were assessed by analysing blank samples (6 per level) fortified with 3 different concentrations (IND: 50, 100, 200 µg kg^{-1}, SK: 100, 250, 500 µg kg^{-1}, AEON: 250, 500, 1000 µg kg^{-1}). To evaluate both parameters relative standard deviations (RSD, %) were calculated. Limits of detection and quantification (LOD and LOQ) were determined as the concentrations, which generate chromatographic peaks with a signal-to-noise ratio of 3:1 and 10:1, respectively, according to ISO 17025 [259, 260].

2.7. Analysis of cooked ham and dry fermented sausage samples

In a pilot study, boar taint positive carcasses were selected at the slaughter line by means of the soldering iron method as optimized by Bekaert et al. [30]. To confirm the presence of IND, SK and/or AEON a neck fat sample from each carcass was analysed according to Bekaert et al. [23]. After slaughter, commercial meat companies, located in Belgium, produced cooked ham and dry fermented sausage from the selected carcasses. Subsequently, a sample was taken from each meat product and analysed in duplicate using the newly developed methods.

3. RESULTS AND DISCUSSION

3.1. Development of sample pre-treatment procedure

Previous reports concerning the extraction of boar taint compounds from adipose tissue served as a starting point for the optimization of the protocol for the extraction of the boar taint compounds from different meat matrices [23, 25, 27]. Initially, extraction and clean-up of all meat products was carried out by the adapted protocol of Bekaert et al., which consisted of a melting step followed by liquid-liquid extraction [23]. However, the results obtained for cooked ham, minced meat, tenderloin, bacon, cutlets, blade loin, and dry-cured ham showed poor reproducibility and an insufficient yield for the extraction of AEON (data not shown). Therefore, experimental changes to the extraction protocol were evaluated for these products, leading to better results, in case the analytes were extracted directly with methanol. After these initial experiments, both extraction protocols were further optimized.

For those meat products for which the protocol with direct extraction of the analytes by methanol proved best (cooked ham, minced meat, tenderloin, bacon, cutlets, dry-cured ham and blade loin), additional optimisation was performed, by testing two alternative methods. In the first method, extraction of the compounds was enhanced by transferring samples to an ultrasonic bath for 10 min followed by one hour in a hot water bath (60 °C). In the second protocol, homogenization and extraction were carried out by using an Ultra-Turrax (IKA® T18 Digital). Based on RSD (%, n = 3) values of 35.5% and 11.13% for the first and second method, respectively, the second method proved to be superior and was selected for the extraction of the boar taint compounds from cooked ham, minced meat, tenderloin, bacon, cutlets, blade loin and dry-cured ham.

Dry fermented sausage and liver paste were extracted following a melting step, however some matrix-specific optimisations to the protocol adapted from Bekaert et al. had to be made [23]. First of all, a larger sample size was used, i.e. 5 g of dry fermented sausage and 8 g of liver paste, to ensure an adequate release of fat. Moreover, also the microwave oven conditions were optimized for each meat product by varying both time and wattage. Because the volume of melted fat in liver paste samples

was too low to allow efficient pipetting, an additional centrifugation step was included in the protocol to separate the fat fraction from the supernatant. For dry fermented sausage, a sufficient volume of fat was obtained after melting in the microwave oven. Consequently, no further concentrating step was needed.

3.2. UHPLC and MS parameters

The UHPLC-MS method was adopted from Bekaert et al., who developed the procedure for the simultaneous quantification of the three known boar taint compounds in adipose tissue [23].

3.3. Method validation

3.3.1. Specificity and selectivity

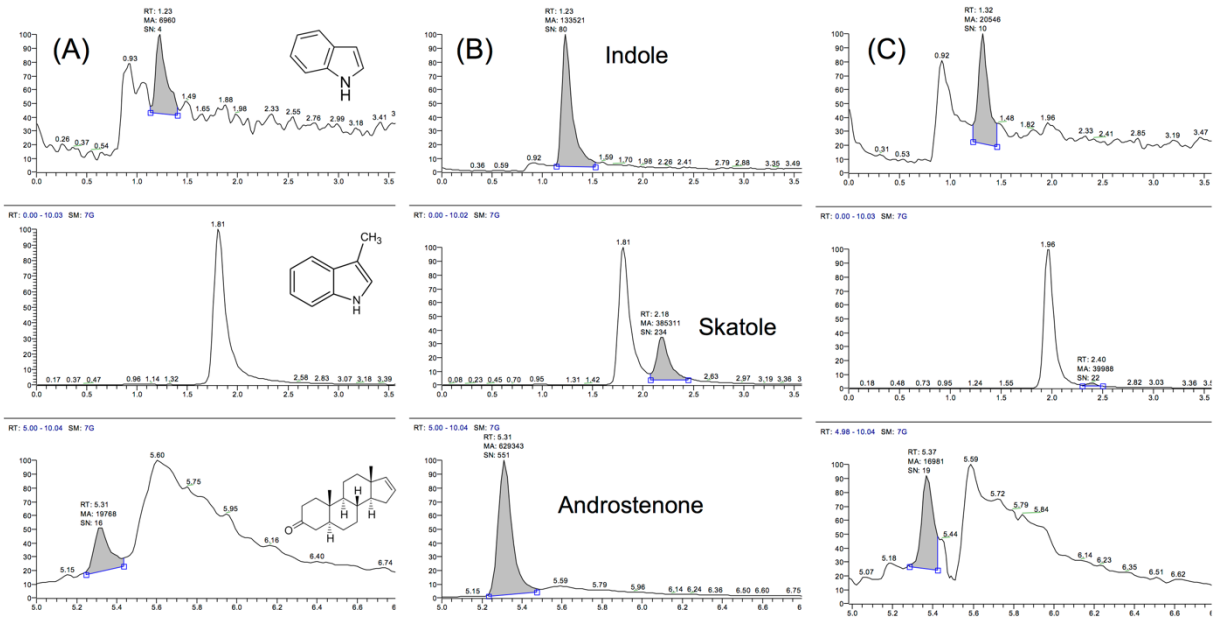

Fig 1 Chromatogram of A) a blank cooked ham sample, B) a sample fortified with 100, 250 and 500 µg kg^{-1} of IND, SK and AEON, respectively and C) a sample fortified at the limits of quantification (LOQs: 5 µg kg^{-1} for IND, SK and AEON), as analysed on the UHPLC-HR-Orbitrap-MS instrument. (RT: retention time; MA: area under the curve; SN: signal-to-noise ratio).

Specificity and selectivity of both methods were evaluated by analysing blank samples as well as blanks fortified with the analytes of interest at a concentration of 100 µg kg^{-1}, 250 µg kg^{-1} and 500 µg kg^{-1} for IND, SK and AEON, respectively. Since no true blank samples were available, different meat products

purchased at the local supermarket were selected containing little or no background of the boar taint compounds. In the samples of cooked ham, bacon, tenderloin, minced meat, cutlets, dry-cured ham and blade loin analysed, low concentrations of IND and AEON were found (Fig1). A possible explanation for this could be the endogenous presence of the boar taint compounds in gilts, sows, barrows and boars. In addition, this theory can be underpinned by the relation between endogenous levels of AEON and the indolic compounds. Compared to gilts, sows and barrows, significantly higher levels of IND and SK can be found in boars, indicating an association between AEON and the other boar taint compound levels [31-33]. In this study, the obtained levels of AEON and IND were higher for minced meat and tenderloin in comparison to those obtained for cooked ham, bacon, cutlets, dry-cured ham, blade loin, and liver paste and could indicate that minced meat and tenderloin originated from boar carcasses.

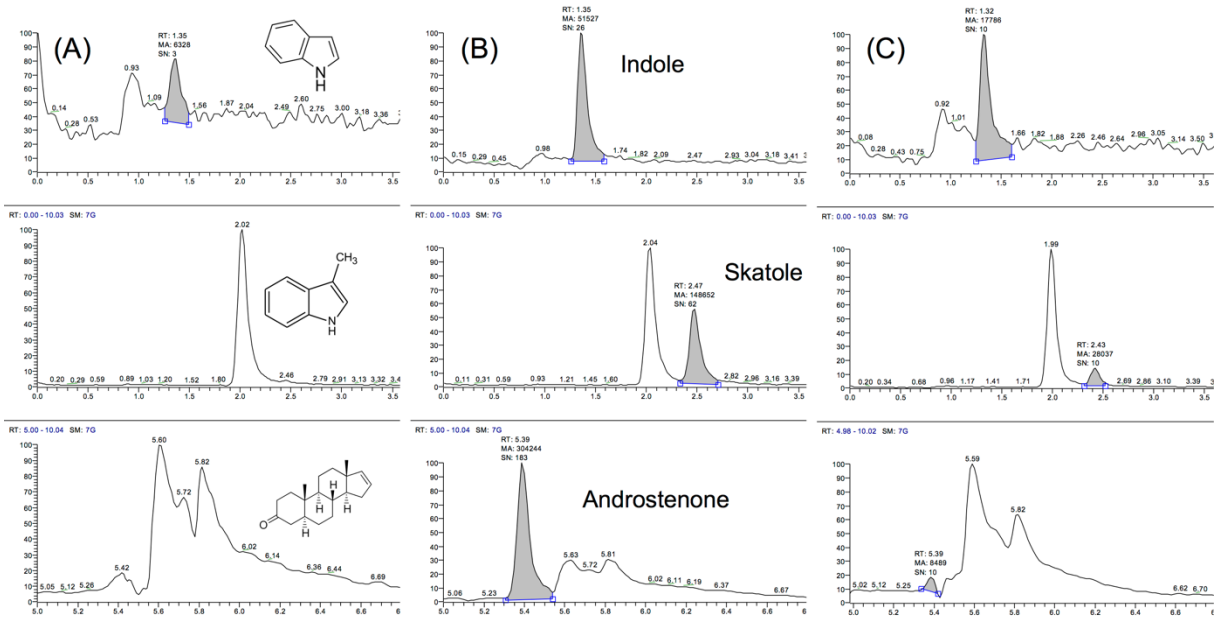

Fig 2 Chromatogram of A) a blank dry fermented sausage sample, B) a sample fortified with 100, 250 and 500 µg kg^{-1} of IND, SK and AEON, respectively and C) a sample fortified at the limits of quantification (LOQs: 10, 25 and 2.5 µg kg^{-1} for IND, SK and AEON, respectively), as analysed on the UHPLC-HR-Orbitrap-MS instrument. (RT: retention time; MA: area under the curve; SN: signal-to-noise ratio).

When fortifying the blank samples, a significant increase in peak area intensity of the chromatographic peaks at their specific retention times could be observed, taking into account a signal-to-noise ratio of at least 3. At the specific retention times of the compounds, no other interfering substances could be

found. However, non-interfering peaks close to the exact retention time of AEON were noticed (Fig1 & 2). The optimized method was found to be specific for IND, SK and AEON in the presence of other matrix compounds. Since analytes were identified based on their accurate mass (signal-to-noise ratio (S/N) ≥ 3; mass deviation ≤ 5 ppm or IND ≤ 0.60 mDa, SK ≤ 0.66 mDa, AEON ≤ 1.37 mDa) and relative retention time, i.e. the ratio between the retention time of the analyte and its internal standard, also high selectivity for both methods is ensured.

3.3.2. Linearity

Linearity of both methods was evaluated by setting up 14-point based calibration curves in matrix for the compounds of interest, this in three replicates. The blank samples were fortified with concentrations ranging from 0-5000 µg kg^{-1}. The linearity and lack of fit of the developed method were verified by building a univariate linear regression model (SPSS version 22.0, IBM, USA). The calibration concentrations were set as independent variables whereas the respective area ratios were set as dependent variables. Since low background concentrations of IND and AEON were observed in the blank samples of cooked ham (IND: 3 µg kg^{-1}, AEON: 8 µg kg^{-1}), bacon (IND: 6 µg kg^{-1}, AEON: 15 µg kg^{-1}), cutlets (IND: 7 µg kg^{-1}, AEON: 14 µg kg^{-1}), dry-cured ham (IND: 4 µg kg^{-1}, AEON: 8 µg kg^{-1}), blade loin (IND: 8 µg kg^{-1}, AEON: 12 µg kg^{-1}), tenderloin (IND: 2 µg kg^{-1}, AEON: 5 µg kg^{-1}) and minced meat (IND: 3 µg kg^{-1}, AEON: 13 µg kg^{-1}), these levels were accounted for. To this end, the mean endogenous concentrations in 20 blank samples were calculated and then subtracted from the retrieved area ratios for each calibration level. Moreover, because of the wide range of the calibration curves, the low calibration levels only minorly influenced the linear regression model. This could also be observed from the residual plot and the heteroscedasticity test ($p < 0.001$), indicating that the error term of the regression model approximated a heterogeneous distribution. This was counteracted through weighted least squares linear regression (WLSLR), by giving equal weight to all calibration levels. Different weighing factors ($1/x^{power}$) were evaluated and the power range was varied between -2 and 2 in steps of 0.5. For each compound, the factor leading to the lowest sum of relative errors was used

as a weighing factor. In Table 1, a comparison was made between 1 and $1/x^{power}$ as weighting factors. Because WLSLR gives equal weight to all calibration levels, a more accurate estimation of the low (10 µg kg^{-1}) and medium (2000 µg kg^{-1}) boar taint levels could be made. For this reason, WLSLR was used to build the final regression model.

Table 2 Calibration equations for IND, SK and AEON obtained by weighted linear regression.

	IND	SK	AEON
Cooked ham	$y = 0.007 + 1.10E^{-3}x$	$y = 0.036 + 1.36E^{-3}x$	$y = 0.008 + 1.09E^{-3}x$
Minced meat	$y = 0.022 + 1.14E^{-3}x$	$y = 0.054 + 1.55E^{-3}x$	$y = 0.063 + 1.00E^{-3}x$
Tenderloin	$y = 0.023 + 1.10E^{-3}x$	$y = 0.064 + 1.70E^{-3}x$	$y = 0.012 + 8.05E^{-4}x$
Bacon	$y = 0.011 + 1.17E^{-3}x$	$y = 0.044 + 1.55E^{-3}x$	$y = 0.008 + 8.58E^{-4}x$
Cutlets	$y = 0.004 + 1.08E^{-3}x$	$y = 0.005 + 1.35E^{-3}x$	$y = 0.029 + 1.74E^{-3}x$
Dry-cured ham	$y = 0.015 + 1.02E^{-3}x$	$y = 0.028 + 1.43E^{-3}x$	$y = 0.021 + 4.64E^{-4}x$
Blade loin	$y = 0.007 + 1.17E^{-3}x$	$y = 0.022 + 1.28E^{-3}x$	$y = 0.007 + 1.72E^{-3}x$
Liver paste	$y = 0.024 + 1.05E^{-3}x$	$y = 0.013 + 1.23E^{-3}x$	$y = 0.071 + 1.81E^{-3}x$
Dry fermented sausage	$y = 0.007 + 1.07E^{-3}x$	$y = 0.017 + 1.43E^{-3}x$	$y = 0.041 + 1.39E^{-3}x$

The obtained regression models showed good linearity ($R^2 \geq 0.99$) and no lack of fit (95% confidence interval; F-test, $p > 0.05$) (Tables 3-5), the regression equations can be consulted in Table 2. Determination coefficients for IND and SK were comparable to those obtained by Rius et al. for loin samples; However, a larger linear dynamic range (0 µg kg^{-1} – 5000 µg kg^{-1}) was obtained with our newly developed methods compared to Rius et al. (50 µg kg^{-1} – 400 µg kg^{-1}) [34].

Table 3 Summary of the method validation performance characteristics as determined for cooked ham.

Analyte	Nominal Concentration (µg kg^{-1})	Recovery Mean ± SD n = 18	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	Precision		Linearity R^2
					Repeatability RSD (%) n = 18	Within-laboratory Reproducibility RSD (%) n = 24	
IND	50	109 ± 7			6.6	6.0	
	100	106 ± 7	1	5	6.3	7.2	0.993
	200	107 ± 6			3.8	5.8	
SK	100	97 ± 10			10.7	9.9	
	250	101 ± 15	2.5	5	14.3	12.6	0.998
	500	98 ± 7			7.5	6.9	
AEON	250	89 ± 13			14.9	17.2	
	500	95 ± 10	1	5	11.0	15.3	0.992
	1000	100 ± 10			9.6	9.3	

Table 1 Accuracy at low (10 µg kg^{-1}), medium (2000 µg kg^{-1}) and high (5000 µg kg^{-1}) concentration levels obtained using unweighted (w=1) and weighted (w=1/x^{power}) linear regression for IND, SK and AEON in all different meat matrices.

	IND				SK				AEON			
		Bias (%)				Bias (%)				Bias (%)		
	W_i	Low	Medium	High	W_i	Low	Medium	High	W_i	Low	Medium	High
Cooked ham	1	5.07	3.79	8.26	1	5.24	3.92	8.54	1	6.55	4.90	10.68
	$1/x^2$	0.58	3.75	9.80	$1/x^{1.5}$	1.04	4.2	11.13	$1/x^2$	7.01	5.75	15.02
Minced Meat	1	0.87	8.15	1.81	1	0.88	0.82	1.83	1	1.04	0.97	2.14
	$1/x^2$	0.02	1.52	3.21	1/x	0.90	0.84	1.77	$1/x^{1.5}$	0.07	0.20	4.05
Tenderloin	1	1.87	1.74	3.86	1	0.86	0.80	1.78	1	1.80	1.68	3.72
	$1/x^{0.5}$	1.12	1.05	5.84	1/x	0.90	0.84	1.76	1/x	0.46	0.53	7.72
Bacon	1	0.97	0.90	2.00	1	1.38	1.15	2.86	1	0.74	0.69	1.52
	$1/x^{1.5}$	0.04	0.12	2.38	$1/x^{0.5}$	0.75	0.78	3.90	$1/x^{1.5}$	0.05	0.15	3.06
Cutlets	1	1.79	1.67	3.70	1	2.21	2.07	4.59	1	6.06	5.65	1.25
	$1/x^{1.5}$	0.11	0.30	6.10	1/x	0.44	0.51	7.38	1/x	1.09	1.27	1.85
Dry-cured ham	1	0.90	0.84	1.87	1	2.13	1.99	4.42	1	0.49	0.46	1.02
	$1/x^{1.5}$	0.06	0.16	3.32	1/x	0.30	0.35	5.03	$1/x^{0.5}$	0.29	0.27	1.50
Blade loin	1	4.74	4.42	9.81	1	5.51	5.14	1.14	1	11.04	10.30	22.62
	$1/x^2$	0.09	0.73	15.50	$1/x^{1.5}$	0.28	0.77	1.59	$1/x^{1.5}$	0.54	1.49	30.68
Dry fermented sausage	1	3.15	2.36	5.14	1	5.82	4.35	9.48	1	17.36	12.99	28.29
	$1/x^2$	0.35	2.86	7.51	$1/x^2$	5.37	4.40	11.51	$1/x^{1.5}$	3.19	13.01	34.35
Liver paste	1	1.42	1.14	2.79	1	5.40	4.15	8.30	1	5.42	4.27	9.99
	$1/x^2$	0.02	0.85	4.30	$1/x^{1.5}$	0.38	3.06	15.51	$1/x^{1.5}$	0.25	2.21	11.25

Table 4 Summary of the method validation performance characteristics as determined for dry fermented sausage.

Analyte	Nominal Concentration (µg kg^{-1})	Recovery Mean ± SD n = 18	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	Precision		Linearity R^2
					Repeatability RSD (%) n = 18	Within-laboratory Reproducibility RSD (%) n = 24	
IND	50	100 ± 6			5.9	6.5	
	100	98 ± 6	5	10	6.2	5.6	0.999
	200	101 ± 3			3.4	3.2	
SK	100	100 ± 3			2.9	2.6	
	250	94 ± 10	2.5	25	10.1	8.9	0.999
	500	99 ± 3			3.4	3.3	
AEON	250	104 ± 6			6.4	6.7	
	500	103 ± 6	1	2.5	5.9	5.6	0.996
	1000	109 ± 11			10.5	10.2	

3.3.3. Trueness and precision

As no certified reference material was available, trueness was assessed as recovery by fortifying blank meat matrices containing little or no traces of the analytes of interest. Six replicates of each of the three spike levels were analysed. To evaluate the precision of the method, the repeatability and within-laboratory reproducibility were determined. Both validation parameters were evaluated by calculating the relative standard deviations (RSD, %). For evaluating the repeatability, three series of six replicates fortified at three different levels (Table 3 & 4) were analysed. This was carried out on different occasions by the same operator under repeatable conditions. A similar set-up (four series of six replicates at the three mentioned concentrations) was elaborated to evaluate the within-laboratory reproducibility. However, the series' replicates were now executed by different lab technicians on another day, whereby environmental conditions consequently altered. The recoveries calculated for both extraction methods meet the permitted levels (-20% to +10%) (Tables 3-5) but were higher than those reported by Rius et al., especially for IND [34]. This could possibly be due to the endogenous presence of the boar taint compounds [31-33]. The RSD values calculated for the repeatability (RSD < 14.9% and < 10.5%) were below 15%, indicating a good repeatability according to the criteria of the European Commission [28]. Moreover, the repeatability calculated for tenderloin was much lower

(RSD < 3.6%) compared to by Rius et al. (RSD < 12.59%) [34]. For the within-laboratory reproducibility, RSD values (RSD < 17.2% and < 10.2% for cooked ham and dry fermented sausage, respectively) were below the performance limits as calculated with the Horwitz equation, indicating a satisfactory precision of both methods [28].

Table 5 Summary of the method validation performance characteristics as determined for different meat products by their respective analysis method.

	Recovery Mean ± SD n = 6			Repeatability RSD (%) n = 6			Linearity R^2		
	IND	**SK**	**AEON**	**IND**	**SK**	**AEON**	**IND**	**SK**	**AEON**
Minced Meat	103 ± 5	101 ± 1	102 ± 1	4.5	1.0	1.0	0.998	0.999	0.999
Tenderloin	104 ± 4	101 ± 1	101 ± 2	3.6	0.9	1.7	0.999	1.000	0.998
Bacon	110 ± 3	102 ± 2	103 ± 5	3.0	1.7	4.5	0.999	0.999	0.999
Cutlets	109 ± 4	106 ± 3	102 ± 6	3.2	2.9	5.7	0.999	0.999	0.996
Dry-cured Ham	104 ± 2	99 ± 0.4	99 ± 0.6	1.7	0.4	0.6	0.999	0.999	0.999
Blade Loin	110 ± 4	103 ± 0.4	104 ± 6	3.5	0.4	6.0	0.993	0.991	0.991
Liver Paste	101 ± 3	100 ± 0.8	100 ± 1	2.7	0.8	1.2	0.999	0.993	0.997

3.3.4. Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were set as the concentrations, which generate chromatographic peaks with a signal-to-noise ratio of minimum 3:1 (LOD) and 10:1 (LOQ). These values were theoretically calculated and were then confirmed (Table 3 & 4) based on the outcome of blank samples fortified at the levels calculated for LOD and LOQ (Fig 1 & 2). Since the blank samples of cooked ham (only IND), bacon, cutlets, dry-cured ham, blade loin, tenderloin and minced meat contained low endogenous levels of IND and AEON, the influence of these levels was investigated. The levels of IND and AEON in cooked ham, bacon, cutlets, dry-cured ham and blade loin were below the respective LOQs: 5 µg kg^{-1} for both IND and AEON and therefore only minorly affected the calibration curves. The indolic levels calculated in tenderloin and minced meat amounted to 5 µg kg^{-1} and 6 µg kg^{-1}, respectively. AEON levels in cooked ham, tenderloin and minced meat were calculated at 6 µg kg^{-1}, 9 µg kg^{-1} and 10 µg kg^{-1}, respectively. Because of the wide range of the calibration curves (0-5000 µg kg^{-1}), these endogenous levels only minorly affected the calibration curves.

3.4. Analysis of cooked ham and dry fermented sausage samples

To illustrate the applicability of the newly developed methods, cooked ham and dry fermented sausage samples produced with meat from boar taint positive carcasses were analysed. In the cooked ham samples, all boar taint compounds could be found (Fig 3A). However, levels obtained for the meat matrix were low in comparison to analysis of the neck fat sample. A possible explanation for this finding is the distribution and storage of the boar taint compounds in pork tissue. IND, SK and AEON possess high partition coefficients (LogP IND: 2.14; LogP SK: 2.60; LogP AEON: 4.9), which translates into a strong lipophilic character. For this reason, the boar taint compounds are mainly present in adipose tissue and to a lesser extent in muscle tissue [35, 36]. In the dry fermented sausage samples, all boar taint compounds were detected with signal-to-noise ratios > 10 and good peak shape was observed (Fig 3B). These findings show the applicability of the newly developed method on dry fermented sausage samples produced from boar-tainted meat.

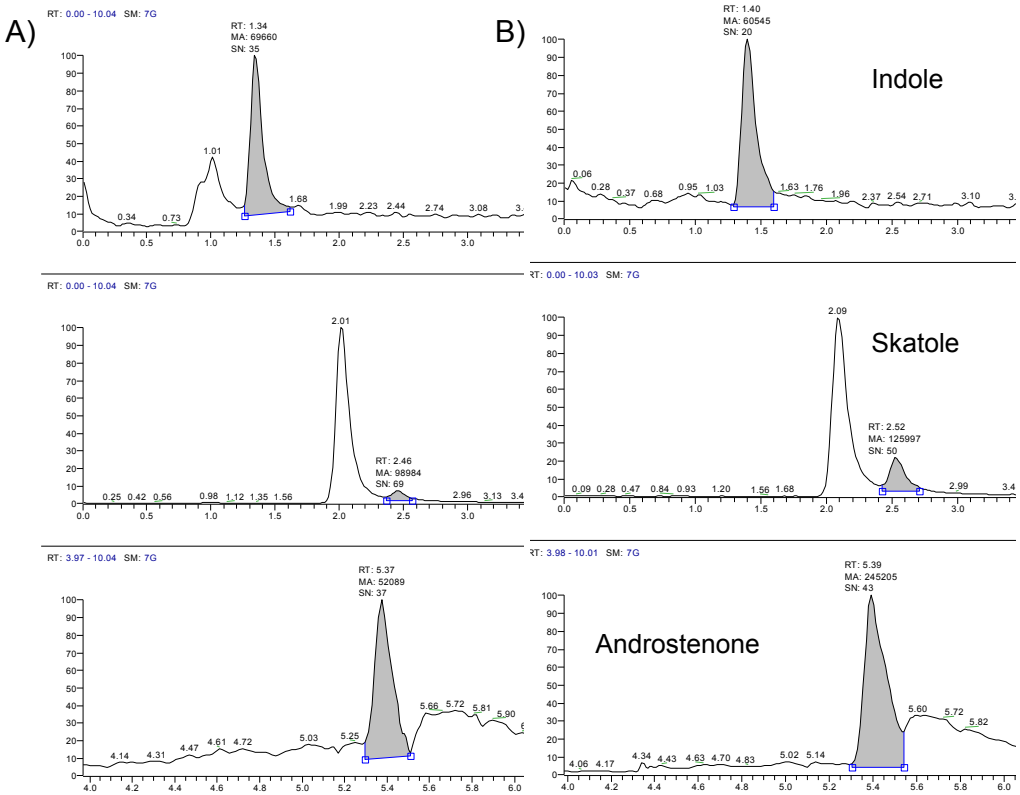

Fig 3 Chromatogram of a A) cooked ham sample and B) dry fermented sausage sample produced from a carcass affected with boar taint.

4. CONCLUSIONS

For the first time, robust, specific and selective extraction and UHPLC-HRMS analysis methods for the simultaneous quantification of IND, SK and AEON in a wide variety of meat products were developed and validated according to the criteria of the European Commission [28]. The applicability of the methods on a wide variety of matrices provides additional value for the evaluation of consumer acceptance towards boar taint affected meat and determining odour thresholds for boar taint related compounds in comparison to existing methods, that mostly focus on adipose tissue as a sample matrix.

Two extraction protocols were optimized for the detection of the boar taint compounds in cooked ham, minced meat, tenderloin, bacon, cutlets, dry-cured ham and blade loin on the one hand and dry fermented sausage and liver paste on the other. Both methods showed a large linear range and good accuracy. Repeatability was proven satisfactory for cooked ham and good for all other meat products. However, given the wide variety of meat products included in this study acceptable precision was obtained for both methods. Additionally, analysis of cooked ham and dry fermented sausage samples from boar taint positive carcasses founded the applicability of both methods for routine analysis.

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CHAPTER III

SENSORY EVALUATION OF BOAR MEAT PRODUCTS BY TRAINED EXPERTS

Adapted from:

Wauters J.[*], Verplanken K.[*], Vercruysse V., Ampe B., Aluwé M., Vanhaecke L. (2017). Food Chemistry 237:516-524. [*] shared first authors

ABSTRACT

Rearing entire male pigs, one of the alternatives for surgical castration, entails the possible occurrence of boar taint. This study aimed at the investigation of the acceptability of meat from entire male pigs in 8 different meat products (cutlets, bacon, blade loin, tenderloin, dry fermented sausage, cooked ham, dry-cured ham and minced meat) by trained assessors. Generally, the sensory evaluation of meat samples was affected the most in the AEON group, indicating that AEON is the most offensive boar taint compound in case of sensitive assessors for the samples included in this study. Differences between the meat products showed the highest potential for processing tainted meat in cold meat products, which was most likely due to the serving temperature on the one hand and production related influences on the other. However, more insights regarding reducing and masking effects of production related factors on boar taint are necessary.

1. INTRODUCTION

Increasing awareness on animal welfare during the past decades led to the voluntary agreement of European Member States to abandon surgical castration of pigs by 2018. Surgical castration of piglets is a widely used practice and primarily intended to eliminate boar taint, an off-odour and flavour that may occur when cooking boar meat, evoking negative consumer reactions [1]. Boar taint is mainly attributed to the accumulation of AEON, SK and to a lesser extent IND in adipose tissue [2].

Rearing entire male pigs, one of the alternatives for surgical castration, offers advantages in terms of animal welfare but also provides an important economic and ecological added value [3, 4]. Research showed that the production of entire male pigs is more efficient given the lesser fat deposition. In addition, compared to castrates less feed is needed in order to achieve the same final weight, whereby also fewer nutrients are emitted to the environment [4, 5]. However, one of the benefits of the surgical castration of pigs is the elimination of boar taint. Consequently, the production of entire male pigs is accompanied by the re-occurrence of boar taint in 4-25% of pig carcasses [3]. Since up until now no reduction strategy such as the use of different races, adjustments of feed and hygiene conditions and slaughtering at lower weight guarantees a complete elimination of boar taint, the presence of the latter in meat could cause negative consumer reactions and possibly lead to severe economic losses in pig husbandry [4, 6-9]. Complete conversion to rearing entire male pigs, however, is only possible if boar taint free meat products can be guaranteed. To this end, on the one hand, it is important to detect aberrant carcasses at the slaughter line and secondly to process tainted carcasses in a technically and economically full-fledged manner without evoking negative consumer reactions [10].

Several studies on the sensory evaluation of boar meat indicate that there is a risk for consumers to reject meat from entire male pigs. As such, in fresh meat products, Meier-Dinkel et al. showed that very sensitive assessors perceived significantly more AEON odour and flavour in boar loins in comparison to loins of gilts and castrates [11]. Less sensitive assessors were less able to discriminate between boars, gilts and castrates. Moreover, consumers who disliked the AEON odour also indicated

a higher disliking of boar loins. These findings were similar to other studies, where loin samples with high AEON and SK levels were found unacceptable by sensitive consumers [12, 13]. Also Blanch et al. observed a higher disliking of the *longissimus dorsi* muscle containing high AEON levels in more sensitive consumers [14]. In processed meat products, such as sausages, masking of boar taint seemed more feasible. Indeed, different studies indicated that smoking combined with the addition of spices had a positive impact as a masking strategy in frankfurter sausages [15, 16]. Also fermented sausages seemed promising to process tainted meat, due to the effect of different starter cultures on aroma formation [17, 18]. As such, in raw-smoked fermented sausages, up to 100% tainted raw material could be used without impairing the mean overall liking. In boiled sausages on the other hand, this percentage dropped to 50% [19]. Also in bacon and pork belly roll, the perception of boar taint could be masked as no significant influence on consumer liking was observed [20]. In other meat products such as cooked and dry-cured ham on the other hand, the eating quality was negatively affected by the presence of boar taint. However, generally a higher acceptance was observed for dry-cured ham in comparison to cooked ham [21, 22]. Finally, in minced meat, high boar taint levels provoked more adverse reactions in comparison to gilts and castrates [23]. Results obtained in different studies indicate that boar taint perception is strongly related to the type of meat product under investigation. Moreover, results between studies can also differ as the sensory perception of boar taint also depends on different factors including boar taint compound levels, cooking conditions, context of perception (home test versus controlled environment), serving temperature, consumers' profile (gender, age, country of origin, olfactory acuity), AEON sensitivity, and appreciation towards AEON [13, 14, 24-27]. Consequently, as these factors are not always standardized, it is hard to compare between studies and draw general conclusions [24]. Moreover, most studies merely focus on the evaluation of 1 or 2 meat products, which complicates the comparison of different meat types to mask boar taint. For this reason, this study aimed to investigate the general acceptability of meat from entire male pigs in a variety of meat products, for which consensus is currently lacking. In total, 8 different meat products (cutlets, bacon, blade loin, tenderloin, minced meat, dry fermented sausage, cooked ham, dry-cured

ham) were selected and for each product, both odour and flavour characteristics were evaluated by trained assessors. Additionally, in order to investigate the influence of meat production processes on the perception of boar taint, two types of cooked ham were evaluated in this study, rendering new insights in the possibilities for processing tainted boar meat. Additionally, the boar taint compound levels were determined in both adipose tissue and meat with a validated in-house method [28].

2. MATERIALS AND METHODS

2.1. Chemical analysis

After production of the different meat products under investigation in this study, the boar taint compound levels were determined in fresh and processed meat products (cutlets, bacon, blade loins, tenderloins, minced meat, dry fermented sausage, cooked and dry-cured ham) derived from the tainted boar and gilt carcasses. All meat was stored at -20 °C until analysis. With the exception of tenderloin, minced meat and dry fermented sausage, both the fat fraction of the meat products and muscle tissue or meat fraction were subjected to analysis. For tenderloin, minced meat and dry fermented sausage, the product itself was analysed without separation of the fat and meat fraction. Afterwards, the boar taint compound levels in the fat fraction of minced meat and dry fermented sausage were extrapolated from the levels obtained in adipose tissue of boar and gilt carcasses, taking into account the fraction of boar and gilt fat used during production of the latter meat products. Details on the reagents and chemicals, and analysis methods for fat tissue and the meat products can be consulted in Chapter II (section 2.1), Bekaert et al. and Verplanken et al., respectively [28, 29].

2.2. Sensory evaluation

2.2.1. Training of expert panels

Thirty-nine candidate boar taint experts, 24 men and 15 women, were subjected to a well-described selection process based on the protocols by Bekaert et al. and Meier-Dinkel et al., in which training occurred by use of solutions or paper strips, respectively [11, 30]. For details on the exact training

procedure is referred to Wauters et al., [31]. In short, first, the candidates' sensitivity for AEON was evaluated. Candidates that were able to correctly identify the AEON (0.17 mg ml^{-1}) containing bottles at least 3 times were withheld for further training (n=30) whereas others not passing the test were excluded from further participation. Training was proceeded in different steps. In a first step, the remaining candidates were trained to discriminate between low and high concentrations of AEON and SK. A candidate-expert was allowed to proceed to the next step if each odd sample (AEON or SK sample) was identified as such on at least 3 different occasions. During this second step, the candidates were challenged to rank 4 concentrations of AEON and SK (0.01 – 0.1 – 0.5 – 2.0 mg l^{-1}). Further training occurred similarly, however, randomly mixed batches of AEON and SK bottles were presented to the candidates who were additionally asked to identify each bottle. If this was performed correctly (allowing two mistakes in compound identification), the candidate-experts were selected for the final training program based on the olfactory assessment of boar fat samples applying the soldering iron melting method described by Bekaert et al. [30]. In total, 19 experts successfully completed the training program.

2.2.2. Sensory evaluation of meat products by trained experts

Sensory evaluation of the meat products occurred in a controlled laboratory with individual booths for each panellist and the environmental conditions were kept as constant as possible. Each expert was foreseen with mineral water and tasteless crackers to clear their palate in between samples.

Preparation of the meat products for testing occurred in a kitchen separated from the evaluation room, eliminating possible interference of baking odours. All warm-served products were baked on grills and for each boar and gilt, a separate grill (2000 W) was used to avoid contamination between samples. The core temperature of meat samples was monitored until it reached 70 °C, after which the meat products were served to the experts. The cold-served meat products were prepared during the morning of each session day and kept refrigerated and sealed with plastic until the beginning of the evaluation session. In each session, 2 meat products (1 hot and cold-served product) were evaluated

by a minimum of 6 trained experts. Each product was evaluated in duplicate on different days. Because of practical limitations, the expert panels did not consist of the same panellists on each session day; however, each panellist followed the same training course. Moreover, in total, each meat product was evaluated by a minimum of 12 experts over the 2 executive evaluation days.

Of each meat product, 14 samples were evaluated whereof 5 gilt samples and 1 sample of each boar (9 in total). The panellists were informed about the first sample, being a gilt, which served as a reference point. The other samples were served to the panellists in randomized order and were evaluated blindly. The panellists scored different characteristics that described the odour, taste and texture of each sample on a 150 mm scale subdivided from 0 to 4, with 0 being absence of the described attribute and 4 the presence of the latter. Odour characteristics that were described included the general odour (overall odour of the meat product under investigation) boar taint odour (a general term using experience in assessing meat from entire male pigs), urinary odour (odour of urine, related to the presence AEON) and manure odour (odour of manure or a barn, related to the presence of SK). Flavour characteristics included the general flavour (overall flavour of the meat product under investigation), boar taint flavour (a general term using experience in assessing meat from entire male pigs), urinary flavour (flavour of urine, related to the presence of AEON) and manure flavour (flavour of manure, related to the presence of SK). Afterwards, also the overall sensory quality and acceptance were scored. The acceptance of each sample was evaluated on a scale of 0 to 10 with 0 being not consumable and 10 perfectly consumable. Moreover, experts were also asked whether or not they considered the meat samples as consumable (YES/NO), in order to obtain a dichotomous variable.

2.3. Carcass selection

For each selection round three hundred carcasses of intact male pigs were screened at the slaughter line (Debra Meat slaughterhouse, Tielt, Belgium) on the occurrence of (intense) boar taint by a trained expert, based on olfactory evaluation using the soldering iron method (RDS 80, Kurtz Ersa, Wertheim,

Germany). For each round of experiments, 45 carcasses were retained for analytical determination of the three main boar taint compounds AEON, SK and IND. Analysis was performed on back fat samples by UHPLC-HRMS analysis.

After chemical analysis, 9 tainted carcasses were ranked either based on the highest concentration of (only) AEON (3 carcasses; group 1; AEON ≥ 500 µg kg^{-1}), on the highest concentration of (only) indolic (i.e. SK and IND) compounds (3 carcasses, group 2; IND ≥ (±) 100 µg kg^{-1} and SK ≥ (±) 200 µg kg^{-1}), or on the combination of the highest concentrations of both AEON and indolic compounds (3 carcasses; group 3; AEON ≥ 500 µg kg^{-1}, IND ≥ 100 µg kg^{-1} and SK ≥ 200 µg kg^{-1}). However, due to study limitations and the relatively low prevalence of tainted boar carcasses, only 1 boar with high AEON and indolic compound levels could be selected. Two other boars with AEON, SK and IND levels that approximated the set thresholds the most were selected. AEON levels for these boars were 458 and 444 µg kg^{-1} for boar 3.2 and boar 3.3, respectively. The SK levels were 59 and 157 µg kg^{-1}, for boar 3.2 and boar 3.3, respectively. IND levels for all 3 boars in the AEON+SK category amounted 100 µg kg^{-1} (Table 1). The 9 retained carcasses, subdivided into 3 classes were used for meat production. Apart from the 9 retained boar carcasses, also 1 gilt carcass was selected at the slaughter line for production of meat products free from boar taint.

2.4. Production of different meat products

After the analysis of back fat and subsequent carcass selection, the boars were hand-cut at the slaughterhouse, preventing mixing up of boars and allowing proper labelling with identification of each piece of meat/fat. Cutlets, bacon, blade loins and tenderloins were transported to the laboratory under cooled conditions, vacuum-packed and frozen at -20 °C until analysis.

The raw materials required to make dry fermented sausage (shoulder and back fat), cooked ham (hind leg; cooked-in), dry-cured ham (hind leg; Cobourg ham) and minced meat (meat and fat) were transported to cooperative commercial meat-processing companies. From every individual boar, 4 processed meat products (cooked ham, dry fermented sausage, minced meat and dry-cured ham) and

4 fresh meat products (tenderloin, cutlets, blade loin and bacon) were produced, for which is referred to Wauters et al. [31]. In the first batch of meat products (for round 1 of the expert panels), a fraction of gilt fat was used for the manufacturing of both minced meat and dry fermented sausage. No sufficient fat amounts were delivered for minced meat production, consequently leading to a varying fraction of added gilt fat (41-82%) between boars. Boar fat was supplied in abundance for dry fermented sausage production, but the consistency of the fat (extremely soft in comparison with gilt fat) prevented its proper use in the production of dry fermented sausage. Therefore, a constant fraction of boar fat (22%) was supplemented with gilt fat (78%). The softer consistency of boar fat is explained by Lundstrom et al., indicating that boar fat might soften due to the higher fraction of unsaturated fatty acids in combination with the higher water content [32]. Additionally, it should be noted that insufficient acidification was obtained for the three dry fermented sausages derived from the carcasses with high AEON levels. Consequently, because of the lack of decrease of pH, these products were more prone to bacterial development (*Listeria monocytogenes*) and were for this reason excluded from sensory evaluation.

Due to intellectual property concerns, only minor details can be provided on the specific manufacturing conditions of each meat product. After mincing and assembling the raw materials for dry fermented sausage, with addition of a common microbial starter culture including a mix of *Lactobacillus* and *Staphylococcus spp.*, fermentation was allowed for 2-3 days at 24 °C and 90-95% relative humidity, followed by a 3-4-week ripening period at 14 °C and 80% relative humidity. For cooked ham, the hind legs were deboned and appropriately trimmed. Subsequently, the hams were injected with a brine solution consisting of salt, glucose syrup, dextrose, seasoning and herbs. Sodium ascorbate and sodium nitrite were added for their respective anti-oxidative and conserving properties. Subsequently, the hams were tumbled at 2 °C for 24h. Afterwards, the hams were transferred into aluminium forms prior to cooking. Cooking was performed in heated water at 69 °C. After approximately 10h, the required core temperature of 67 °C was reached, after which the hams were immediately cooled in a ventilated refrigerator at 2 °C for a minimum of 18h. For dry-cured ham, the

hind legs were trimmed, with omission of bony material, to a ham weight (Cobourg) between 4.2 and 4.9 kg. The hams were brined for 8 days at a temperature below 5 °C. The brine contained a solution of 16.5% NaCl, 675 mg kg^{-1} NaNO2, 450 mg kg^{-1} KNO3, 5.6% lactic acid, 0.22% glucose, and was further adapted to a density of 21 degrees Baumé by addition of nitrite pickled salt. After brining, salting of the hams was continued by osmotic infiltration during 24 days at 2-4 °C, with a relative humidity decreasing from 75% to 60%. The hams were than individually shaped in aluminium containers for 48h at 4 °C. After removal of the containers, the hams were ripened for 37h, including a smoking period of 10h at 30 °C and 60% relative humidity.

In the second experiment, the influence of two different production processes for cooked ham on its sensory quality was evaluated. Cooked ham was produced following two different procedures, a cooked-in (closed aluminium container) and open-cook (open aluminium container) protocol, which might result in different findings regarding acceptance. During the cooked-in process, the hams were cooked and cooled in a closed aluminium recipient, possibly retaining the boar taint compounds. The hams following an open-cook process on the other hand were cooked in an open aluminium recipient.

2.5. Data analysis

Statistical analysis of the data was performed in R 3.3.1 and a significance level of 0.05 was taken into account. All data was assumed to be sufficiently normally distributed based on the graphical examination (QQ-plot and histogram) of the residuals. In order to avoid repeated measures on identical samples, statistical analysis was performed on aggregated data (mean sample score for all trained experts).

2.5.1. Differences in boar taint compound levels between two types of cooked ham

Differences in boar taint compound levels between two types of cooked ham (cooked-in and open cook) were assessed by building a general linear model including the boar taint compound levels

(AEON, SK and IND) as dependent variables and the type of cooked ham as independent variable or fixed factor. To correct for confounding effects, carcass category and boar were included as covariables. Two-way interactions were taken into account.

2.5.2. Differences between boars with high boar taint compound levels and gilts

In a first experiment, principal component analysis (PCA) was performed to identify differences between carcass categories (gilt, SK, AEON, SK+AEON) in terms of the general odour, boar taint odour, urinary odour, manure odour, general flavour, boar flavour, urinary flavour, manure flavour, overall sensory quality and acceptance.

2.5.3. Differences between meat products

In a second experiment, differences between meat products were investigated through PCA analysis for the general odour, boar taint odour, urinary odour, manure odour, general flavour, boar flavour, urinary flavour, manure flavour, overall sensory quality and acceptance. In order to account for differences in importance of the boar taint compounds between meat products, also the interaction term between the boar taint compounds and meat products was included. Non-significant interactions were excluded from the final models. In case of a significant effect of the meat products on the principal components, a post-hoc Tukey test on the principal component was performed to make pairwise comparisons between the meat products.

2.5.4. Influence of production process on cooked ham perception

In a final experiment, the difference in boar taint, boar flavour, overall sensory quality and acceptance between two types of cooked ham was investigated. To this end, the type of cooked ham (open-cook, cooked-in), carcass type (gilt, SK, AEON, SK+AEON) and test day were included in a general linear model as fixed effects. To correct for clustering on boar level, the boar number was included as a random effect.

3. RESULTS AND DISCUSSION

3.1. Chemical analysis

Table 1 Concentration (µg kg^{-1}) of androstenone (AEON), skatole (SK) and indole (IND) in neck fat of nine selected boar carcasses.

	AEON			SK			AEON+SK		
Concentration (µg kg^{-1})	Boar 1.1	Boar 1.2	Boar 1.3	Boar 2.1	Boar 2.2	Boar 2.3	Boar 3.1	Boar 3.2	Boar 3.3
AEON	1613	1969	3131	131	147	187	1187	458	444
SK	63	21	16	199	279	320	521	59	157
IND	75	85	49	98	106	101	340	114	182

Table 2 Concentration (µg kg^{-1}) of androstenone (AEON), skatole (SK) and indole (IND) obtained in the fat fraction of different meat products.

		AEON category			SK category			AEON + SK category		
		Boar 1.1	**Boar 1.2**	**Boar 1.3**	**Boar 2.1**	**Boar 2.2**	**Boar 2.3**	**Boar 3.1**	**Boar 3.2**	**Boar 3.3**
Minced meat	AEON	863	1167	97	1301	66	58	569	115	20
	SK	34	13	12	7	83	83	246	188	< LOQ
	IND	41	51	21	41	32	60	161	21	< LOQ
Cutlets	AEON	1692	1405	1981	206	264	388	1278	245	524
	SK	110	34	33	244	341	383	451	73	199
	IND	176	69	34	121	163	159	316	53	231
Bacon	AEON	845	690	1157	144	247	308	850	438	491
	SK	15	< LOQ	< LOQ	165	237	241	434	52	103
	IND	< LOQ	< LOQ	207	141	188	107	259	110	136
Blade loin	AEON	1321	921	1271	132	175	196	790	217	348
	SK	89	13	8	124	385	319	682	73	185
	IND	148	87	38	70	154	130	402	101	171
Cooked ham	AEON	1955	3137	6176	21	125	188	1501	1012	328
	SK	12	< LOQ	< LOQ	41	183	180	511	45	46
	IND	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	244	45	< LOQ
Dry-cured Ham	AEON	987	2015	2740	112	136	264	747	302	485
	SK	71	29	42	190	313	347	447	75	149
	IND	162	152	94	148	187	164	315	141	160
Dry fermented sausage	AEON	362	442	700	33	37	44	268	106	102
	SK	15	6	5	46	63	72	117	15	36
	IND	18	20	12	23	25	24	77	27	42

*** < LOQ: below limit of quantification**

Minced meat was produced with 22% boar and 78% gilt fat due to logistic shortcomings.

Based on chemical analysis of the boar taint compounds in neck fat, nine carcasses were selected and subdivided in three categories (1: AEON, 2: SK, 3: AEON + SK) (Table 1). For each product, also the boar taint compound levels in the fat fraction of the meat and /or meat fraction were analysed (Tables 2 &

3). In general, high boar taint compound levels were obtained in the fat fraction of the different meat products, while lower levels were found for the meat fraction of the different cuts and products. However, it should be noted that for minced meat, a dilutive effect was observed due to supplementation with gilt fat, resulting in lower boar taint compound levels in the fat and meat fraction of minced meat. For cooked ham, high AEON levels were observed in the fat fraction (pork rind) of the hams, which was most likely attributable to redistribution of the boar taint compounds during the cooking process and accumulation in the pork rind [31, 33]. For a more detailed discussion on these results we refer to Wauters et al. [31].

Table 3 Concentrations (µg kg^{-1}) of androstenone (AEON), skatole (SK) and indole (IND) obtained in the meat fraction of different meat products.

		AEON			SK			AEON + SK		
		Boar 1.1	**Boar 1.2**	**Boar 1.3**	**Boar 2.1**	**Boar 2.2**	**Boar 2.3**	**Boar 3.1**	**Boar 3.2**	**Boar 3.3**
Minced meat	AEON	1597	1454	93	1296	210	65	921	234	296
	SK	46	25	29	21	75	92	317	168	109
	IND	74	57	35	27	54	48	222	75	79
Cutlets	AEON	364	386	774	34	34	68	278	38	98
	SK	< LOQ	< LOQ	< LOQ	5	33	9	112	< LOQ	9
	IND	26	14	13	17	22	24	94	15	30
Bacon	AEON	1234	753	1598	68	74	261	721	114	193
	SK	37	< LOQ	< LOQ	60	83	178	281	9	44
	IND	75	33	30	40	43	80	193	34	52
Blade loin	AEON	352	274	707	39	25	100	257	72	130
	SK	< LOQ	< LOQ	< LOQ	38	49	96	133	< LOQ	45
	IND	37	14	11	14	22	44	95	26	37
Tenderloin	AEON	86	68	352	10	13	22	148	16	13
	SK	< LOQ	< LOQ	< LOQ	< LOQ	22	16	63	< LOQ	13
	IND	29	15	14	15	18	17	64	15	21
Cooked ham	AEON	62	56	34	23	32	35	65	22	19
	SK	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	38	< LOQ	< LOQ
	IND	9	< LOQ	< LOQ	< LOQ	13	11	28	11	6
Dry-cured ham	AEON	109	131	157	< LOQ	< LOQ	34	181	7	71
	SK	< LOQ	< LOQ	< LOQ	< LOQ	17	19	70	< LOQ	< LOQ
	IND	15	8	5	9	10	21	70	7	6
Dry fermented sausage	AEON	444	574	958	< LOQ	< LOQ	40	568	202	19
	SK	< LOQ	< LOQ	< LOQ	28	50	68	139	< LOQ	16
	IND	21	13	10	14	21	27	76	17	21

*** < LOQ: below limit of quantification**

In a second experiment, the boar taint compound levels of two different production processes of cooked ham, i.e. a cooked-in and open-cook procedure, were evaluated in 9 additional carcasses (Table 4). No significant differences in boar taint compound levels in the fat fraction of the hams were observed between the two types of hams ($p > 0.05$). However, the effect of carcass category was significant for AEON ($p = 0.004$), indicating that the difference in AEON levels between different carcass categories was larger than the differences in AEON levels between the two types of cooked hams. No significant interaction between carcass category and type of cooked ham was observed. For the AEON category, lower levels were observed for the cooked-in process in meat fat (pork rind) in comparison to the open-cook procedure. However, the levels of IND, SK and AEON in the meat fraction of both types of cooked ham were comparable (Table 4). For the SK category, higher SK and lower AEON levels were observed in meat fat from the open-cook procedure. In the meat fraction, boar taint compound levels were comparable. Finally, for the AEON+SK category, higher boar taint compound levels were observed in the meat fat from the cooked-in procedure, while levels in the meat fraction were comparable for the cooked-in and open-cook procedures. It is hypothesized that the fat fraction of cooked hams produced using the cooked-in process contains higher boar taint compound levels as a result of redistribution of the boar taint compounds from the intramuscular to subcutaneous fat during cooking of the hams in a closed environment. Indeed, due to the high temperatures during cooking of the hams, the boar taint compounds may migrate to the outer surface of the hams (pork rind) and evaporate. However, evaporation is hampered in case of a cooked-in process due to the use of closed containers. Consequently, the boar taint compounds remain present in the fat fraction (pork rind) of the hams, which explains the higher levels [31, 33]. Further research taking into account the boar taint compound levels in the hams prior and after cooking is necessary to elucidate this hypothesis.

Table 4 Concentrations of androstenone (AEON), skatole (SK) and indole (IND) in cooked ham following 2 production procedures in neck fat, meat fat and meat.

		AEON			SK			AEON + SK		
		Boar 4.1	**Boar 4.2**	**Boar 4.3**	**Boar 5.1**	**Boar 5.2**	**Boar 5.3**	**Boar 6.1**	**Boar 6.2**	**Boar 6.3**
Neck fat										
	AEON	961	1213	866	461	128	629	795	1566	4468
	SK	96	31	59	150	239	419	178	241	438
	IND	12	13	15	7	68	89	73	61	145
Cooked ham (cooked-in)										
Fat fraction	AEON	604	1021	386	185	54	729	657	1028	4790
	SK	78	17	19	49	87	450	178	224	504
	IND	30	50	57	48	66	139	104	96	218
Meat fraction	AEON	17	25	27	5	< LOQ	< LOQ	19	53	136
	SK	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	5	< LOQ	< LOQ	5
	IND	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	11
Cooked ham (open cook)										
Fat fraction	AEON	710	1551	601	254	137	452	384	564	2769
	SK	85	7	30	150	281	363	117	122	291
	IND	54	51	59	102	138	155	72	59	137
Meat fraction	AEON	39	49	31	20	7	19	26	40	104
	SK	< LOQ	< LOQ	< LOQ	11	18	23	< LOQ	< LOQ	17
	IND	< LOQ	< LOQ	< LOQ	< LOQ	6	8	< LOQ	< LOQ	8

*** < LOQ: below limit of quantification**

3.2. Differences between boars with high boar taint compound levels and gilts

PCA analysis demonstrated that 84% and 6% of the variability in the model was explained by the first (PC1) and second component (PC2), respectively, which were retained for further investigation (Fig 1). PC1 was mostly influenced in a negative way by the general odour, boar odour, urinary odour, manure odour, general flavour, boar flavour, urinary flavour and manure flavour, and in a positive way by the overall sensory quality. A positive PC1 indicates a positive evaluation of the meat products. This indicates the negative influence of both AEON and SK on the sensory evaluation of meat products. PC2 on the other hand was mainly related to the urinary odour and flavour (positive) and manure odour and flavour (negative), indicating a discrimination between AEON and SK perception. This was confirmed in the PCA score plot, which demonstrated a good discrimination between the AEON and

gilt group, whereby the AEON group was more related to the negative part of PC1 or boar taint related characteristics. The higher influence of boar taint in the AEON group may be attributed to the higher AEON levels. Compared to the latter group, AEON levels in the AEON+SK and SK group were much lower (Fig 1A). Moreover, this finding suggests that AEON was perceived as the most offensive tainting compound for the carcasses selected in this trial. Indeed, in literature, the presence of AEON was found as more potent in comparison to SK, however, only in the case of consumers or experts sensitive to AEON [13, 34]. Moreover, this finding is not conclusive as other studies found SK to be more potent in comparison to AEON [35]. Furthermore, as in this study, a trained panel was used, the effect of AEON on the sensory perception of boar meat might be overestimated in comparison to real-life, as in real-life consumers are less sensitive to AEON and are often not aware of the existence of boar taint. Consequently, in contrast to studies using trained panels, often no or only minor significant differences between boar and gilt meat products are observed in consumer panels [23, 24]. In comparison to the AEON group, the gilt group was more related to the positive part of PC1, indicating that the meat samples of the gilt group represented good overall sensory characteristics. Also, no urinary and manure odour deviations were observed in the gilt group. In comparison to the AEON group, more overlap was observed between the SK, SK+AEON and gilt group, suggesting less obvious differences in the sensory attributes related to PC1 (Fig 1A). A possible explanation for this finding is the level of the boar taint compounds in the meat products, as this is an important factor in the olfactory perception of boar taint [27]. For example, in the SK and SK+AEON group, the overlap between the latter and gilt group is most likely attributable to the lower AEON levels that were observed in the SK and SK+AEON group, which results in an improved perception of the meat products. Differences between the latter groups were more obvious for PC2, indicating that the trained assessors were able to discriminate between the presence of AEON and SK in meat samples, although to a limited extent. Another possible explanation is the low sample power in this study due to the limited number of carcasses (n=9) included in the study. Consequently, no significant difference could be demonstrated. One way to increase the power of the statistical analysis design is increasing sample size. However, due to

budgetary and logistic limitations, only 9 carcasses could be selected. Another alternative for increasing power is to apply a statistical test that can handle data of a repeated measures design. However, in order to prevent unnecessary complication of the study design, it was opted to work on aggregated data.

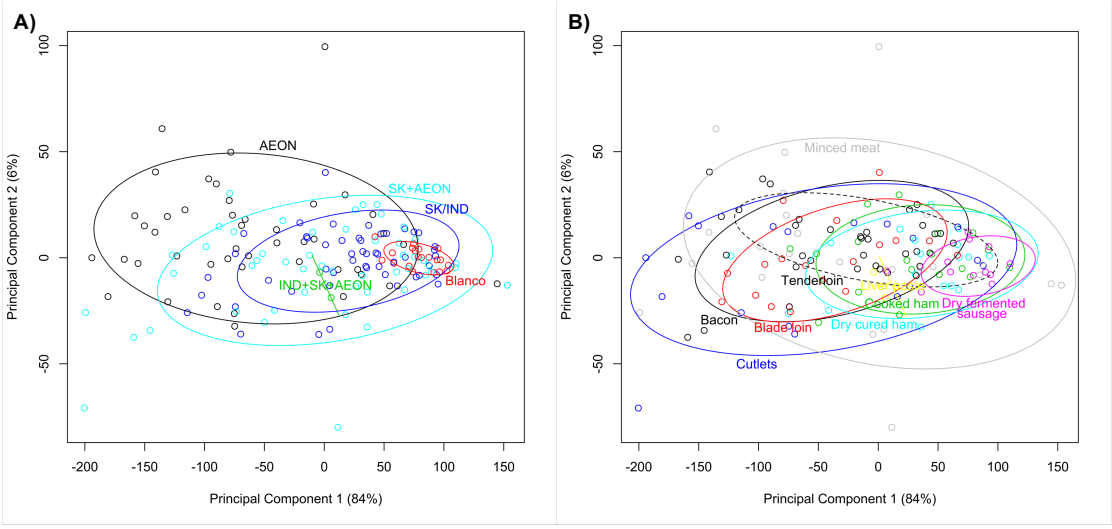

Fig 1 A) Principal component analysis score plot for the different carcass categories (AEON, SK/IND, AEON+SK, Blank = gilt) upon sensory evaluation of meat samples by trained experts, and B) Principal component analysis score plot for the different meat products upon sensory evaluation of meat samples by trained experts.

3.3. Differences between meat products

In order to demonstrate differences between the meat products, the latter was included as a factor in the models with PCA1 and PCA2 as outcome variable. Moreover, also the interaction term between the boar taint components and meat products were taken into account. For PC1, the interaction term between AEON and SK levels in neck fat and the product type were p=0.288 and p=0.494, respectively, indicating a similar effect of AEON and SK for each meat product. A significant effect was observed for meat product ($p < 0.001$) and the levels of AEON ($p < 0.001$) and SK ($p=<0.001$) in neck fat. Moreover, although not significant the results suggested that in comparison to tenderloin, blade loin, bacon and cutlets, the cold consumed meat products and minced meat showed more potential for masking boar taint without impairing likeability, as the latter meat products were more related to the positive part

of PC1 (Fig 1B). These results were confirmed by a Tukey test, which showed significant differences for PC1, whereby a higher preference was observed for the cold consumed meat products (dry fermented sausage, dry-cured ham and cooked ham) compared to the warm-consumed meat products including cutlets, bacon and blade loin. No significant differences were observed between minced meat and tenderloin, for which an intermediate preference was observed. Within the warm consumed meat products, the highest preference was observed for minced meat, followed by tenderloin, blade loin, bacon and cutlets. These results indicate that of all warm-consumed meat products, minced meat and tenderloin showed the highest potential for the processing of boar meat without evoking negative consumer perceptions. A possible explanation for the masking of boar taint and flavour in minced meat is the partial use of gilt fat during production due to logistic shortcomings. Other reasons for the lower amount of adverse reactions in minced meat are seasoning of commercially produced minced meat and the general likeability of minced meat by consumers [36]. In tenderloin, the lesser influence of boar taint, although still perceived, on the sensory evaluation of the meat products by a trained expert panel is most likely attributable to its lean character in comparison to other meat products such as cutlets, bacon and blade loin. Indeed, due to the higher fat content of cutlets, bacon and blade loin, higher boar taint compound levels are to be expected in these meat products in comparison to tenderloin (Table 3). Consequently, also a more pronounced effect of the presence of the boar taint compounds on the sensory evaluation of these meat products is expected. For cutlets, similar findings were observed by Bonneau et al. [12].

As cooked ham, dry-cured ham and dry fermented sausage were situated in the positive part of PC1, the latter products show more potential for the masking of tainted boar meat. Consequently, this indicates that the presence of the boar taint compounds had little or no influence on the quality of the latter products evaluated by a trained experts panel. For dry fermented sausage, this could be attributed to the partial use of gilt fat during production on the one hand but could also be explained by the reducing and masking effect of fermentation, whether or not followed by smoking, on the perception of boar taint in meat [18, 32, 37]. Moreover, Stolzenbach et al. demonstrated that

fermentation through fast acidification by cultures of *S. xylosus* and *S. carnosus* was very efficient to reduce boar taint [18]. However, this reduction was still insufficient to completely mask boar taint perception. Moreover, it should be noted that during production of dry fermented sausage, the three carcasses of the AEON category were identified as dark-firm-dry (DFD). The DFD meat in this category hindered acidification during the fermentation process leading to an insufficient decrease of the pH. Consequently, dry fermented sausage in the AEON group was more prone to bacterial development causing a potential risk for the occurrence of *Listeria monocytogenes* and for this reason was excluded from evaluation by the expert panel.

Overall, the results obtained in this study indicated a more pronounced influence of boar taint in the warm-served meat products in comparison to the cold-served meat products. Consequently, the cold-consumed meat products show the biggest potential for the processing of tainted boar meat without evoking negative perceptions. This finding can be supported by different influential factors that impact the production or consumption of meat products. A first is the serving temperature, which plays an important role in the release of boar taint compounds. Although IND, SK and AEON are semi-volatile compounds, they are only released to a high degree when meat and/or fat is heated. Consequently, boar taint and boar taint related characteristics are perceived more strongly in warm-served meat products [21, 32, 38]. Second, also production related processes might influence the perception of boar taint in meat products. Indeed, previous studies demonstrated that consumer acceptance increases when applying different production processes such as seasoning, fermentation, drying, smoking and cooking in comparison to non-processed fresh meat [16, 18, 19, 32, 37, 39, 40]. Dry fermented sausage for example undergoes fermentation, which results in aroma changes and might reduce or mask boar taint present in the final meat product [18]. Dry-cured ham on the other hand undergoes salting and drying during its production process. Because of oxidation of the fat fraction during the drying process and the pro-oxidative effect of salt, it has been hypothesized that the boar taint compounds oxidize to a certain degree during this process [21]. Consequently, drying may reduce boar taint levels in the final product. Finally, including a cooking step during production may also result

in decreased levels of the boar taint compounds in for example cooked ham; however, consensus on the influence of cooking remains unreached [21, 22, 32].

For PC2, the interaction term between AEON and product type was borderline significant (p=0.07), indicating that AEON has a more pronounced effect in some meat products. Moreover, a significant effect of SK level on PC2 was observed, this in contrast to the AEON levels and product type. Although no significant differences were observed, tenderloin was situated in the positive part of PC2, indicating a more pronounced effect of AEON (urinary odour and flavour) on sensory perception in comparison to SK. For the other meat products, less discrimination related to PC2 was observed (Fig 1B). This was in line with results obtained in previous studies, which indicated that mostly AEON is responsible for the disliking of boar meat [34, 41].

3.4. Influence of production process of cooked hams

As the production process is also expected to impact the reduction of boar taint in the final product, the influence of the latter was investigated for two types of cooked ham. Cooked ham was produced using a cooked-in or an open-cook procedure and afterwards, the difference in boar taint and flavour perception, overall sensory quality and acceptance were assessed. Significant differences in the latter parameters were observed between the two types of cooked ham, throughout the three boar groups (Fig 2). Indeed, differences in boar taint, boar flavour, overall sensory quality and acceptance between the cooked hams were less obvious in the gilt category (Fig 2). In the hams produced following a cooked-in process, significantly more boar taint odour (p=0.008) and boar taint flavour (p=0.01) were observed in comparison to the open-cook process. Consequently, because of the higher boar taint and flavour perception, a 16% lower overall sensory quality (p=0.007) and 15% lower acceptance (p=0.006) were attributed to the cooked-in process, indicating a preference for the open-cook process. This is most likely due to the release of the boar taint compounds from the meat during cooking. When applying a cooked-in procedure, the boar taint compounds may redistribute from the intramuscular to the subcutaneous fat of the ham. However, due to the closed environment in which the ham is

cooked, the boar taint compounds remain present in the end product, while in an open-cooking process, they are released more effectively from the ham [31, 33].

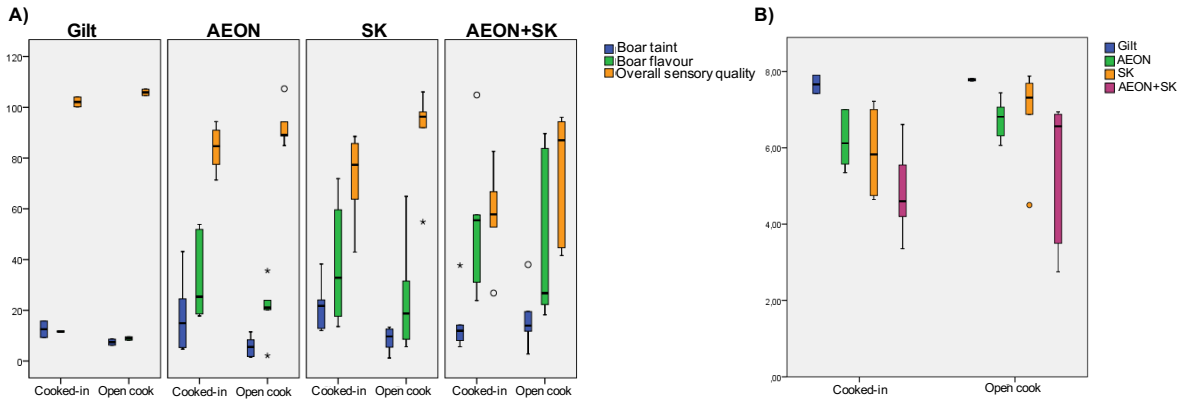

Fig 2 A) Box plots for the gilt, AEON, SK and AEON+SK carcass categories for boar taint, boar flavour and overall sensory quality as quality parameters for the evaluation of cooked hams following a cooked-in or open-cook procedure. All parameters were scored on a scale from 0 (neutral) to 150 (very strong) and B) Box plots for the gilt, AEON, SK and AEON+SK carcass categories for acceptance as a quality parameter for the evaluation of cooked hams following a cooked-in or open-cook procedure. Acceptance was scored on a scale from 0 (not consumable) to 10 (perfectly consumable).

4. CONCLUSIONS

In this study, the sensory evaluation of meat products by trained experts was more impaired by meat samples of the AEON group in comparison to the SK, SK+AEON and gilt group. Consequently, it might be advisable for the meat industry to screen carcasses at the slaughter line and subdivide tainted carcasses in different categories prior to processing. Moreover, this study also demonstrated that cold-served meat products, especially dry fermented sausage and dry-cured ham, are the most promising for processing tainted boar meat regardless of the boar category. Moreover, for cooked ham it was demonstrated that different production processes influence the perception of boar taint related attributes. Therefore, production processes should be optimized in order to completely mask the presence of boar taint in the final end products.

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CHAPTER IV

SENSORY EVALUATION OF BOAR-TAINT-CONTAINING MINCED MEAT, DRY-CURED HAM AND DRY FERMENTED SAUSAGE BY A TRAINED EXPERT PANEL AND CONSUMERS

Adapted from:

Verplanken K.[*], Wauters J.[*], Vercruysse V., Aluwé M., Vanhaecke L. (2017). Food Chemistry 233:247-255. [*] shared first authors

ABSTRACT

One of the main issues related to entire male pigs is the occurrence of boar taint, an off-odour, which compromises the consumability. In this study, rejection thresholds by a trained expert panel (IND: 24-65 µg kg^{-1}, SK: 44-89 µg kg^{-1}, AEON: 121-342 µg kg^{-1}) for the boar taint compounds were estimated for the meat fraction of minced meat, dry fermented sausage and dry-cured ham, based on the results and dataset obtained in chapter III. Afterwards, sensory evaluation of these products containing 10% tainted meat (minced meat and dry fermented sausage) or moderate boar taint compound levels (dry-cured ham) occurred. The beneficial effect of diluting tainted meat was demonstrated, as no significant difference in acceptance/consumability was observed between gilts and 10% tainted meat by experts as well as consumers. Also dry-curing proved a promising technique for masking boar taint and preventing consumer dissatisfaction. The obtained results demonstrate the applicability of the estimated thresholds in meat products as a tool for identifying masking and reducing strategies on the perception of boar taint.

1. INTRODUCTION

Increasing awareness on animal welfare during the past decades led to a voluntary intent to abandon surgical castration of pigs by 2018 [1]. Since the surgical castration of pigs was primarily intended to eliminate boar taint, an off-odour and flavour mainly caused by the accumulation of AEON, SK and to a lesser extent IND in pig adipose tissue, rearing entire male pigs consequently implies the re-occurrence of boar taint [2-4]. Moreover, up until now no reduction strategy guarantees a complete elimination of boar taint [5-10]. Consequently, the presence of boar taint in entire male pig carcasses could cause negative consumer reactions and lead to economic losses in pig husbandry. In order to increase the marketing potential of meat from entire male pigs, more insights regarding consumer acceptance are necessary.

Several studies on the sensory evaluation of boar meat indicate that there is a high rejection risk, attributable to the presence of boar taint [11-14]. Consequently, tainted meat is unfit for marketing, this represents on average 6.5% and 3% of boar meat, which is rejected based on boar taint and flavour, respectively [15]. However, boar taint perception varies among different tested meat products [13, 16]. Generally, fresh meat products such as loins and cutlets are accompanied with a higher rejection risk, especially when heated, in comparison with processed meat products such as dry fermented sausage, cooked and dry-cured ham [11, 12, 15, 17-19]. Processed meat products show more potential for commercializing tainted boar meat due to the masking and reducing effects of seasoning, dry curing, cooking, fermenting and smoking [13, 16, 20, 21]. However, these techniques mostly do not guarantee 100% prevention of adverse consumer reactions caused by the presence of boar taint [13, 16, 20]. Among all masking strategies, smoking and fermenting offer the most potential for commercializing tainted boar meat [20, 21]. As such, for the production of smoked sausages, up to 25% severely tainted meat could be used during production. However, this percentage dropped to 6 to 12% if the sausages were consumed heated [16, 22]. In fermented sausages, up to 50% of tainted meat with varying boar taint compound levels (AEON: 950 – 13,140 µg kg^{-1}, SK: 160 – 610 µg kg^{-1}, IND:

60 – 650 µg kg^{-1}) could be used [21]. In sausages, type frankfurter and bologna, 75% and 50% tainted meat, respectively, could be processed without compromising the quality of the end product [17]. Consequently, apart from smoking and fermenting, also diluting tainted with untainted meat seems a promising strategy to prevent adverse consumer reactions. Moreover, taking into account an average natural prevalence of 3% severely tainted (sensory score > 4) carcasses and an observed prevalence varying from 0 to 8% or 14% on individual Belgian and Dutch farms, a maximal mixing percentage of approximately 10% tainted meat may be anticipated [23, 24].

In order to investigate the effect of diluting tainted meat (minced meat, dry fermented sausage) and dry-curing (dry-cured ham) in combination with smoking, different aims were envisaged in this study. First, for each meat product, i.e. minced meat, dry-cured ham and dry fermented sausage, different boar taint related descriptors, the overall sensory quality and acceptance were evaluated by trained assessors, sensitive for AEON, and rejection thresholds were established for each meat product through receiver operating characteristic (ROC) analysis. Finally, the acceptability of minced meat and dry fermented sausage containing 10% tainted meat and dry cured ham with moderate boar taint levels (AEON: 500 – 2000 µg kg^{-1}, SK: 200 – 300 µg kg^{-1}) was assessed by consumers, which allowed evaluation of the applicability of the established rejection thresholds as estimated by a trained expert panel.

2. MATERIALS AND METHODS

In this study, three experiments were set up to gain understanding in the feasibility of processing tainted boar meat (Table 1). To this end, first rejection thresholds were estimated by a trained expert panel for the meat fraction of minced meat, dry-cured ham and dry fermented sausage by evaluation of meat products containing 100% tainted boar meat. Afterwards, taking into account the obtained thresholds and natural boar taint prevalence, minced meat and dry fermented sausage containing 10% tainted boar meat and dry-cured ham containing moderate boar taint compound levels (AEON: 500 – 2000 µg kg-1; SK: 200 – 300 µg kg-1) were evaluated by trained assessors. Finally, the validity of the

rejection thresholds obtained by a trained expert panel was evaluated by consumer panels for minced meat and dry fermented sausage containing 10% tainted boar meat and dry-cured ham containing moderate boar taint compound levels. For each experiment, carcasses were selected at the slaughter line and their boar taint compound levels in neck fat and the meat products itself were determined by UHPLC-HR-Orbitrap-MS analysis (Tables 2 & 3) [25].

Table 1 Overview of the experimental set-up.

	Type of evaluation	Meat product	Diluting percentage	Number of samples
Experiment 1: Assessment of odour thresholds	Trained expert panel	Minced meat	100 % tainted boar meat	9
		Dry fermented sausage	100 % tainted boar meat	
		Dry-cured ham	100% tainted boar meat	
Experiment 2: Evaluation of boar meat by trained experts	Trained expert panel	Minced meat	10 % tainted boar meat	9
		Dry fermented sausage	10% tainted boar meat	
		Dry-cured ham	100 % (moderately) tainted boar meat	
Experiment 3: Evaluation of boar meat by consumers	Consumer panel	Minced meat	10 % tainted boar meat	9
		Dry fermented sausage	10 % tainted boar meat	
		Dry-cured ham	100 % (moderately) tainted boar meat	

Table 2 Overview of the boar taint compound levels (µg kg^{-1}) in neck fat and in the different meat products for the different boar carcasses selected for the assessment of odour detection thresholds (experiment 1) and sensory evaluation by a trained expert panel (experiment 2).

			Carcass Category								
			AEON			SK			AEON + SK		
			Boar 1	Boar 2	Boar 3	Boar 4	Boar 5	Boar 6	Boar 7	Boar 8	Boar 9
Experiment 1:	**Carcass (neck fat)**	AEON	1613	1969	3131	121	147	187	1187	458	444
Odour thresholds		SK	63	21	16	199	279	320	521	59	157
		IND	75	85	49	98	106	101	340	114	182
	Minced Meat (meat)	AEON	1597	1454	93	1296	210	65	921	234	296
		SK	46	25	29	21	75	92	317	168	109
		IND	74	57	35	27	54	48	222	75	79
	Dry fermented sausage	AEON	444	574	958	< LOQ	< LOQ	40	568	202	19
	(meat)	SK	< LOQ	< LOQ	< LOQ	28	50	68	139	< LOQ	16
		IND	21	13	10	14	21	27	76	17	21
	Dry-cured ham	AEON	109	131	157	< LOQ	< LOQ	34	181	7	71
	(meat)	SK	< LOQ	< LOQ	< LOQ	17	19	70	< LOQ	< LOQ	< LOQ
		IND	15	8	5	9	10	21	70	7	6
Experiment 2:	**Carcass (neck fat)**	AEON	961	1213	866	461	128	629	795	1566	4468
Trained experts		SK	96	31	59	150	239	419	178	241	438
		IND	12	13	15	7	68	89	73	61	145
	Minced Meat	AEON	17	39	14	15	< LOQ	14	43	45	69
	(10% boar meat)	SK	10	7	2	14	15	18	20	13	18
		IND	6	5	5	10	10	10	14	9	12
	Dry fermented sausage	AEON	24	68	25	16	15	21	23	52	117
	(10% boar meat)	SK	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
		IND	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
	Dry-cured ham	AEON	49	59	23	14	< LOQ	< LOQ	22	25	183
	(meat)	SK	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
		IND	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	9	< LOQ	< LOQ

*** Selected carcasses were subdivided into different categories containing high AEON levels (AEON), SK levels (SK) or AEON and SK levels (AEON+SK). < LOQ = below limits of quantification.**

Table 3 Overview of the boar taint compound levels (µg kg^{-1}) in neck fat of the selected boar carcasses and the different meat products for sensory evaluation by consumers (Experiment 3).

			AEON	SK	IND
Experiment 3:	**Carcass (neck fat)**	Boar 1	962	44	37
Consumer panel		Boar 2	258	431	85
		Boar 3	584	230	95
	Minced Meat (10% boar meat)	Pooled sample of boar 1, 2 & 3	40	<LOQ	2
	Dry fermented sausage (10% boar meat)	Pooled sample of boar 1, 2 & 3	<LOQ	16	7
	Dry-cured ham (meat)	Boar 1	44	<LOQ	<LOQ
		Boar 3	15	<LOQ	<LOQ

*** Selected carcasses were subdivided into different categories containing high AEON levels (AEON), SK levels (SK) or AEON and SK levels (AEON+SK). < LOQ = below limits of quantification.**

2.1. Chemical analysis

After selection of carcass at the slaughter line, the boar taint compound levels were determined in neck fat by means of UHPLC-HR-Orbitrap-MS as described by Bekaert et al. [25]. After production of the different meat products under investigation in this study, the boar taint compound levels were determined in the meat fraction of the processed meat products (minced meat, dry fermented sausage and dry-cured ham) derived from the tainted boar and gilt carcasses. All meat was stored at -20 °C until analysis. Details on the reagents and chemicals, and analysis methods for fat tissue and the meat products can be consulted in Chapter II (section 2.1), Bekaert et al. and Verplanken et al., respectively [25, 26].

2.2. Sensory evaluation

2.2.1. Training of experts

For the assessment of rejection thresholds in the meat products, minced meat, dry fermented sausage and dry-cured ham containing 100% tainted boar meat, were evaluated by a trained expert panel. To this end, first thirty-nine candidate experts were trained. In total, 24 men and 15 women, were subjected to a well-described selection process based on the protocols by Bekaert et al. and Meier-Dinkel et al., in which training occurred by use of solutions or paper strips, respectively [27, 28]. For details on the exact training procedure we refer to Wauters et al. [29]. In short, first, the candidates' sensitivity for AEON was evaluated. Candidates that were able to correctly identify AEON containing bottles were withheld for further training (n = 30) whereas others not passing the test were excluded from further participation. Training was proceeded in different steps. In a first step, the remaining candidates were trained to discriminate between low and high concentrations of AEON and SK. A candidate-expert was allowed to proceed to the next step if each odd sample was identified as such. During this second step, candidates were challenged to rank 4 concentrations of AEON and SK. Further training occurred similarly, however, randomly mixed batches of AEON and SK bottles were presented to the candidates who were additionally asked to identify each bottle. If this was performed correctly

(allowing two mistakes in compound identification), the candidate-experts were selected for the final training program based on the olfactory assessment of boar fat samples applying the soldering iron melting method described by Bekaert et al. [27]. In total, 14 experts successfully completed the training course.

2.2.2. Sensory evaluation of meat products by trained experts

Sensory assessment occurred in a controlled room with individual booths for each panellist and the environmental conditions were kept as constant as possible. Each expert was foreseen with mineral water and tasteless crackers to clear their palate in between samples. Preparation of the meat products for testing occurred in a kitchen separated from the evaluation room, eliminating possible interference of baking odours. Minced meat patties were baked on grills (2000 W) without seasoning and for each boar and gilt, a separate grill was used to avoid contamination between samples. The cold-served meat products (dry-cured ham and dry fermented sausage) were prepared during the morning of each session day and kept refrigerated (4 °C) until the beginning of the evaluation session. In each session, evaluation of the meat products occurred by a minimum of 6 trained experts and each product was evaluated in duplicate on different days. Because of practical limitations, the expert panels did not consist of the same panellists on each session day; however, each panellist followed the same training course. Of each meat product, 14 samples were evaluated, whereof 5 gilt samples and 1 sample from each boar (9 in total). The panellists were informed about the first sample, being from a gilt, which served as a reference point. The other samples were served to the panellists in a randomized order and were blindly evaluated.

2.3. Assessment of rejection thresholds by a trained expert panel

The panellists were then asked to score the consumability (YES/NO) of the meat products (minced meat, dry fermented sausage, dry-cured ham) on a scale of 0 to 10, with 0 being not consumable and 10 perfectly consumable. Based on these scores, the rejection thresholds were estimated by ROC analysis.

2.4. Descriptive analysis by trained experts

Taking into account the obtained rejection thresholds by trained experts and maximum natural boar taint prevalence of 10% on individual pig farms, in a second experiment minced meat and dry-fermented sausage containing 10% tainted boar meat and dry-cured ham containing moderate boar taint compound levels were evaluated by a trained expert panel. During sensory assessment, the panellists scored several characteristics describing the odour (general odour, boar taint, urinary odour, manure odour), taste (general flavour, boar flavour, urinary flavour, manure flavour) and general quality (overall sensory quality, acceptance) of each sample on a 150 mm scale subdivided from 0 to 4, with 0 being absence of bad odour or taste and 4 a very pronounced presence of the latter. Finally, the panellists also scored the acceptance of each meat product on a scale of 0 to 10, with 0 being not consumable and 10 perfectly consumable and they were also asked to indicate whether the meat product was consumable (YES/NO) in order to obtain a dichotomous variable. Each meat product was evaluated in duplicate on two different test days.

2.5. Sensory evaluation by consumers

In experiment 3, sensory evaluation of minced meat, dry fermented sausage and dry cured ham occurred by consumers. Several demographic data (nationality, gender, age, smoker, having a cold, eating pork) of the consumers were collected (Table 4). Before evaluation of the meat products, consumers were not informed on the goal of the study nor tested on their sensitivity towards AEON. Sensory evaluation occurred in a controlled room according to ISO 4120 (2004) guidelines for sensory evaluation [30]. Each meat product was evaluated by 200 consumers using a comparative external benchmark test. In this test, the consumers were challenged to assess the sensory quality of 3 samples (2 different gilts and 1 boar), whereby the three samples were presented to the consumers simultaneously. For the evaluation of dry-cured ham, 4 samples were presented to the consumers (2 different gilts and 2 different boars). Minced meat samples were presented to the consumers as grilled patties. Dry fermented sausage and dry-cured ham were presented cold as cubes and slices,

respectively. The consumers were asked to score different evaluation variables (first overall impression, aroma, first impression: flavour, taste: freshness, specific flavour, aftertaste, overall taste, divergent aroma, divergent flavour) on a 5-point Likert scale.

Table 4 Experiments 3: Demographic data of the consumer panel for the sensory evaluation of minced meat, dry fermented sausage and dry-cured ham.

	Minced meat	Dry fermented sausage	Dry-cured ham
Nationality (%)			
Belgian	92.5%	96.0%	95.0%
French	7.0%	3.5%	5.0%
Dutch	0.5%	0.5%	0.0%
Sex			
Man	32.5%	35.0%	31.0%
Women	67.5%	65.0%	69.0%
Age (%)			
<20	6.0%	8.0%	3.0%
21-35	36.5%	37.0%	50.5%
36-55	55.5%	52.0%	44.5%
>55	2.0%	3.0%	2.0%
Smoker (%)	0%	0%	0%

2.6. Carcass selection

For the three different experiments performed in this study, carcasses were selected at the slaughter line. In all carcass selection rounds, 300 carcasses of entire male pigs were screened at the slaughter line (Debra Meat slaughterhouse, Tielt, Belgium) on the occurrence of (intense) boar taint by a trained expert, based on olfactory evaluation using the soldering iron method (RDS 80, Kurz Ersa, Wertheim, Germany). Forty-five carcasses were retained for chemical analysis of the three main boar taint compounds AEON, SK and IND. Analysis was performed on back fat samples by UHPLC-HRMS (Fig 1). The method parameters can be consulted in Bekaert et al. [25]. After chemical analysis, nine tainted carcasses were retained for meat production and were ranked either based on the highest concentration of (only) AEON (3 carcasses; group 1; AEON ≥ 1500 µg kg^{-1} fat), the highest concentration of (only) indolic (i.e. SK and IND) compounds (3 carcasses, group 2; IND ≥ (±) 100 µg kg^{-1} fat and SK ≥ (±) 200 µg kg^{-1} fat), or the combination of the highest concentrations of both AEON and indolic compounds (3 carcasses; group 3; AEON ≥ 500 µg kg^{-1}, IND ≥ 100 µg kg^{-1} fat and SK ≥ 50 µg kg^{-1} fat).

However, due to study limitations and the relatively low prevalence of tainted boar carcasses, in experiment 1, only 1 boar with high AEON and indolic compound levels could be selected. Two other boars with AEON, SK and IND levels that approximated the set thresholds the most were selected. AEON levels for these boars were 458 and 444 µg kg^{-1} for boar 3.2 and boar 3.3, respectively. The SK levels were 59 and 157 µg kg^{-1}, for boar 3.2 and boar 3.3, respectively. IND levels for all 3 boars in the AEON+SK category amounted 100 µg kg^{-1} (Table 2). For experiment 2, one boar in the SK category was selected containing only 150 µg kg^{-1} SK. Also in the AEON+SK category, one boar only contained 178 µg kg^{-1} of SK (Table 2). Apart from 9 tainted boar carcasses, also 1 gilt carcass was selected for the production of blank meat.

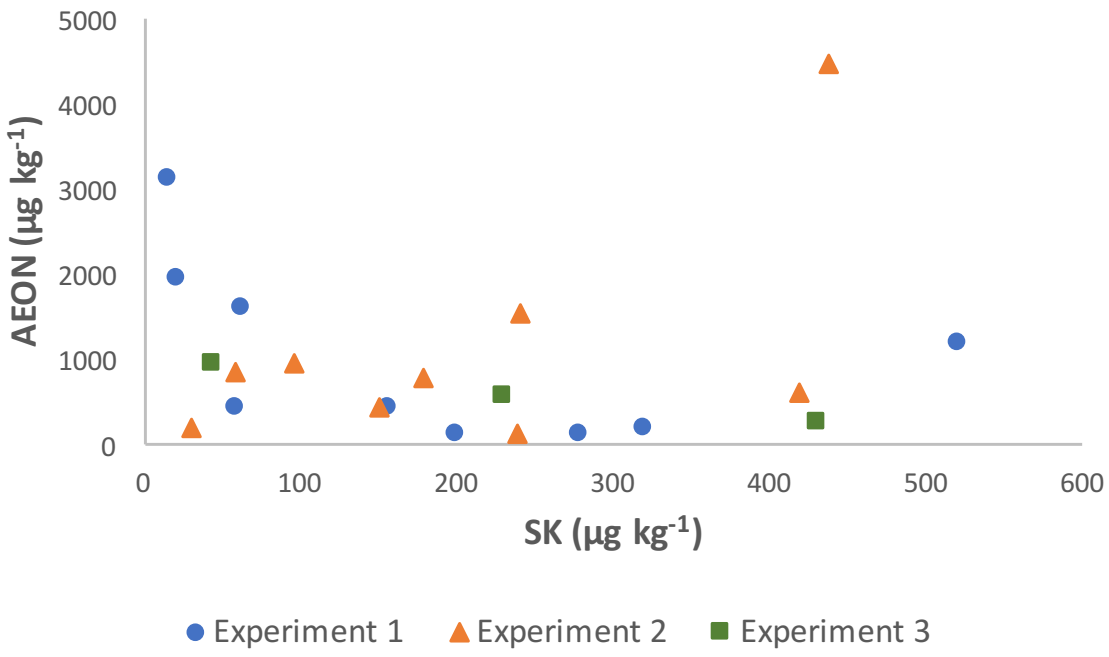

Fig 1 Scatter plot of the androstenone (AEON) and skatole (SK) neck fat levels of the selected boar carcasses used in experiment 1 (assessment of odour thresholds), experiment 2 (sensory evaluation by trained assessors) and experiment 3 (sensory evaluation by consumers).

2.7. Production of different meat products

Following analysis of the back fat samples and subsequent carcass selection, boars were hand-cut at the slaughterhouse, preventing mixing of boars and allowing proper labelling with identification of each piece of meat/fat. The raw materials required to produce minced meat (meat and fat), dry fermented sausage (shoulder and back fat) and dry cured ham type Cobourg (hind leg) were transported to commercial meat-processing companies. For experiment 1 & 2, from every individual boar, a batch of minced meat, dry fermented sausage and dry-cured ham were produced. For experiment 3, from the 3 selected carcasses, a mixed batch or pooled sample of minced meat and dry-fermented sausage was produced and 2 carcasses containing high AEON and SK & AEON levels were selected for dry-cured ham production (Tables 2 & 3). In experiment 1, a fraction of gilt fat was used for the manufacturing of minced meat and dry fermented sausage. Insufficient amounts of fat were delivered for minced meat production, consequently leading to a varying fraction of added gilt fat (41-82%) between boars. Boar fat was supplied in abundance for dry fermented sausage production, but the consistency of the fat (extremely soft in comparison to gilt fat) prevented its proper use in the production of dry fermented sausage. Therefore, a constant fraction of boar fat (22%) was supplemented with gilt fat (78%). Minced meat was stored frozen (-20 °C) in commercial plastic recipients until sensory evaluation of the meat products. Dry fermented sausage and dry-cured ham were stored refrigerated (6 °C) until evaluation.

2.8. Data analysis

Statistical analysis of the data was performed in SPSS version 22 (IBM corporation, NY, USA) and a significance level of 0.05 was taken into account. All data was assumed to be sufficiently normally distributed based on the graphical examination (QQ-plot and histogram) of the residuals. In order to avoid repeated measures on identical samples, statistical analysis was performed on aggregated data (mean sample score for all trained experts).

ROC analysis was used to estimate the rejection thresholds by trained experts for all boar taint compounds in each meat product separately, whereby the boar taint compound levels obtained in neck fat and meat were set as test variables and acceptance (dichotomous variable) as the state or outcome variable (experiment 1). In a second experiment, in order to identify significant differences between different carcass categories (gilt, SK, AEON, SK+AEON), a linear model was built including general odour, boar taint, urinary odour, manure odour, general flavour, boar flavour, urinary flavour, manure flavour, overall sensory quality and acceptance as fixed factors. This was carried out for each meat product separately. In case of significant differences, a post-hoc Tukey test was performed to make pairwise comparisons between the different carcass categories. Moreover, in order to correct for variation on different test days, the test day was included in the model as a covariable. Additionally, to evaluate which boar taint related descriptors (general odour, boar taint, urinary odour, manure odour, general flavour, boar flavour, urinary flavour and manure flavour) significantly influenced the overall sensory quality and acceptance of boar meat, linear models were built for each meat product. In addition to the boar taint related descriptors, the carcass category (gilt, SK, AEON, SK+AEON) and test day were included in the model as covariables to correct for variation among the latter. Moreover, as the different boar taint related descriptors were highly correlated ($|r| = 0.29\text{-}0.93$), separate models were constructed for each of the latter. Finally, in a third experiment, assessing the consumers' preference (experiment 3) occurred by assigning ranks to each sample (2 gilts, 1 boar), whereby the rank value was set as the weighted average of the evaluated variables computed as: 5 x overall taste, 2 x aftertaste and 1x other evaluation variable (first general impression, aroma, first impression of flavour, taste, specific flavour, divergent aroma, divergent flavour). All sensory descriptors were scored on a scale from 0 (neutral) to 5 (very strong). The overall score for each product was evaluated on a scale from 0 (neutral) to 10 (very high). After evaluation, differences between the 10% boar and two gilt categories were evaluated through one-way repeated measures ANOVA, whereby the carcass category (10% boar, gilt 1, gilt 2) was set as independent variable and the different evaluation variables (ranking value, overall taste, overall score, overall first impression, overall aroma, first impression

(flavour), taste: freshness, specific flavour, aftertaste, overall taste, flavour (divergent), aroma (divergent), preference) as dependent variables.

3. RESULTS AND DISCUSSION

3.1. Assessment of rejection thresholds in meat

Currently, general agreement on the acceptable levels of the boar taint compounds in meat is lacking. For this reason, rejection threshold levels for all boar taint compounds were established for each meat product through ROC analysis based on the evaluation of meat samples by trained assessors (Fig 2). With the exception of dry-cured ham, the obtained ROC models showed sufficient accuracy (AUC: 0.642-0.804) to predict the acceptance outcome based on the obtained boar taint compound levels in neck fat and meat (Table 5). However, since only a limited number of boar carcasses (n = 9) was included due to budgetary and logistics reasons, this study is associated with a relatively low sample power.

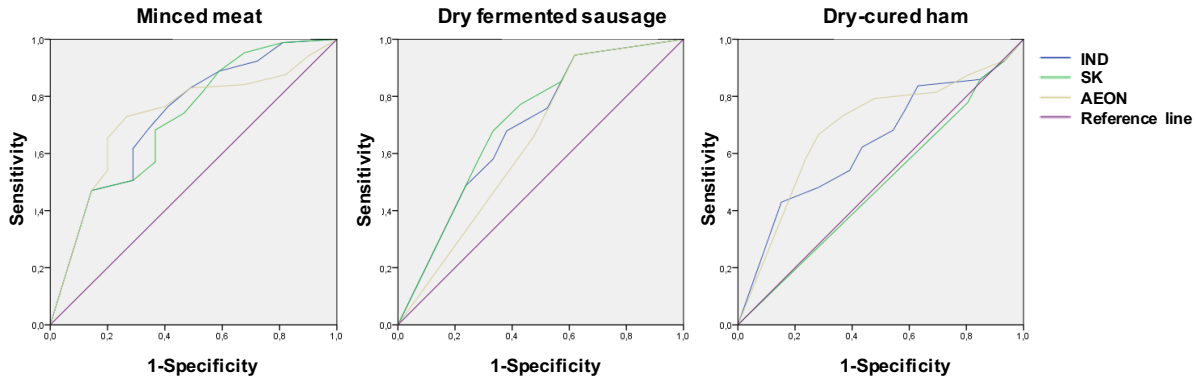

Fig 2 ROC curves for the estimation of rejection thresholds for indole (IND), skatole (SK) and androstenone (AEON) in minced meat, dry fermented sausage and dry-cured ham.

Apart from increasing the sample size to increase the power of the study, also applying a statistical test able to handle data from a repeated measures design could be applied. However, in order to prevent unnecessary complication of the study design, it was opted to use ROC analysis on aggregated data to give an estimation of the rejection thresholds by trained experts of the boar taint compounds

in different meat products. In order to check the validity of our results, with the exception of minced meat due to a dilution effect with gilt fat, also odour thresholds in neck fat were estimated and compared to literature [160].

Table 5 Experiment 1: Estimation of rejection thresholds of the boar taint compounds androstenone (AEON), skatole (SK) and indole (IND) in neck fat and the meat products for minced meat, dry fermented sausage and dry-cured ham through receiver operating characteristic (ROC) analysis after sensory evaluation of 100% tainted boar meat by trained assessors.

			AUC	Confidence interval (95%)	Threshold (µg kg^{-1} fat)	Sensitivity	1-Specificity
Minced meat	**Neck fat**	AEON	0.709	0.655-0.764	823	0.765	0.400
		SK	0.705	0.651-0.759	239	0.818	0.533
		IND	0.656	0.598-0.713	110	0.812	0.722
	Meat	AEON	0.728	0.673-0.783	342	0.829	0.489
		SK	0.716	0.663-0.769	83	0.818	0.533
		IND	0.731	0.678-0.783	65	0.829	0.489
Dry fermented sausage	**Neck fat**	AEON	0.682	0.585-0.779	451	0.846	0.571
		SK	0.712	0.616-0.808	239	0.772	0.429
		IND	0.692	0.596-0.788	110	0.753	0.524
	Meat	AEON	0.642	0.536-0.748	121	0.846	0.571
		SK	0.712	0.616-0.808	59	0.852	0.571
		IND	0.693	0.597-0.789	24	0.852	0.571
Dry-cured ham	**Neck fat**	AEON	0.709	0.640-0.778	1400	0.867	0.543
		SK	0.592	0.518-0.666	239	0.778	0.804
		IND	0.570	0.495-0.645	110	0.785	0.783
	Meat	AEON	0.688	0.617-0.759	170	0.926	0.935
		SK	0.489	0.412-0.565	44	0.926	0.935
		IND	0.634	0.562-0.707	46	0.926	0.935

The obtained threshold levels based on the concentration at neck fat level for the 3 meat products were 110, 239 and 451-1400 µg kg^{-1} fat for IND, SK and AEON, respectively. With the exception of the level found for AEON, these levels are comparable to the general acceptable levels of the boar taint compounds in literature (i.e. IND: 100 µg kg^{-1} fat, SK: 250 µg kg^{-1} fat, AEON: 500 to 1000 µg kg^{-1} fat), confirming the applicability of the models to estimate rejection threshold levels in meat [16, 17, 31-33]. For AEON, relatively high variability was observed for the obtained threshold level in neck fat. This could be explained by variation in AEON perception between meat products or might be due to the variation in AEON sensitivity among trained experts [16]. Moreover, in literature similar trends are observed as no consensus on the acceptable AEON levels in neck fat has been reached yet. Previous studies reported AEON cut-off levels up to 2000 and 3000 µg kg^{-1} fat in the absence of SK [14, 18].

Furthermore, research showed that the combined presence of the indolic compounds and AEON could fortify the perception of the boar taint compounds in comparison to its perception when present separately [34]. However, it should be noted that because of the limited number of carcasses included in this study (3 for each boar category), a possible interaction between the indolic compounds and AEON in the perception of boar taint was not taken into account. Consequently, the obtained threshold levels in meat might be an underestimation of olfactory perception as previous research reported an additive effect of the boar taint compounds when present in concentrations below the rejection threshold levels [34]. Compared to neck fat, the obtained general acceptable levels for the boar taint compounds in meat were on average a factor 2, 4 and 4 lower for minced meat, dry fermented sausage and dry-cured ham respectively. This can most likely be explained by the fact that the boar taint compounds accumulate in adipose tissue. Consequently, as high neck fat levels are associated with relatively lower levels of the boar taint compounds in the meat product, the rejection thresholds of the boar taint compounds determined in meat products are also expected to be lower in comparison to those in neck fat [29]. Furthermore, despite the fact that SK is slightly more lipophilic than IND, and that SK is considered as more contributing to boar taint, lower thresholds were observed for IND in comparison to SK (Table 5). Research suggests however, that the perception of SK as more disturbing is independent from the rejection thresholds of IND and SK as in neck fat, IND and SK are associated with rejection thresholds of 100 and 200-250 µg kg^{-1}, respectively [31]. Consequently, similarly to neck fat, for meat products, the rejection threshold of IND was also expected to be lower in comparison to that of SK. In minced meat, thresholds of 65, 83 and 342 µg kg^{-1} fat were obtained for IND, SK and AEON, respectively. In dry fermented sausage, lower threshold levels were obtained, i.e. 24, 59 and 121 µg kg^{-1} fat for IND, SK and AEON, respectively. Finally, for dry cured ham the threshold levels in meat, i.e. 46, 44 and 170 µg kg^{-1} fat for IND, SK and AEON, respectively, and were comparable to those obtained in dry fermented sausage. Since differences in boar taint perception are observed among different meat products, also variation in rejection thresholds were expected. Different consumer studies indeed indicate a higher boar taint perception in heated meat products, whereby consequently

lower rejection thresholds are expected [13, 16, 17, 35]. Although minced meat investigated in this study was heated, the obtained rejection threshold levels were highest in the latter meat product, this against all expectations as the perception of boar taint generally depends on the temperature. A possible explanation for this is the standardized environment that was used during evaluation by trained experts and the cooking of minced meat in a separate room, avoiding any interference of baking odours. Consequently, as no baking odours were perceived by the trained experts, this may lead to a discrepancy with cooking minced meat at home [13, 14].

Good sensitivity (AUC > 0.753) was observed for the estimated rejection thresholds in meat products (Table 5). However, in some cases low specificity was observed (AUC < 0.500). This indicates that the estimated thresholds are very sensitive, thus leading to a very low proportion of false negative results. The lower specificity on the other hand indicates that a higher proportion of false positive results will be produced, using these thresholds [36]. In contrast to false negatives, false positives will not result in loss of consumers' confidence in pork industry. However, since tainted boar meat is often subject to penalty fees, a large number of false positives may have an important economic impact on pork industry [37].

3.2. Sensory evaluation by trained assessors

In a first experiment, differences in boar taint related descriptors, the overall sensory quality and acceptance between boars and gilts were investigated by evaluation of meat products by trained assessors. For none of the meat products, a significant difference in odour characteristics (general odour, boar taint, urinary and manure odour) was observed between the different carcass categories ($p > 0.05$) (Table 6). Similarly, with the exception of manure flavour in dry-cured ham, no significant differences were observed in flavour characteristics (general, boar, urinary and manure flavour) between the different carcass categories ($p > 0.05$) (Table 6).

Table 6 Linear models for the comparison of different boar taint related descriptors, overall sensory quality and acceptance of the meat products of the different carcass categories (Gilt, SK, AEON, SK+AEON).

	Carcass category	Minced meat	Dry-cured ham	Dry fermented sausage
General odour	**Gilt**	66	78	22
	SK	78	73	64
	AEON	74	72	47
	SK+AEON	95	52	44
Boar taint	**Gilt**	26	21	0
	SK	44	25	8
	AEON	30	25	0
	SK+AEON	55	10	14
Urinary odour	**Gilt**	9	5	1
	SK	13	6	8
	AEON	22	13	0
	SK+AEON	35	3	5
Manure odour	**Gilt**	15	5	0
	SK	31	6	0
	AEON	13	0	0
	SK+AEON	30	10	14
General flavour	**Gilt**	104	96	89
	SK	100	109	113
	AEON	117	110	108
	SK+AEON	112	110	118
Boar flavour	**Gilt**	61	62	66
	SK	54	79	63
	AEON	73	72	76
	SK+AEON	60	74	89
Urinary flavour	**Gilt**	24	19	31
	SK	0	60	38
	AEON	65	41	56
	SK+AEON	43	37	27
Manure flavour	**Gilt**	41	$7^{a,b}$	42
	SK	48	4^{a}	44
	AEON	25	$6^{a,b}$	28
	SK+AEON	39	22^{b}	89
Overall sensory quality	**Gilt**	48	48	43
	SK	56	38	37
	AEON	37	44	38
	SK+AEON	48	37	46
Acceptance	**Gilt**	4.4	4.1	3.7
	SK	4.7	3.8	3.8
	AEON	3.6	3.5	3.8
	SK+AEON	3.5	3.6	4.0

$^{a, b}$ indicate significant and * no significant differences between carcass categories per meat product and descriptor ($\alpha = 0.05$). Odour and flavour descriptors were scored on a scale from 0 (neutral) to 150 (very strong), acceptance was scored on a scale from 0 (not consumable) to 10 (perfectly consumable).

For dry-cured ham, a significantly higher manure flavour was observed in the SK+AEON category in comparison to the SK boar category, but this did not result in a significant difference in overall sensory quality, nor acceptance. Differences in manure flavour between the SK+AEON and gilt and AEON

categories were not found to be significant. These results may indicate that the perception of boar taint can be masked in minced meat and dry fermented sausage containing 10% tainted boar meat and dry-cured ham containing moderate boar taint compound levels. This is most likely due to diluting tainted boar meat on the one hand and the masking and reducing effects of smoking, curing and drying on the perception of boar taint on the other. However, it should be noted that because of the limited number of carcasses included in this study (3 for each boar category), the sample power might be too low in order to demonstrate a significant effect between the different carcass categories. However, in contrast to this study, previous studies indicated that masking strategies including smoking, curing and drying were insufficient to completely eliminate perception of boar taint [11, 13, 16-18]. This emphasizes the importance of the initial boar taint compound levels in carcasses and the need for unambiguous rejection thresholds for the latter in meat products and setting guidelines for the processing of tainted boar meat.

Table 7 Experiment 2: p-values of linear models constructed for the evaluation of the influence of different boar taint related descriptors on the overall sensory quality and acceptance of minced meat, dry-cured ham and dry fermented sausage through sensory assessment by a trained expert panel.

	Minced meat		Dry-cured ham		Dry fermented sausage	
	Overall sensory quality	Acceptance	Overall sensory quality	Acceptance	Overall sensory quality	Acceptance
General odour	0.051	0.012	0.356	0.369	0.123	0.828
Boar taint	0.025	0.007	0.774	0.166	0.554	0.012
Urinary odour	0.014	0.005	0.501	0134	0.776	0.497
Manure odour	0.094	0.099	0.588	0.014	0.724	0.720
General flavour	<0.001	<0.001	<0.001	0.002	<0.001	<0.001
Boar flavour	0.005	<0.001	0.004	0.109	0.001	0.002
Urinary flavour	0.006	<0.001	0.117	0.018	0.013	0.037
Manure flavour	0.612	0.337	0.042	0.236	0.039	0.058

In a second experiment, designed to investigate the influence of different boar taint related descriptors on the overall sensory quality and acceptance of meat products, different linear models were constructed. The test day and carcass categories were included in these models as covariables. With the exception of dry fermented sausage, the test day did not significantly influence ($p > 0.05$) the

overall sensory quality and acceptance. This indicates that despite the use of different trained experts on each evaluation day, assessment of the meat products was very repeatable. Only for general flavour of minced meat, a significant influence ($p = 0.037$) of the test day was observed. For dry fermented sausage, the test day had a significant influence on the acceptance and overall sensory quality, indicating that more or less sensitive experts may have been present on the different test days. However, by including the test day as a covariable, this variation was accounted for. In general, for minced meat both the odour and flavour characteristics influenced the overall sensory quality and acceptance (Table 7). For dry-cured ham and dry fermented sausage on the other hand, mostly the flavour characteristics affected the overall sensory quality and acceptance (Table 7). A possible explanation lies in the increased importance of the perception of boar taint and related odours when meat is heated. Indeed, boar taint odour is perceived more strongly in heated meat products as the boar taint compounds are volatile and thus released when meat or fat of boars is heated [33, 38]. For this reason, in warm-consumed meat products a similar contribution of odour and flavour characteristics is observed [39, 40]. In cold-consumed meat products on the other hand, it appears that boar odour is not perceived, this in contrast to boar flavour [41]. Consequently, it can be stated that it is easier to completely avoid adverse reactions due to the presence of boar taint related odour and flavour in cold-consumed meat products.

3.3. Sensory evaluation by consumers

For none of the meat products, significant differences in overall results (ranking value, overall taste and overall score) nor a difference in product preference between boar and gilt meat products were observed. Moreover, no significant differences in scores for the different evaluation variables were observed between boars and gilt as well (Table 8).

Table 8 Experiment 3: Comparative external benchmark study for the sensory assessment of minced meat, dry fermented sausage and dry-cured ham by evaluation of different sensory descriptors by consumers.

		10% Boar		Gilt 1		Gilt 2	
		Mean	TopBox (%)	Mean	TopBox (%)	Mean	TopBox (%)
Minced meat	Ranking value	5.3		5.1		5.2	
	Overall taste	3.4*	48*	3.3	37	3.3	44%
	Overall score	6.7*	58	6.4	45	6.5	54%*
	Overall first impression	3.3*	41	3.1	35	3.4*	48%*
	Overall aroma	3.2	37	3.2	30	3.3*	39%*
	First impression (flavour)	3.5*	53*	3.3	41	3.4	50%*
	Taste: freshness	3.6	57	3.5	51	3.5	56%
	Specific flavour	3.5	53	3.4	48	3.4	50%
	Aftertaste	3.4*	47	3.3	40	3.3	43%
	Overall taste	3.4*	48*	3.3	37	3.3	45%
	Flavour (divergent)	3.2	44	3.1	37	3.3	42%
	Aroma (divergent)	3.3	45	3.1	39	3.2	43%
	Preference	31%		27%		23%	
Dry fermented sausage	Ranking value	5.5		5.4		5.4	
	Overall taste	3.5	59	3.4	51	3.5	53%
	Overall score	6.8	62	6.7	57	6.7	62%
	Overall first impression	3.6	65	3.6	58	3.6	60%
	Overall aroma	3.5	55	3.5	53	3.5	51%
	First impression (flavour)	3.5	56	3.5	54	3.5	56%
	Taste: freshness	3.6	61	3.6	59	3.6	59%
	Specific flavour	3.6	63	3.5	55	3.5	57%
	Aftertaste	3.5	59	3.4	51	3.5	52%
	Overall taste	3.5	59	3.4	51	3.5	53%
	Flavour (divergent)	3.4	58*	3.3	48	3.3	52%
	Aroma (divergent)	3.2	55	3.1	51	3.1	53%
	Preference	33%		22%		32%	

		Boar 1		Boar 2		Gilt 1		Gilt 2	
		Mean	TopBox (%)	Mean	TopBox (%)	Mean	TopBox (%)	Mean	TopBox (%)
Dry-cured ham	Ranking value	5.7		5.7		5.7		5.6	
	Overall taste	3.6	57.5	3.6	53	3.6	59	3.5	54
	Overall score	7.2*	79	7.1	68.5	7.1	72.5	7.0	67
	Overall first impression	3.9*	78	3.9	76.5	3.8	72	3.6	58
	Overall aroma	3.8	71	3.8	71.5	3.7	71	3.7	62.5
	First impression (flavour)	3.7	62	3.6	57	3.7	59.5	3.6	58
	Taste: freshness	3.8	76.5	3.8	69.5	3.7	70	3.7	70
	Specific flavour	3.7	68.5	3.8	68	3.8	72	3.6	66
	Aftertaste	3.6	55	3.6	54	3.6	56	3.6	55
	Overall taste	3.6	57.5	3.6	53	3.6	59	3.5	54
	Flavour (divergent)	3.7	61.5	3.7	62	3.7	65	3.6	55
	Aroma (divergent)	3.7	67	3.7	65.5	3.7	63.5	3.6	62
	Preference	30%		17%		20%		19%	

***: Indicative difference (α = 0.1), TopBox = % good/very good = Ratio of % samples scored as good to % samples scored as very good. The overall taste, overall first impression, overall aroma, first impression (flavour), taste: freshness, specific flavour, aftertaste, overall taste, flavour (divergent) and aroma (divergent) were scored on a scale from 0 (neutral) to 5 (very strong). The overall score was evaluated on a scale from 0 (neutral) to 10 (very high). The rank value was set as the weighted average of the evaluated variables computed as: 5 x overall taste, 2 x aftertaste and 1x other evaluation variable (first general impression, aroma, first impression of flavour, taste, specific flavour, divergent aroma, divergent flavour)**

Despite the lack of significant differences, for minced meat, an indicative preference (significance level = 0.1) regarding the overall first impression and aroma was observed for boar and gilt 2. Additionally, a higher score for the first impression on flavour for the boar and gilt 2 compared gilt 1 and a higher score for overall taste for the boar compared to the gilt samples was observed for minced meat. However, it should be noted that the consumers did not perceive baking odours as evaluation of the minced meat samples occurred in a separate room. Consequently, the perception of unpleasant odours released during cooking due to the presence of boar taint in the minced meat samples were not taken into account but could attribute to the overall depreciation of minced meat [13]. For dry fermented sausage, no indicative preference for either category was observed for all evaluation variables. However, for the 10% tainted boar category, the flavour was scored good more often than very good. Finally, for dry-cured ham, an indicative preference was observed for boar 1 regarding overall score and overall first impression (Table 8). Since no significant differences were observed between the boar and gilt samples for either one of the meat products, these results confirm that minced meat and dry fermented sausage containing up to 10% tainted meat and dry-cured ham containing moderate boar taint compound levels, i.e. AEON up to 962 µg kg^{-1} and SK up to 230 µg kg^{-1} in neck fat, were found to be consumable. Furthermore, these results stipulate that diluting tainted meat and dry curing in combination with smoking can mask the presence of unpleasant odours and flavours for consumption by consumers. This in contrast to evaluation by trained experts, which are considered to be more sensitive than consumers [13].

4. CONCLUSIONS

In this study, it was demonstrated that the rejection thresholds (µg kg^{-1} meat) obtained for the boar taint compounds in meat products are a factor 2 to 4 lower in comparison to the generally accepted threshold levels for neck fat. Evaluation of the consumability of these meat products, containing 10% tainted boar meat (minced meat and dry fermented sausage) in accordance to the maximum observed prevalence of boar taint in individual Belgian farms, indicated that there was no significant difference

between gilt and 10% tainted boar meat containing products for the carcasses selected in these trials. In dry-cured ham, although not diluted, a significant difference between boars and gilts was only observed for the manure odour by the trained expert panel. However, no significant differences were observed by consumers, indicating that preferably moderately tainted carcasses could be used for the production of dry-cured ham. Overall, dilution of tainted boar carcasses up to 10% in minced meat and dry fermented sausage shows potential to valorise tainted carcasses when rearing entire male pigs. Furthermore, the results obtained in this study demonstrate the applicability of the obtained rejection thresholds in minced meat, dry-cured ham and dry fermented sausage and their utility in the processing of tainted boar meat without evoking adverse reactions.

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PART II

DEVELOPMENT OF FAST DETECTION METHODS FOR BOAR TAINT

CHAPTER V

RAPID METHOD FOR THE SIMULTANEOUS DETECTION OF BOAR TAINT COMPOUNDS BY MEANS OF SOLID PHASE MICROEXTRACTION COUPLED TO GAS CHROMATOGRAPHY/MASS SPECTROMETRY

Adapted from:

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ABSTRACT

Because of animal welfare issues, the voluntary ban on surgical castration of male piglets, starting January 2018 was announced in a European Treaty. One viable alternative is the fattening of entire male pigs. However, this can cause negative consumer reactions due to the occurrence of boar taint and possibly lead to severe economic losses in pig husbandry. In this study, headspace solid phase microextraction (HS-SPME) coupled to GC-MS was used in the development and optimization of a candidate method for fast and accurate detection of the boar taint compounds. Remarkably fast extraction (45 s) of the boar taint compounds from adipose tissue was achieved by singeing the fat with a soldering iron while released volatiles were extracted *in-situ* using HS-SPME. The obtained method showed good performance characteristics after validation according to CD 2002/657/EC and ISO/IEC 17025 guidelines. Moreover, cross-validation with an in-house UHPLC-HR-Orbitrap-MS method showed good agreement between an in-laboratory method and the new candidate method for the fast extraction and detection of SK and AEON, which emphasizes the accuracy of this new SPME-GC-MS method. Threshold detection of the boar taint compounds (IND: 100 µg kg^{-1}, SK: 200 µg kg^{-1}, AEON: 500 µg kg^{-1}) on a portable GC-MS could not be achieved. However, despite the lack of sensitivity obtained on the latter instrument, a very fast method with run-to-run time of 3.5 min for the detection of the boar taint compounds was developed.

1. INTRODUCTION

In light of the impending ban on the surgical castration and conversion to rearing immunocastrates or entire male pigs, there is a need for fast detection methods for boar taint at the slaughter line. Indeed, adequate at-line detection of the boar taint compounds makes it possible to identify tainted carcasses and thus prevent negative consumer reactions [1].

A large number of in-laboratory methods for the simultaneous detection of IND, SK and AEON have already been developed [2-7]. These methods require sampling and are characterized by excessive sample pre-treatment followed by relatively long analysis times. Consequently, these methods cannot achieve the high-throughput needed at the slaughter line. Over the past years, research has been devoted to the development of boar taint detection methods applicable at the slaughter line. Sensory methods such as the soldering iron method, directly applicable at-line, are widely practiced and provide a fast and holistic detection of boar taint [8, 9]. However, results provided with these methods are relying on the sensory score of one or more trained assessors [10]. Moreover, other bottlenecks such as habituation, fatigue, and inter-individual variation were recently revealed [8].

The use of other techniques such as parasitic biosensors [11, 12], chemical sensor array technology [13, 14], colorimetric analysis [15], and gas-phase spectrometry [16] show great potential as applications in a slaughterhouse environment. However, sensitivity and specificity obtained with these techniques remain questionable. Moreover, these methods are often poorly validated, lack at- line but more importantly do not meet the industrial necessities with regard to analysis time and automation capability [17].

Recently, Sørensen et al. used surface-enhanced Raman scattering combined with multivariate data analysis for targeted quantification of the boar taint compounds [18]. This method shows great potential for optimization into an on-line application with regard to low equipment cost, acquisition time and portability. However, extensive extraction taking at least 60 min per sample remains necessary, which limits its possibilities for at-line implementation. Moreover, the observed prediction

errors amounted to 87% and 352% around the odour threshold for SK and AEON, respectively, which hampers accurate quantification of the boar taint compounds.

The use of LC or GC combined with MS has proven to be a powerful tool for highly accurate in-laboratory detection of boar taint in adipose tissue [2-4, 6]. More recently, a high-throughput GC-MS protocol with the possibility for automation was developed [19]. This method provides a quantitative result from the first carcass within 24 min followed by results from sequential carcasses every 6 min. Although the method requires sampling of back fat and as a consequence cannot be applied at-line, GC-MS has proven to be a powerful tool for fast and accurate analysis of the boar taint compounds. Use of a portable GC-MS instrument combined with HS-SPME would allow an even higher throughput and would eliminate the current time-consuming sampling procedures. In this study, a HS-SPME extraction protocol was developed and was tested on a person-portable GC-MS for the rapid detection of the three known boar taint compounds in neck fat. Sample extraction was optimized using a D-optimal and central composite face-centred (CCF) design. Afterwards, the method was validated according to the CD 2002/657/EC and ISO/IEC 17025 guidelines [20, 21]. Finally, the fast HS-SPME-GC-MS method was cross-validated with an in-laboratory UHPLC-HR-Orbitrap-MS method and transferred to a portable GC-MS instrument [2].

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

The reference standards IND (2,3-benzopyrrole, CAS 120-72-9), SK (3-methylindole, CAS 83-34-1) and AEON (5α-androst-16-ene-3-one, CAS 18339-16-7) and the internal standards 2-methylindole (2-MID, CAS 95-20-5) and androstadienedione (1,4-androstadiene-3,17-dione, ADD, CAS 897-06-3) were obtained from Sigma Aldrich (St. Louis, MO, USA). For each compound a stock solution was prepared in methanol at a concentration of 1 mg ml^{-1}. Solutions were stored in dark glass bottles at -20 °C. Reagents were of analytical grade and were obtained from VWR International (Merck, Darmstadt, Germany). SPME fibres were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Samples

Boar taint positive carcasses were selected at the slaughter line by means of the soldering iron method optimized by Bekaert et al. and a neck fat sample was taken [8]. All samples were cooled during transport to the lab and were immediately stored upon arrival at -20 °C until analysis.

2.3. Optimization of sample pre-treatment

2.3.1. SPME fibre selection

In this study four different SPME fibres (polydimethylsiloxane (PDMS) 100µm, divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 µm, carboxen/polydimethylsiloxane (CAR/PDMS) 75 µm, polyacrylate (PA) 85 µm) for the extraction of IND, SK and AEON from fat were compared by building equilibration curves for each fibre. In order to imitate adipose tissue as a sample matrix, corn oil was used during SPME fibre selection. To this end, 2 g of corn oil was spiked with IND, SK, and AEON at 1000, 2000, and 10,000 µg kg^{-1}, respectively. Prior to extraction, corn oil solutions were equilibrated at 80 °C for 2 min. Extraction of the boar taint compounds on the SPME fibres was carried out in a headspace volume of 20 ml at 80 °C for 1, 5, 15, 30 and 60 min. Repeatability (n = 3) of each fibre was evaluated at an extraction time of 15 min. All analyses were conducted with an MPS® autosampler with HS-SPME unit.

2.3.2. D-optimal and Central Composite Face-centred (CCF) design

In order to select the best matrix for optimization of the HS-SPME protocol, a comparison between corn oil solutions, blank neck fat of sow carcasses containing little or no background levels of the boar taint compounds, and boar neck fat samples was made. To this end 2 g of corn oil or blank neck fat (minced or not-minced) were fortified at 100, 200, and 500 µg kg^{-1} by addition of 100 µl of a standard solution containing 2, 4 and 10 ng $µl^{-1}$ of IND, SK, and AEON, respectively. After fortification, the samples were left at room temperature for 30 min to allow distribution of the added compounds into the matrix. Boar neck fat with comparable levels of boar taint compounds (IND: 88.4 µg kg^{-1}, SK: 108

µg kg^{-1}; AEON: 588 µg kg^{-1}) was selected and quantified with a validated in-house method [2]. All samples were then subjected to HS-SPME extraction. Extraction occurred in a headspace volume of 20 ml and a DVB/PDMS 65 µm fibre was used. The samples (2 g) were not equilibrated and were extracted for 2 min at 200 °C. Afterwards, the SPME fibre was immediately injected into the GC-MS. All analyses were conducted in triplicate with an MPS® autosampler with HS-SPME unit.

Because of the complex nature of extraction of IND, SK and AEON from adipose tissue due to the relatively low volatility of the compounds, further optimization occurred by using an experimental design. Based on literature, different variables (extraction time, extraction temperature, sample size, headspace volume, desorption time and fibre type) that may significantly influence the extraction were selected, taking into account the relevance of each variable in an at-line environment. All variables were screened in a D-optimal design including 3 centre points and a total of 19 runs. The obtained areas of the chromatographic peaks for IND, SK, and AEON were used to generate response surface plots. During execution of the D-optimal design, extraction of the samples was carried out in a confined environment using headspace vials. The samples were not equilibrated prior to extraction and were heated using an oven tray, while exposed to the SPME fibre, in order to achieve the required extraction temperature. Afterwards, the SPME fibre was immediately injected into the GC-MS for analysis. Ranges of all parameters were adjusted according to the settings of the experimental design and handling of the samples and SPME fibre occurred manually.

The variables extraction temperature (100 °C to 400 °C), sample size (0.5 g to 2 g), and desorption time (30 to 150 s) were subjected to further optimization using a central composite face-centred design (CCF), in which main effects and two-way interaction terms were investigated. The experimental design consisted of 17 runs including 3 centre points. During execution of the CCF design, the extraction time was set at 45 s and the DVB/PDMS 65 µm fibre was used for extraction of the boar taint compounds. Although SPME extraction usually occurs in a confined environment, e.g. use of headspace vials and oven tray, such an environment is difficult to simulate at the slaughter line, as

sampling is avoided in order to keep analysis times as short as possible. For this reason, use of an oven tray was replaced by use of a soldering iron (RDS 80, Kurtz Ersa, Wertheim, Germany) to heat the samples and obtain an even higher extraction temperature, further facilitating release of the boar taint compounds from their matrix. Extraction occurred by singeing the samples for 45 s at the required temperature with the soldering iron. The SPME fibre was placed directly above and in close proximity (1 cm) of the soldering iron to allow mass transfer of the boar taint compounds. Extraction was carried out manually under a fume hood to ensure a constant airflow. Other parameters were adjusted according to the settings of the experimental design. After optimization, the optimal settings (extraction time: 45 s, extraction temperature: 400 °C, sample size: 1.5 g, desorption time: 100 s) of the extraction protocol were applied for all further experiments (validation and cross-validation). Thereby extraction occurred using the soldering iron as described for the CCF statistical design.

Both D-optimal and CCF designs were conducted on a benchtop HP 6890 GC HP 5973 quadrupole MS. To evaluate the quality of the statistical models, a one-way ANOVA test was performed. All analyses were conducted in Modde 5.0 software (Umetrics, Umea, Sweden) and a p-value below 0.05 was considered as significant.

2.4. Instrumentation

2.4.1. Benchtop GC-MS

Development and validation of the GC-MS method initially occurred on a Hewlett Packard 6890 GC coupled to a Hewlett Packard 5973 quadrupole MS (Agilent Technologies, Palo Alto, CA) equipped with an MPS® autosampler with HS-SPME unit (Gerstel, Mülheim an der Ruhr, Germany). Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. Splitless injection was applied and the injector and transfer line temperatures were kept at 250 °C and 280 °C, respectively. Chromatographic separation was carried out on a zebron-semivolatiles column (30 m x 0.25 I.D. x 0.25 film thickness) with 5%-phenyl-arylene phase (Phenomenex, Utrecht, Belgium) and by the following oven temperature program: 80 °C (2 min) to 180 °C at 40 °C min^{-1}, to 220 °C at 10 °C min^{-1}, to 280 °C at 40 °C min^{-1} and held for 2 min.

Mass spectra were recorded in selected ion monitoring (SIM) modus (IND: m/z 90, m/z 117; SK: m/z 103, m/z 130; AEON: m/z 257, m/z 272) with a solvent delay of 3 min and ionizing electron energy of 70 eV. Data analysis was carried out with MSD Chemstation Data Analysis Application software (Agilent Technologies, Palo Alto, CA).

2.4.2. Portable GC-MS

After development and validation, the HS-SPME-GC-MS method was transferred to the portable Tridion™-9GC-TMS instrument (Torion Technologies, PerkinElmer, American Fork, UT) employable at the slaughter line. Helium was used as carrier gas with an inlet pressure of 25 psi. Split injection was applied with a split ratio of 10 and the injector and transfer line temperatures were kept at 270 °C and 250 °C, respectively. Chromatographic separation was carried out on an MXT-5 column (5 m x 0.1 mm I.D. x 0.4 µm film thickness) with a diphenyl dimethylpolysiloxane stationary phase (Restek Corporation, Bellefonte, PA) and by the following temperature program: 50 °C (10 s) to 270 °C at 2 °C s^{-1} and held for 60 s. Mass spectra were recorded in full scan modus (m/z 45 – 500) at 10 full scans s^{-1} and the resolution was set at 1 amu FWHM over the complete scan range. Data analysis was carried out with CHROMION software (Torion Technologies, PerkinElmer, American Fork, UT).

2.4.3. UHPLC-HR-Orbitrap-MS

Cross-validation occurred by analysing boar samples on the newly developed HS-SPME-GC-MS method and an in-house UHPLC-HR-Orbitrap-MS method. An overview of the method characteristics for the latter can be consulted in Bekaert et al. [2].

2.5. Validation

The optimized method was validated according to the criteria of the European Commission (CD 2002/657/EC) by evaluating specificity, selectivity, linearity, trueness, precision, and ruggedness [20]. Since the levels of boar taint compounds are not restricted by maximum residue limits, the limits of

detection and quantification were determined according to the general requirements for testing and calibration, ISO/IEC 17025 [21].

In order to check the specificity and selectivity of the method, blank as well as samples fortified with the analytes of interest at a concentration of 100, 200 and 500 µg kg^{-1} of IND, SK and AEON, respectively were analysed. Interferences on the retention times for the boar taint compounds were checked and boar taint compounds were identified based on their retention time and ion ratios (S/N ≥ 3), also their ion ratio tolerance was taken into account. For this purpose, the expected ion ratios of the boar taint compounds were determined in standard solutions (n = 6) fortified with 100, 200 and 500 µg l^{-1} of IND, SK and AEON, respectively. Afterwards, the observed ion ratios were determined in blank samples (n = 18) fortified at 100, 200 and 500 µg kg^{-1} for IND, SK and AEON, respectively. Quantification was based on 9-point matrix matched calibration curves with following concentration levels: 0, 25, 50, 100, 200, 500, 1000, 2000 and 5000 µg kg^{-1}. Because of the wide linear range of the calibration curves, the low calibration levels only have a minor influence on the linear regression model. This was counteracted through performing weighted least squares linear regression (WLSLR), by giving equal weight to all calibration levels. Different weighting factors ($1/x^{power}$) were evaluated and the power of the range was varied between -2 and 2 in steps of 0.5. For each compound, the factor leading to the lowest sum of relative errors was used as a weighting factor. To assess linearity, a univariate linear regression model was built (SPSS version 22.0, IBM, USA), in which the calibration concentrations were set as independent and the areas as dependent variables. Furthermore, the lack of fit was determined through an F-test. As no certified reference material was available for boar neck fat, trueness was assessed as recovery by fortifying blank samples containing no traces of the analytes of interest. To evaluate the precision of the method, repeatability and within-laboratory reproducibility were determined. Both validation parameters were evaluated by calculating the relative standard deviations (RSD, %). For assessing repeatability, three series of six replicates fortified at three different levels were analysed. This was carried out by the same operator under repeatable conditions. A similar set-up (four series of six replicates at three concentration levels) was elaborated to evaluate the within-laboratory

reproducibility. However, the series' replicates were now analysed by different lab technicians on different days, whereby environmental conditions consequently altered. Finally, the limits of detection and quantification (LOD and LOQ) were determined as the concentrations that generate chromatographic peaks with a signal-to-noise ratio of 3:1 and 10:1 respectively, according to ISO/IEC 17025 and were experimentally confirmed. Ruggedness of the method was evaluated by altering different experimental and sample pre-treatment conditions that could be subject to fluctuations (soldering iron, SPME fibre, environmental temperature, operator) [20]. Moreover, analyses were carried out on different days to account for any day-to-day variations for which was not anticipated but may occur.

2.6. Analysis of boar samples: cross-validation

Fifty boar taint positive carcasses were selected at the slaughter line by means of the soldering iron method as optimized by Bekaert et al. and neck fat samples were taken [8]. Each sample was analysed with a validated in-house UHPLC-HR-MS method to confirm the presence of IND, SK and/or AEON [2]. Afterwards, the neck fat samples were analysed with the newly developed HS-SPME-GC-MS method.

None of both methods serves as a reference method for the detection of boar taint in adipose tissue, as does the method of Buttinger et al. [22]. For this reason, neither the in-house UHPLC-HR-MS and HS-SPME-GC-MS method provides unequivocally correct measurements, whereby their degree of agreement was assessed. To this end the Pearson correlation between the two methods was calculated as well as the limits of agreement, assessed as the bias estimated by the mean difference and the standard deviation of the differences between the two methods.

2.7. Statistical analysis

To evaluate the influence of the matrix on the amount of extracted boar taint compounds, a one-way analysis of variance (ANOVA) test was performed with matrix type as a fixed factor and recovery of the boar taint compounds as the dependent variable. Normality of the standardized residuals was checked

through QQ-plots and the Shapiro-Wilk test. Homogeneity of variance was evaluated through the Levene's test and standardized residuals vs. predicted plot. Post hoc analysis (Bonferroni test) was executed for pairwise comparisons.

Cross-validation of the HS-SPME-GC-MS method with the UHPLC-HR-Orbitrap-MS method was carried out by calculating correlation coefficients and the limits of agreement. To this end a simple linear regression model was built with the concentration levels detected by the UHPLC-HR-MS method as the independent variable and by the HS-SPME-GC-MS method as dependent variable. It was assumed that there was a linear relationship between the dependent and independent variables, also independence of the error term was assumed. Normal distribution of the error term was verified through QQ-plots and the Shapiro-Wilk test. Homoscedasticity of the error term was evaluated by plotting the standardized residuals versus the predictor variable. The limits of agreement were evaluated by the Bland-Altman plot and set as the bias estimated by the mean difference (d) and the standard deviation (s) of the differences between the two methods. The limits of agreement were then calculated as d ± 2s. The precision of these estimates was expressed by the standard error and 95% confidence interval around the estimates. The significance level for all analyses was set at 0.05, only for the Levene's test a significance level of 0.01 was used. All analyses were conducted in SPSS version 22.

3. RESULTS AND DISCUSSION

3.1. Optimization of sample extraction

3.1.1. SPME fibre selection

To select the best SPME fibre type for extraction of the boar taint compounds from corn oil, equilibrium curves and repeatability for 4 types of SPME fibres were compared (Fig 1). When applying the PA 85 µm fibre, the boar taint compounds could not be detected. This could be explained by the very low affinity of the boar taint compounds towards the polar polyacrylate coating. Use of the PDMS 100 µm

fibre type resulted in very low chromatographic peak areas for IND, SK, and AEON and as a consequence, the latter fibre would probably be inadequate for threshold extraction of the boar taint compounds from neck fat at the slaughter line. The low sensitivity obtained with the PDMS fibre can potentially be attributed to a competition effect between the apolar PDMS coating and the fatty matrix. Indeed, SPME is exhaustive but is based on diffusion of the analytes from the sample matrix and equilibration between two or multiple phases. The semi-volatile boar taint compounds accumulate in adipose tissue and are not easily released from this fatty matrix, possibly explaining the low sensitivity obtained with the PDMS fibre [23]. The CAR/PDMS 75 µm fibre proved to have the highest sensitivity towards IND and SK after an extraction time of 30 min. However, equilibrium was reached faster with the DVB/CAR/PDMS 50/30 µm fibre for all boar taint compounds, resulting in a better extraction efficiency at short extraction times compared to the CAR/PDMS 75 µm fibre. Generally, thicker coatings have a higher extraction capacity, resulting in a higher sensitivity. But extraction occurs slower when compared to thinner coatings, resulting in a higher sensitivity of the DVB/CAR/PDMS 50/30 µm fibre at short extraction times [23]. Moreover, better repeatability was obtained with the DVB/CAR/PDMS 50/30 µm fibre at an extraction time of 15 min (RSD, %: IND = 2.23; SK = 4.28; AEON = 16.67) in comparison to the CAR/PDMS 75 µm fibre (RSD, %: IND = 23.37; SK = 26.77; AEON = 39.08), most likely due to the larger experimental error when working in pre-equilibrium circumstances.

Because of the higher sensitivity of the DVB/CAR/PDMS 50/30 µm fibre at short extraction times and better repeatability, use of this fibre for rapid determination of the boar taint compounds was preferred. It appeared that the higher sensitivity at short extraction times was mainly due to the bipolar divinylbenzene phase in the coating of the DVB/CAR/PDMS 50/30 µm fibre. For this reason, it was decided to include both DVB/CAR/PDMS 50/30 µm and DVB/PDMS 65 µm fibres in the experimental design for optimization.

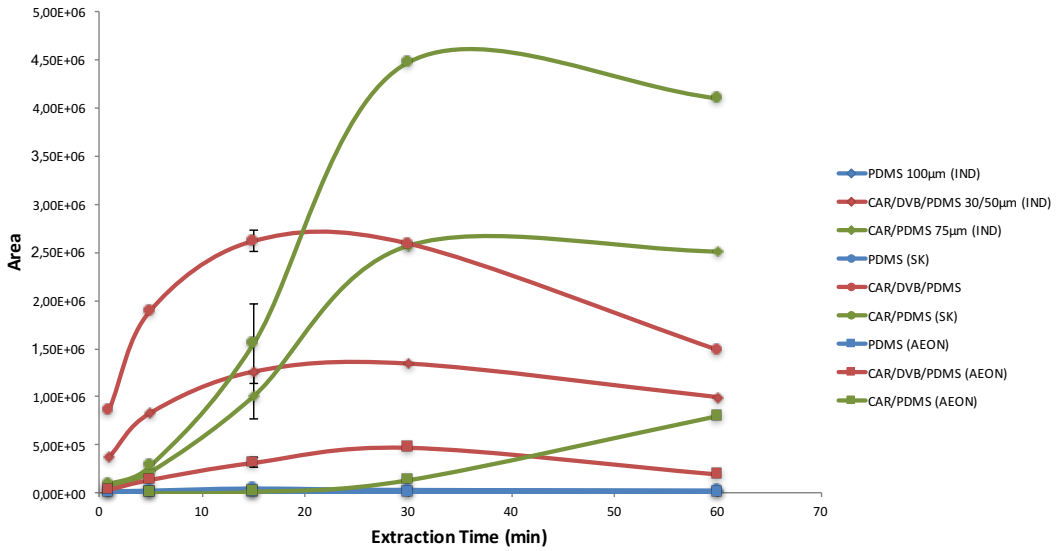

Fig 1 Equilibrium curves for the boar taint compounds in corn oil using different SPME fibres.

3.1.2. D-Optimal and Central Composite Face-Centred (CCF) design

Since no certified reference material for boar fat is available, careful consideration was given to the choice of matrix during development and optimization of methods. For this reason, a comparison of fortified corn oil, fortified blank fat (minced or non-minced), and boar fat for HS-SPME extraction was made. One-way ANOVA analysis indicated a significant influence of the matrix type on the recovery for all boar taint compounds ($p < 0.05$). Multiple comparison analysis (Bonferroni) of the matrix type showed that minced blank fat best resembled boar fat since a significant difference was observed only for the recovery of SK ($p < 0.001$). For corn oil, significant differences with boar fat were observed for both IND ($p = 0.010$) and SK ($p = < 0.001$), whereby the extraction yield from corn oil was significantly higher. Between non-minced blank fat and boar fat, significant differences were observed for all boar taint compounds (IND: $p = 0.002$, SK: $p < 0.001$, AEON: $p = 0.003$), whereby non-minced blank fat was associated with a significantly lower extraction yield. Between minced and non-minced blank fat, no significant differences were observed for IND ($p > 0.05$); however, a significantly higher extraction efficiency, more comparable to boar fat, was observed for SK ($p < 0.001$) and AEON ($p = 0.004$) in minced blank fat in comparison to non-minced blank fat. Although significant differences were observed between boar fat and minced blank fat, the latter matrix was selected for all further

experiments. To allow sufficient distribution of the boar taint compounds within the blank matrix, the samples were rested for 30 min prior to extraction.

Extraction time, soldering iron temperature, sample size, headspace volume, desorption time and fibre type were selected as variables for the HS-SPME extraction protocol in a D-optimal design (Table 1). Soldering iron temperature was identified as the main significant factor for all boar taint compounds, with p-values ranging from 0.007, 0.036, and 0.010 for IND, SK, and AEON, respectively. Desorption time did not prove a significant factor for SK ($p = 0.620$). For AEON on the other hand this factor was identified as a positive significant factor ($p = 0.048$). This result was to be expected since a longer desorption time results in a higher release of analytes from the fibre and thus in higher peak areas. However, for IND, although not significant ($p = 0.060$), a higher desorption time resulted in lower peak areas. This was most likely due to a limited carry-over effect, which was not observed for the other boar taint compounds. Indeed, all samples in the D-optimal design were analysed in randomized order, in which case the desorption time was not always sufficiently high to completely desorb IND from the fibre. Sample size was not identified as a significant factor for SK ($p = 0.428$). However, for IND ($p = 0.072$) and AEON ($p = 0.070$) it was almost significant and therefore included in the CCF design for further optimization. Extraction time and SPME fibre type were not identified as significant factors to include for further optimization. However, use of the DVB/PDMS 65 µm fibre resulted in a better chromatographic peak shape and less matrix interferences. As a result, this fibre type was preferred for extraction of IND, SK and AEON from a fatty matrix.

In the CCF experimental design, 3 factors influencing the extraction (soldering iron temperature, sample size and desorption time) were included for further optimization (Table 1). Best results (Fig 2) for IND were obtained at a soldering iron temperature of 400 °C, sample size of 1.5 g and desorption time of 120 s. For SK and AEON, the best results were obtained with a soldering iron temperature of 400 °C, sample size of 1.5 g and desorption time of 100 s. It should be noted that only the soldering iron temperature proved to be significant for the recovery of the boar taint compounds (IND: $p < 0.001$,

SK: p = 0.014, AEON: p = 0.014). No significant carry-over effect was observed for the boar taint compounds at a desorption time of 100 or 120 s. Also for sample size, no significant difference was observed within the range of the experimental design. Since the soldering iron has been widely applied for the sensory analysis of boar taint whereby it sufficiently releases the boar taint compounds, it was also applied as a heating device for HS-SPME extraction of the boar taint compounds [8, 9]. Use of the soldering iron resulted in very fast extraction of the boar taint compounds (45 s) and optimal extraction was achieved at a soldering iron temperature of 400 °C for all boar taint compounds, desorption time of 100 s and sample size of 1.5 g.

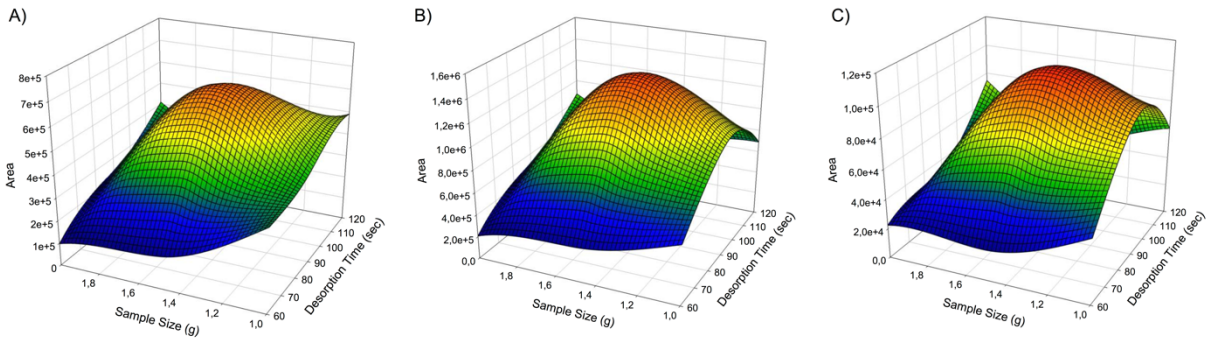

Fig 2 Response surface plots for A) indole, B) skatole and C) androstenone obtained by executing a central composite face-centred design at an extraction temperature of 400 °C.

Table 1 Overview of the coefficients and p-values of the boar taint compounds in relation to the variables obtained with a D-optimal and Central Composite Face-Centred (CCF) design.

	Factor	Type	Settings	Indole		Skatole		Androstenone	
				Coeff. SC	p	Coeff. SC	p	Coeff. SC	p
D-Optimal design	Extraction time (s)	Quantitative	30-60	6561	0.657	-24224	0.454	-1504	0.740
	Soldering iron temperature (°C)	Quantitative	100 - 200	46450	0.007	74085	0.036	13602	0.010
	Sample size (g)	Quantitative	1-2	28467	0.072	25697	0.428	8827	0.070
	Headspace Volume (ml)	Multilevel	15,20	18000	0.240	-19452	0.550	2416	0.599
	Desorption time (s)	Quantitative	60-120	-29967	0.060	-15927	0.620	9770	0.048
	Fibre type	Qualitative	PDMS/DVB	11871	0.431	57051	0.096	-4350	0.350
			CAR/DVB/PDMS	-11871	0.431	-57051	0.096	4350	0.350
CCF design	Soldering iron temperature (°C)	Quantitative	200-400	372688	< 0.001	296224	0.014	-16450	0.014
	Sample size (g)	Quantitative	1-2	-669027	0.140	-835954	0.507	-33195	0.702
	Desorption time (s)	Quantitative	60-120	870204	0.063	1740690	0.181	139347	0.127
	Soldering iron temperature*Soldering iron temperature			-233317	0.819	-1039230	0.726	-46689	0.820
	Sample size*Sample size			17541	0.986	-486722	0.869	13701	0.947
	Desorption time*Desorption time			-409131	0.689	-1378260	0.642	-81789	0.690
	Soldering iron temperature*Sample size			-25237	0.948	-345016	0.752	26041	0.730
	Soldering iron temperature*Desorption time			297393	0.449	129086	0.908	-26220	0.736
	Sample size*Desorption time			361219	0.361	782753	0.490	66021	0.403

3.2. Validation

Analysis of boar samples without use of internal standards resulted in high RSD values (35 to 50%). This finding is to be expected since extraction of the boar taint compounds occurred before reaching equilibrium [24]. To normalize for any given variation, it was decided to use internal standards for the analysis of boar samples.

3.2.1. Specificity and selectivity

Specificity and selectivity were evaluated by analysing blank samples as well as fortified blanks. When fortifying blank samples, a significant increase in peak area intensity of the chromatographic peaks at their specific retention times could be observed, taking into account a signal-to-noise ratio of at least 3. At the specific retention times of the compounds, no other interfering substances could be found, indicating good specificity of the method (Fig 3). Since mass spectra were recorded in SIM modus on the benchtop GC-MS, also high selectivity was obtained. Furthermore, the presence of the boar taint compounds was confirmed by their expected ion ratios and maximum tolerance window. The observed ion ratios lied within the maximum permitted tolerance (15% for IND and 10% for SK and AEON) for all boar taint compounds, confirming good specificity and selectivity of the method (Table 2).

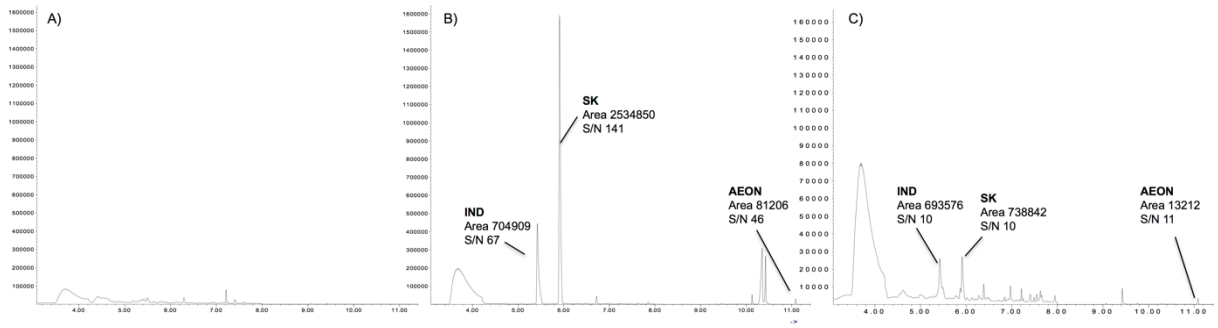

Fig 3 Selected ion chromatograms of the boar taint compounds obtained on a benchtop GC-MS in A) blank adipose tissue, B) blank adipose tissue fortified with the boar taint compounds at their odour threshold values 100, 200 and 500 µg kg^{-1} for indole (IND), skatole (SK) and androstenone (AEON), respectively, and C) blank adipose tissue fortified at LOQ levels (25, 30 and 100 µg kg^{-1} for IND, SK and AEON, respectively).

3.2.2. Linearity

Linearity was evaluated by fitting 9-point matrix matched calibration curves ranging from 0 to 5000 µg kg^{-1} through weighted least squares linear regression (WLSLR). Because WLSLR gives equal weight to all calibration levels, a more accurate estimation of the low concentration levels could be made. Optimal weighting factors of 2 for IND and 0.5 for SK and AEON were determined. The obtained regression models showed very good linearity ($R^2 \geq 0.99$) (Table 2) and no lack of fit (95% confidence interval; F-test, $p > 0.05$). The determination coefficients were comparable to those obtained by other analytical methods for the simultaneous detection of IND, SK and AEON; however, a larger linear range (0 to 5000 µg kg^{-1}) was obtained for the indolic compounds with our newly developed HS-SPME-GC-MS method [2-4, 6, 19].

3.2.3. Trueness and precision

Trueness was evaluated through recovery and precision of the method was assessed through repeatability and within-laboratory reproducibility. The recoveries met the permitted levels (-20% to +10%) (Table 2) and were comparable with previously reported accuracy measurements [2, 4]. However, the recoveries obtained for IND at level 1 and 2, for SK at level 2 and for AEON at level 1 and 3 were relatively high compared to Fischer et al. [3]. In practice, higher recovery values will lead to overestimation of the boar taint compound levels in pig carcasses and thus to more false positive results compared to false negatives. However, false positive results will not result in negative consumer reactions, this in contrast to false negative results. Consequently, false positives will have a less important impact on consumer acceptance than false negatives. Moreover, recovery can be considered as the systematic error or bias of the method. As this systematic error is known, it can be compensated and corrected for. The RSD values calculated for the repeatability (RSD < 14.6%) were below 15%, indicating satisfactory repeatability according to the criteria of the European Commission. For the within-laboratory reproducibility, RSD values (RSD < 19%) (Table 2) were below the performance limits as calculated by the Horwitz equation, indicating satisfactory precision of the HS-

SPME-GC-MS method. Better precision was obtained with previous reported in-laboratory methods for the detection of the boar taint compounds, which could be attributed to the short extraction time applied in this study [2-4, 19]. Extraction occurred for 45 s, thus before reaching partition equilibrium in which case quantification greatly depends on the extraction parameters whereby any variation in the latter can result in a higher experimental error and thus lower precision [25]. Another possible explanation for the higher RSD (%) values could be attributed to the variations in proportions of fat, connective tissue and water in pig adipose tissue, which may affect the sample matrix gas phase partitioning of the boar taint compounds and lead to variations in extraction [17, 26]. This was not the case for other analytical methods as extraction mostly occurred by melting the fat followed by liquid-liquid extraction with methanol [2, 3]. Nevertheless, the newly developed HS-SPME-GC-MS method still delivers reliable results according to the requirements of the European Commission for analytical methods for matrices of animal origin (CD 2002/657/EC) [20]. Moreover, better precision was obtained with our fast HS-SPME extraction method when compared to fast analysis of the boar taint compounds in adipose tissue by means of surface-enhanced Raman scattering (SERS) [18].

3.2.4. Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were theoretically calculated and were then experimentally confirmed (Table 2) based on the outcome of blank samples fortified at the levels calculated for LOD and LOQ (Fig 3). The LOD and LOQ values for the indolic compounds were higher than those reported by Fischer et al. [3] but were still below the odour thresholds for IND, SK and AEON in adipose tissue. The higher LOD and LOQ values can be explained by the limited extraction time under which the SPME extraction occurred. LOD and LOQ values were higher than compared to other analytical methods for the boar taint compounds, which is most likely due to the short extraction time applied in this study [2-4, 19].

3.2.5. Ruggedness

Ruggedness of the method was investigated by altering experimental conditions such as use of different soldering irons, SPME fibres, operators, environmental temperatures and other day-to-day variations. The different factors were modified in order of their relative importance but did not significantly influence the repeatability and within-laboratory reproducibility of the method (RSD < 15% and 20%).

Table 2 Summary of the method validation performance characteristics.

Analyte	**Nominal Concentration** ($\mu g\ kg^{-1}$)	**Specificity and Selectivity** Ion Ratio				**Recovery** Mean ± SD (n = 18)	**LOD** ($\mu g\ kg^{-1}$)	**LOQ** ($\mu g\ kg^{-1}$)	**Precision**		**Linearity** R^2
		Expected	RSD (%) n = 6	Observed	RSD (%) n = 18				Repeatability RSD (%) n = 18	Within-laboratory Reproducibility RSD (%) n = 24	
Indole	50					105 ± 12			14.0	19.3	
	100	0.24	5.1	0.25	3.6	108 ± 7	10	25	10.4	12.7	0.994
	200					94 ± 12			13.3	14.7	
Skatole	100					98 ± 16			14.6	18.1	
	200	0.73	2.4	0.73	2.8	107 ± 13	10	30	10.1	15.1	0.991
	400					102 ± 12			12.1	12.9	
Androstenone	250					109 ± 17			14.4	19.0	
	500	0.86	4.8	0.84	3.3	90 ± 9	25	100	14.4	17.3	0.990
	1000					109 ± 11			13.9	17.3	

3.3. Portable GC-MS

After validation of the HS-SPME-GC-MS method on a benchtop instrument, it was transferred to a portable GC-MS instrument. This portable instrument is employable at the slaughter line and would make at-line screening of boar-tainted carcasses possible. A very fast method with run-to-run time of 3.5 min was developed, which allows high-throughput of samples providing a first result after 4 min 15 s, followed by results every 3.5 min. However, analysis of fortified blank samples showed that sensitivity of the portable GC-MS instrument was insufficient for the detection of the boar taint compounds at threshold levels using the current extraction protocol. IND and SK could be detected (S/N > 3) in adipose tissue at fortification levels of 1000 and 2000 µg kg^{-1}, respectively. AEON could not be detected, even at a fortification level of 10 000 µg kg^{-1} (Fig 4). This lack of sensitivity was most likely due to the use of a narrow bore column on the portable instrument on the one hand and the inability of the instrument for selected ion monitoring (SIM) on the other. Indeed, although use of a narrow bore column technically allows for splitless injections. However, due to its small internal diameter (0.1 µm) compared to the 0.25 µm I.D. of the GC column used on the benchtop instrument, the narrow bore column is easily overloaded and consequently has a lower sample capacity, which reflects in a lower sensitivity [27]. Second, the portable GC-MS instrument is not capable of running in SIM mode, whereby all analyses are performed in full scan. Consequently, matrix effects of adipose tissue cannot be avoided and ion suppression effects become predominant, which also reflected in loss of sensitivity in comparison to a benchtop GC-MS instrument.

Approaches to increase the sensitivity on the portable GC-MS instrument include derivatisation or purge-and-trap [28, 29]. However, given the practical limitations that are encountered at the slaughter line such as the requested high-throughput of carcasses and food safety regulations, the latter alternatives overreach the goal of fast detection of boar taint at the slaughter line and for this reason were not further pursued.

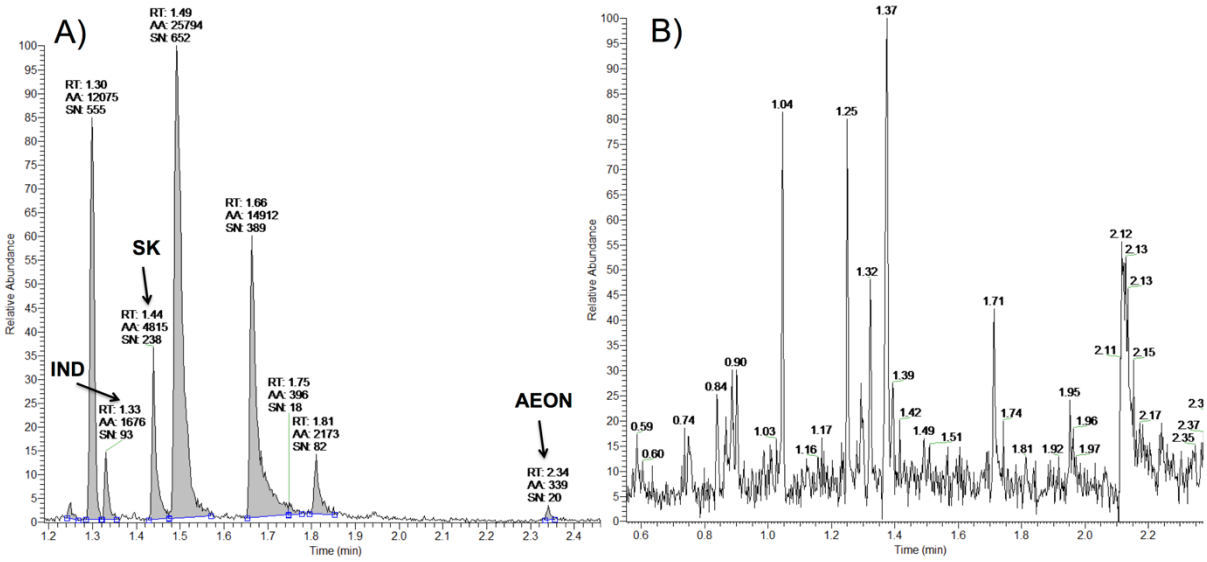

Fig 4 Chromatograms (full scan) of the boar taint compounds obtained on the portable Tridion™-9GC-TMS instrument in A) a standard solution with 100, 200 and 500 µg kg^{-1} of IND, SK and AEON, respectively and B) blank adipose tissue fortified at 100, 200 and 500 µg kg^{-1} of IND, SK and AEON, respectively.

3.4. Cross-validation: analysis of boar samples

In a pilot study, neck samples of 50 boars that tested positive for boar taint were sampled. Afterwards, the presence of the boar taint compounds was confirmed by an in-house UHPLC-HR-MS method prior to analysis by the newly developed HS-SPME-GC-MS method [2]. The two methods were cross-validated by calculating correlations and assessing their degree of agreement.

The very high determination coefficients (Fig 5) obtained between the results obtained by UHPLC-HR-MS and HS-SPME-GC-MS analysis give a first indication of the equality of both methods for the detection of boar taint in neck fat. However, data that seem to be in poor agreement can still produce high correlations since perfect correlation is obtained if the data points lie along any straight line but perfect agreement is only obtained if the data points lie along the line of equality [30]. Moreover, all the data points are clustered near the regression line whereby it is difficult to assess between-method differences. For this reason, also the limits of agreement (Table 3) between the two methods were calculated and the differences between methods against the mean was displayed in a Bland-Altman plot (Fig 5) to investigate any possible relationship between the measurement error and the true value.

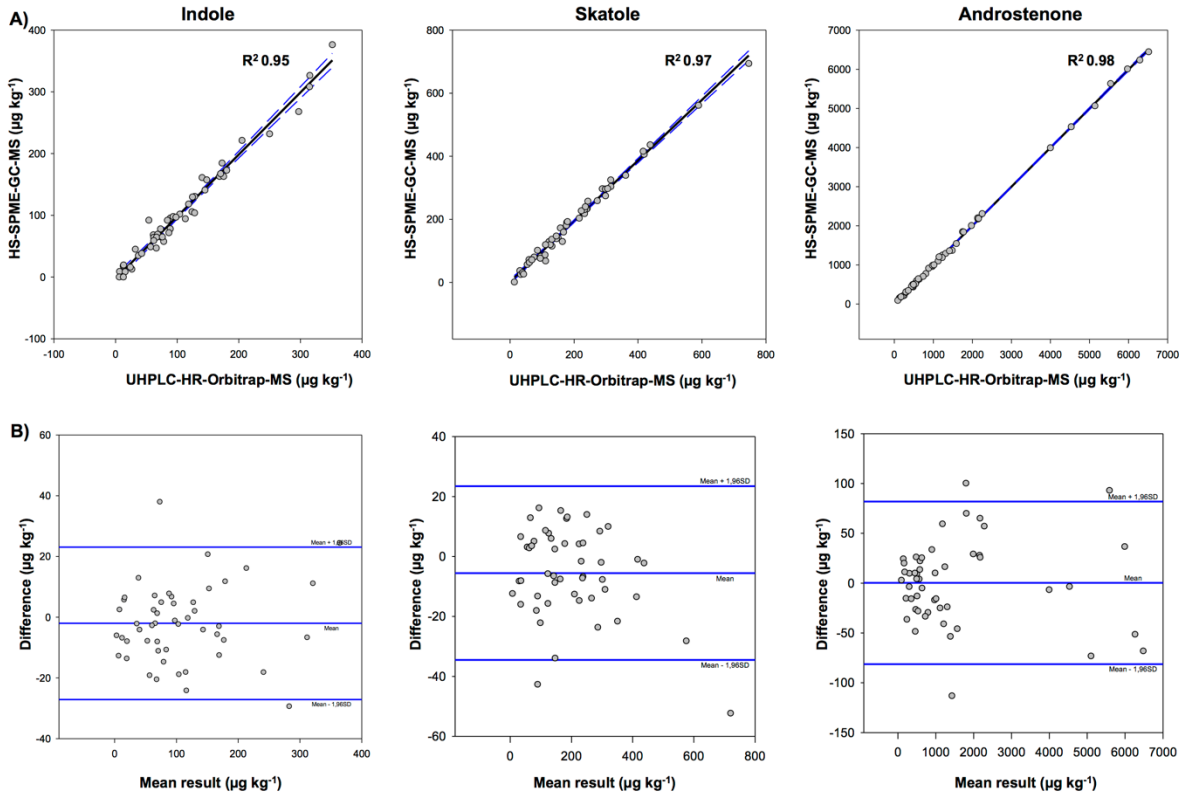

Fig 5 A) Scatter plots of the boar taint compound levels obtained by UHPLC-HR-Orbitrap-MS analysis versus HS-SPME-GC-MS analysis, B) Bland-Altman plots to identify differences between the results obtained by the UHPLC-HR-Orbitrap-MS method and HS-SPME-GC-MS method versus the mean result obtained by the two methods.

The HS-SPME-GC-MS method gave results that were 27 µg kg^{-1}, 35 µg kg^{-1} or 81 µg kg^{-1} below or 23 µg kg^{-1}, 23 µg kg^{-1} or 82 µg kg^{-1} above those obtained by UHPLC-HR-MS analysis, for IND, SK and AEON, respectively. This results in a percentage deviation of the HS-SPME-GC-MS method of 27, 17 and 16% below or 23, 12 and 16% above the odour threshold values of IND, SK and AEON, respectively, in comparison with the UHPLC-HR-MS method. This indicates that with the exception of IND, comparable results were obtained with the fast HS-SPME-GC-MS method and the in-laboratory UHPLC-HR-MS method. Although a very high determination coefficient (R^2 > 0.95) for IND was observed between results obtained with the two methods, a relatively high percentage deviation was observed. However, despite the poor agreement obtained for IND, highly accurate results for the quantification of SK and AEON, the two most important compounds in the contribution to boar taint, in adipose tissue were obtained by fast HS-SPME-GC-MS analysis. Moreover, since our HS-SPME-GC-MS method gives results

comparable with an in-laboratory UHPLC-HR-MS method for the latter compounds, it is a promising tool for highly accurate classification of tainted carcasses as light, moderate or severely tainted, which is important for further processing of the tainted carcasses and preventing negative consumer reactions [31, 32].

Table 3 Summary of the cross-validation parameters for the UHPLC-HR-Orbitrap-MS and HS-SPME-GC-MS methods for adipose tissue.

Compound	Determination coefficient (R^2)	Limits of agreement				Standard error	95% Confidence Interval
		Lower limit	RSD (%)	Upper limit	RSD (%)		
IND	0.95	27	27	23	23	2.02	-5.66 , 1.62
SK	0.97	35	17	23	12	-5.55	-9.75 , -1.35
AEON	0.98	81	16	82	16	0.32	-11.50 , 12.15

4. CONCLUSIONS

In this study, a candidate HS-SPME-GC-MS method for fast and accurate at-line detection of the boar taint compounds was developed and validated. Very fast extraction (45 s) of the boar taint compounds from adipose tissue was achieved by singeing the fat with a soldering iron in combination with SPME. Validation of the HS-SPME-GC-MS method according to the CD 2002/657/EC and ISO/IEC 17025 guidelines proved the quality of the method for the quantification of the boar taint compounds in neck fat. Moreover, analysis of boar samples with our new method raised quantitative results comparable to a validated in-laboratory UHPLC-HR-Orbitrap-MS method, which emphasized the applicability of the HS-SPME-GC-MS method. Furthermore, despite the lack of sensitivity obtained on the portable GC-MS instrument, our fast and accurate HS-SPME extraction protocol is an important step towards achieving high-throughput combined with highly accurate detection of boar taint at the slaughter line.

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CHAPTER VI

MOLECULARLY IMPRINTED POLYMER ARRAY FOR THE DETECTION OF BOAR TAINT COMPOUNDS, WITH SPECIAL EMPHASIS ON SKATOLE AND INDOLE

Adapted from:

Verplanken K., De Middeleer G., Dubruel P., De Saeger S., Wauters J., Vanhaecke L., *In preparation*.

ABSTRACT

Due to the intended ban on the surgical castration of pigs, the possible presence of boar taint, a contemporary off-odour, in pig meat urges the pig industry to take precautionary measures. For this reason, rapid screening methods are required, in which molecularly imprinted polymers (MIPs) can serve as recognition elements. Due to difficulties on developing MIPs against AEON (data not included), this study focussed on SK as a target compound. Different MIPs for SK were developed following a non-covalent precipitation polymerization approach and were characterized through batch-rebinding studies, scanning electron microscopy and dynamic light scattering. All MIPs represented very good recovery (> 87%) in buffer solutions with a pH ranging from 3 to 10, this in contrast to ethanol as a rebinding medium (0-11.4%). Binding isotherms and Scatchard analysis showed low specificity for the MIPs, indicating a lack of imprinting effect. Consequently, the MIPs showed a high degree of cross-reactivity towards structure analogues. However, selectivity significantly increased by combining different MIPs in an array and applying a fingerprinting approach (classification accuracy 87.5%). Finally, application of this array on boar neck fat samples (classification accuracy 82.7%) demonstrated its potential for implementation in high-throughput screening assays for boar taint.

1. INTRODUCTION

Increasing awareness on animal welfare issues led to an intended ban on the surgical castration of piglets by January 2018 [1]. Apart from immunocastration, another viable alternative for the surgical castration is the fattening of entire male pigs. However, since surgical castration was primarily intended to prevent boar taint, an off-odour causing negative consumer reactions, rearing entire males would imply the re-occurrence of boar taint in 4 to 25% of entire male carcasses [2, 3]. Since boar taint causes negative consumer reactions, it is crucial to avoid economic losses by preventing tainted pig carcasses to reach consumers. For this reason, fast and accurate screening strategies for boar taint are in order [4].

At present, screening for tainted carcasses at the slaughter line mostly happens by sensory analysis such as the human nose method. However, as these methods show important disadvantages such as inter-individual and temporal variation, alternative strategies are required [5]. Several candidate techniques including instrumental and sensor-based methods have been proposed for the fast detection of boar taint [6-15]. However, these methods often remain poorly validated or do not meet the required performance characteristics needed for implementation at the slaughter line [16]. Within sensor-based technology, the use of electronic noses, thickness shear mode resonators and parasitoid biosensors have been evaluated for screening boar carcasses. However, an important disadvantage of these sensors is their lack of specificity [6-9, 14]. One of the possibilities to increase specificity is the application of molecularly imprinted polymers (MIPs).

Molecular imprinting is a state-of-the-art technique for the development of synthetic recognition elements for a wide variety of compounds. Since the use of antibodies suffers from some major drawbacks such as poor stability at increased temperatures and pH, MIPs, have gained increased interest because of their superior stability, robustness and higher resistance to extreme environmental and chemical conditions [17, 18]. Moreover, MIPs can be used in various applications ranging from filter systems, sample clean-up procedures and separation sciences to implementation in sensors and

screening assays [19, 20]. The production of these recognition elements occurs through formation of a pre-polymerization complex through interaction between a template and functional monomer molecules. Afterwards, the pre-polymerization complex is stabilized by addition of a cross-linking polymer. During this process, unique three-dimensional cavities complementary in size and shape to the template are formed, which are characterized by a high specificity towards the template [21]. However, since the interactions between the template and functional monomer are primarily non-covalent in nature, the use of templates with small size and limited functionality, such as AEON and SK, remains a challenge due to a high degree of non-specific binding and weak interactions [22, 24]. Since non-covalent imprinting of AEON did not render MIPs with the required recovery, specificity and selectivity for implementation in a screening assay (data not included in this study), this study focussed on the development of MIPs for SK. In literature, a previous study on the imprinting of magnetic MIPs for SK revealed a certain degree of selectivity towards SK in comparison to structure analogues. However, these MIPs demonstrated low binding capacity towards SK, which made them unsuitable for implementation in screening assays for boar taint [25]. For this reason, this study elaborated on the development of a selective MIP array for SK. The morphological characteristics of the obtained MIPs were evaluated through scanning electron microscopy (SEM) and dynamic light scattering (DLS). Moreover, the binding characteristics of the MIPs were evaluated through batch-rebinding studies. These results indicated poor selectivity and specificity of the individual MIPs, which was enhanced by combining them in an array. Finally, the MIP array was successfully applied on boar adipose tissue samples.

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

Methacrylic acid (MAA) (CAS 79-41-4), 4-vinylpyridine (4-VP) (CAS 100-43-6), ethylene glycol dimethacrylate (EGDMA) (CAS 97-90-5), divinylbenzene (DVB) (CAS 1321-74-0), trimethylolpropane trimethacrylate (TRIM) (CAS 3290-92-4), 2,2-dimethoxy-2-phenylacetophenone (DMP) (CAS 24650-42-

8) and 2,2'-azobis(isobutyronitrile) (AIBN) (CAS 78-67-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All functional monomers and cross-linking polymers were purified over aluminium oxide prior to use and were stored at -20 °C. The reference standards of skatole (SK) (CAS 83-34-1), indole (IND) (CAS 120-72-9), 6-methoxyindole (6-MOID) (CAS 3189-13-7), 2-methylindole (2-MID) (CAS 95-20-5), 5-methylindole (5-MID) (CAS 614-96-0) and naphthalene (NAPH) (CAS 91-20-3) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Androstenone (AEON) (CAS 18339-16-7) was obtained from Steraloids Inc (Newport, RI, USA). For each reference standard, a stock solution of 1 mg ml^{-1} was prepared in methanol and stored in dark glass bottles at -20 °C. Toluene, acetonitrile (ACN) and methanol (MeOH) were purchased from VWR International (Merck, Darmstadt, Germany) and were of analytical grade. Methanol of MS grade was purchased from Fischer Scientific (Leichestershire, UK) and was used for mass spectrometric purposes.

2.2. Development and synthesis of molecularly imprinted polymers for skatole

A non-covalent precipitation approach was premised for the imprinting of SK. Different MIPs were developed by use of different functional monomers, cross-linking polymers, porogen solvents, initiators and polymerization conditions (irradiation and duration) (Table 1). The porogen solvent was added in a constant amount (97 %wt). Apart from the use of SK as a template, two additional MIPs were prepared with AEON for implementation in a MIP array (Table 1). Polymerization occurred in borosilicate glass tubes. First, SK or AEON were dissolved in the porogen solvent, after which the functional monomer, cross-linking polymer and initiator were added. Subsequently, the polymerization mixture was vortexed for 30 s and degassed for 10 min in an ultrasonic bath at room temperature (Elmasonic P, Elma Schmidbauer GmbH, Singen, Germany). Afterwards, the polymerization mixture was cleared from oxygen by purging with nitrogen gas for 5 min and sealed off. Finally, polymerization was initiated by placing the polymerization mixtures between UV lights (λ = 365 nm; 150 mW/cm^2) at 0 °C or by thermal initiation in an incubator at 60 °C. Polymerization was

terminated by exposing the borosilicate tubes to oxygen influx and the MIPs were collected after drying on aluminium foil. Finally, the template was removed by Soxhlet extraction (20 h) with MeOH. In parallel with the MIPs, non-imprinted polymers (NIPs) were produced according to the same protocol as a control to study non-specific interactions, except no template was added.

Table 1 Overview of the composition of molecularly imprinted polymers (MIPs) imprinted with SK or AEON.

MIP	Ratio template/monomer/ cross-linker	Template	Functional monomer	Cross-linker	Porogen	Initiator (mmol)	Irradiation
S1	1/8/4	SK	MAA	EGDMA	Toluene	AIBN	60°C, 24h
S2	1/1/5	SK	MAA	TRIM	Toluene	AIBN	60°C, 48h
S3	1/1/3	SK	MAA	TRIM	Toluene	AIBN	60°C, 48h
S4	1/2/10	SK	MAA	TRIM	Toluene	AIBN	60°C, 24h
S5	1/2/10	SK	MAA	TRIM	Toluene	AIBN	UV, 5°C, 48h
S6	1/3/15	SK	MAA	TRIM	ACN	AIBN	UV, 5°C, 48h
S7	2/1/3	SK	MAA	TRIM	Toluene	DMPA	UV, 5°C, 48h
S8	1/3/15	SK	4-VP	TRIM	Toluene	AIBN	60°C, 48h
S9	1/1/5	SK	4-VP	TRIM	Toluene	AIBN	60°C, 24h
S10	1/1/5	SK	4-VP	DVB	ACN	AIBN	60°C, 48h
A8	1/8/25	AEON	MAA	EGDMA	ACN	AIBN	60°C, 8h
A10	1/4/20	AEON	MAA	DVB	ACN	AIBN	60°C, 48h

2.3. Evaluation of molecularly imprinted polymers

2.3.1. Morphological characterization

The morphological characteristics of the MIPs and NIPs, implemented in the final array, were evaluated by SEM and DLS. In first instance, size and shape of the polymers were evaluated through SEM analysis (Phenom SEM desktop device, Phenom, Eindhoven, The Netherlands). Prior to analysis, the samples were sputtered with gold to optimize visualization. Additionally, DLS analysis on a Zetasizer Nano-ZS instrument (Malvern Instruments, Malvern, UK) was performed to determine the MIPs particle size and distribution. Hereto, 5 mg of each polymer was suspended in 1 ml of the porogen solvent and each suspension was further diluted (1/10,000) to avoid a too high polydispersity index. The particle size and distribution were determined in triplicate.

2.3.2. Equilibrium experiments

Binding characteristics of the developed MIPs were determined through equilibrium experiments, (i) recovery studies and (ii) batch-rebinding studies. To this end, an in-house validated UHPLC-HR-Orbitrap-MS method was used, according to Bekaert et al. [26].

2.3.2.1. Recovery

The recovery of each MIP for SK at its odour threshold concentration (0.2 ng μl^{-1}) was determined in ethanol and three different buffer solutions (0.1 M acetate buffer pH 3, 0.1 M acetate buffer pH 7 and 0.1 M bicarbonate buffer pH 10). Of each MIP, 5 mg was transferred to an Eppendorf tube. This was carried out in triplicate. Subsequently, the polymers were spiked with 1 ml loading solution of SK (0.2 ng μl^{-1}). Additionally, blank samples, free of MIP, were included to determine the response of the initial loading solutions. Afterwards, the tubes were vortexed for 30 s and shaken for 20 h on an end-over-end tumbler. Next, the tubes were centrifuged for 10 min at 17,000 x g, 80 µl supernatant was collected and 2-MID (20 µl of 1 ng μl^{-1}) was added as an external standard. Afterwards, the samples were diluted with 900 µl MeOH or 0.05% formic acid in ultrapure water, for the ethanol and buffer solution samples, respectively. Of the diluted samples, 100 µl was taken and 100 µl 0.05% formic acid or MeOH were added prior to UHPLC-MS analysis for the ethanol and buffer solution samples, respectively. Finally, the recovery of the MIPs for SK was calculated as the difference in concentration between the blank solutions and free concentration of SK in the supernatant following equilibration.

As significant differences in recovery were observed for the MIPs between ethanol and buffer solutions as a medium, the type of interaction (H-bound or ion-ion) between SK and the MIPs was evaluated. In this experiment, instead of using a SK solution, a NAPH solution (0.2 ng μl^{-1}) was added to the MIPs.

2.3.2.2. Binding isotherms and scatchard analysis

Two series of MIPs and NIPs, 5 mg of each, were transferred into Eppendorf tubes. Subsequently, the polymers were spiked with 1 ml loading solution in ultrapure water, containing SK in concentrations

ranging from 0 to 0.150 µmol ml^{-1} (0 – 0.005 – 0.0075 – 0.010 – 0.015 – 0.025 – 0.050 – 0.075 – 0.100 – 0.125 – 0.150 µmol ml^{-1}). Blank samples, without polymer, were also included in order to determine the response of the initial loading solutions. Afterwards, the tubes were vortexed for 30 s and subsequently shaken for 20 h on an end-over-end tumbler. Next, the tubes were centrifuged for 10 min at 17,000 x g, then 80 µl supernatant was collected and the external standard 2-MID was added (20 µl of 1 ng $µl^{-1}$). Afterwards, the samples were diluted with 900 µl 0.05% formic acid in ultrapure water. Of the latter solution, 100 µl was taken and 100 µl MeOH was added prior to UHPLC-HRMS analysis.

To construct binding isotherms, the amount of SK bound was determined as the difference in concentration between the blank solution and the free concentration of SK in the supernatant after equilibration. Afterwards, the binding isotherms were transformed by Scatchard analysis to determine the apparent dissociation constant (K_D) and the maximum amount of binding sites (B_{max}).

2.3.2.3. Selectivity

Selectivity of the MIPs was evaluated by comparing the recoveries obtained for SK and its different structure analogues (IND, 6-MOID, 5-MID). Five mg of each MIP was transferred into Eppendorf tubes and loaded with 1 ml of a mixture of SK, IND, 6-MOID and 5-MID (0.2 ng $µl^{-1}$) in ultrapure water. For each MIP, 6 replicates were taken into account. Afterwards, the tubes were shaken for 20 h on an end-over-end tumbler. Next, sample preparation was proceeded according to the protocol described under 2.3.2.1.

Since the MIPs, when applied separately, demonstrated a high degree of cross-reactivity, selectivity was enhanced by combining a selection of MIPs in an array. Furthermore, in order to evaluate the selectivity of the MIP array, linear discriminant analysis (LDA) was performed. The different MIPs were set as predictor variables in order to predict group membership. The different indolic compounds (SK, IND, 6-MOID and 5-MID) were set as the grouping variable.

2.4. Application of the MIP array on boar samples

The use of the proposed MIP array for the detection of the two boar taint compounds IND and SK was evaluated on boar neck fat samples (n = 52). To this end, 28 tainted boar neck fat samples and 24 untainted neck fat samples, containing IND or SK, respectively, above and below the odour threshold values (IND 100 µg kg^{-1}, SK 200 µg kg^{-1}), respectively were collected. Afterwards, all samples were extracted following an in-house protocol based on Bekaert et al. [26].

Two grams of neck fat were taken, cut into small pieces and melted in the microwave for 3 min at 300 W. Afterwards, 150 µl of melted fat was transferred to an Eppendorf tube and 750 µl of methanol was added. Next, the Eppendorf tubes were shaken before placing them in a warm water bath (60 °C) for 60 min to enhance extraction. After extraction, the samples were immediately cooled at -20 °C for 15 min. Next, the samples were centrifuged for 5 min at 17,000 x g and 500 µl supernatant was taken, to which 8 mL of ultrapure water was added. Afterwards, each MIP was spiked with 1 mL of the latter solution and left to equilibrate for 20 h on an end-over-end tumbler followed by centrifugation at 17,000 x g for 10 min. Finally, 100 µl of the supernatant was transferred to an LC-MS vial and 100 µl MeOH was added prior to UHPLC-HRMS analysis. In order to determine the response of the initial loading solutions, an additional sample of each extract was included without addition of polymer. Afterwards, LDA analysis was applied to obtain a predictive model for the classification of blank and SK samples. The different MIPs were set as predictor variables in order to predict group membership (Blank or SK).

2.5. Data analysis

LDA analysis was performed in SPSS version 23 (IBM corporation, NY, USA) and the significance level for all measurements was set at 0.05. The quality of each predictor in the LDA models was evaluated through the equality of group means and canonical discriminant functions. Furthermore, the accuracy of the LDA model was assessed through the Wilk's Lambda test and the sensitivity and specificity of

the obtained model were evaluated through the cross-validated classification results according to a leave-one-out classification.

3. RESULTS AND DISCUSSION

3.1. Morphological characterization

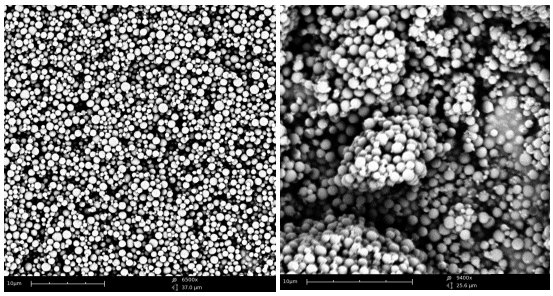

Fig 1 Scanning electron microscopy (SEM) picture of MIP S1 (left) and MIP S10 (right) as visualized on a Phenom SEM desktop device.

Since the particle size and shape are important characteristics influencing the re-binding of their target molecules, the morphological characteristics of the developed MIPs were evaluated through DLS and SEM analysis. DLS analysis revealed particle sizes sub 10 µm, indicating a sufficient total surface area for rebinding of the template (Table 2). For MIP S10 uniform particles were obtained (PdI < 0.200). The other MIPs were characterized by a certain degree of polydispersity (PdI > 0.200), which might negatively affect reproducibility of rebinding of the template to the MIPs [27]. Despite the polydisperse character of most particles, visualization of the MIPs through SEM analysis indicated that for all MIPs, spherical particles were obtained. Examples of SEM analysis results obtained for MIP S1 and S10 can be found in Fig 1.

Table 2 Morphological characterization of molecularly imprinted polymers (MIPs) by means of dynamic light scattering (DLS), presenting the Z-average resulting from 3 separate measurements.

MIP	Z-average ± SD (d.nm)	PdI
MIP S1	2653 ± 169	0.387
MIP S3	3399 ± 465	0.451
NIP S7	7314 ± 338	0.342
MIP S9	2257 ± 584	0.662
MIP S10	5859 ± 350	0.088
MIP A8	5517 ± 394	0.487
MIP A10	6820 ± 462	0.458

3.2. Equilibrium experiments

3.2.1. Recovery

The recovery of the MIPs for SK was evaluated in ethanol and 3 different buffer solutions. In ethanol, all MIPs displayed a very low recovery (0% - 11.4%), whereas in buffer solutions, significantly higher recoveries (87% - 100%) were obtained (Fig 2). Moreover, in buffer solutions, good repeatability of the rebinding of SK to the MIPs was observed (RSD% < 20%). The substantial differences in recovery observed in ethanol as compared to buffer solutions are most likely related to the structure of SK. Indeed, as SK contains one secondary amine function, interaction with the MIPs in ethanol is hypothesized to predominantly attributed to electrostatic interactions (H-bonds). Moreover, as ethanol is a relatively polar rebinding medium, this results in destabilization of the H-bonds and thus low recovery [28-30]. In the presence of aqueous media, an even more pronounced disruption of the hydrogen bonds is expected due to solvation, which in turn should decrease recovery. However, in the case of SK, a shift in interactions takes place. Indeed, as SK (pKa 17.48) contains a protolytic functional group, it is mostly present in its ionised form within a wide pH range. Consequently, the interaction between the template and MIP particles in aqueous solution is driven by ion exchange, leading to stronger rebinding [30]. Additionally, as the MIPs demonstrated a binding capacity towards NAPH (0% - 17%) in buffer solutions and ethanol comparable to the binding capacity towards SK in ethanol, the hypothesis of the presence of ionic interactions between SK and MIPs in buffer solutions was further reinforced (Fig 2) [31]. Similar results were reported by Takeda et al., who observed a decrease in recovery of IND in the presence of ethanol compared to water for a PSf membrane imprinted with indole-3-ethanol [32].

Although a higher recovery of the MIPs for SK was observed in aquatic buffer solutions, the results obtained in this study suggest a lack of imprinting effect. Indeed, NIP S7 and MIP A8 and A10 (imprinted with AEON) demonstrated a very high recovery (87% - 100%) for SK in buffer solutions (Fig 2), indicating that the SK-polymer rebinding interaction is mostly non-specific in nature. This may be attributed to

the stability of the prepolymerization complex, which is influenced by the template/functional monomer ratio, type of crosslinker, porogen, kinetic energy of the system, type of initiator, etc. [29, 30, 33, 34]. Although these parameters were varied in order to optimize the MIP performance (Table 1), comparable recoveries were obtained for each polymer (MIP and NIP) (Fig 2), suggesting a lack of imprinting effect.

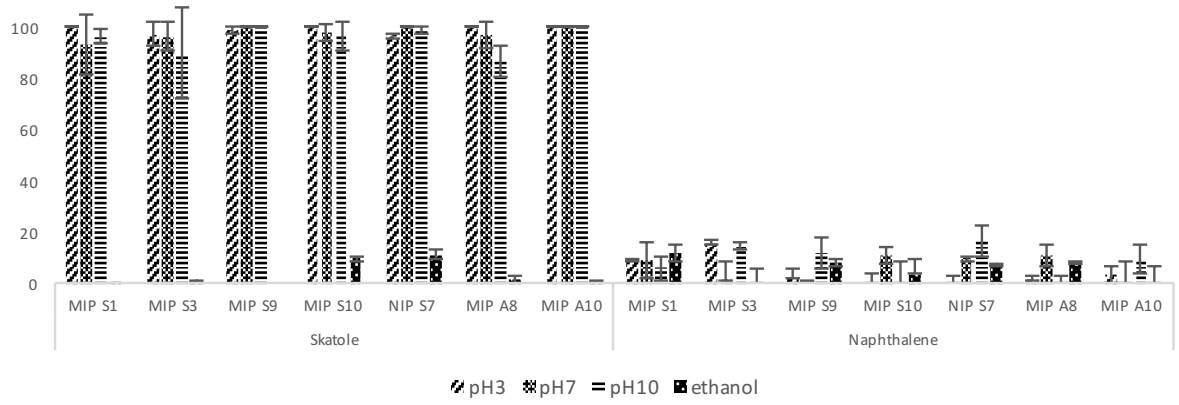

Fig 2 Overview of recoveries of SK (left) and naphtalene (right) obtained for the molecularly imprinted polymers (MIPs) included in the array, in different buffer solutions and ethanol.

3.2.2. Binding isotherms and scatchard analysis

To determine the specificity of the MIPs for SK, binding isotherms were constructed and a comparison between the MIPs and their corresponding NIPs was made (Fig 3). With the exception of MIP S1 and MIP S10, little or no difference was observed between most MIPs and their corresponding NIPs, indicating no clear imprinting effect of the template during polymerization. Consequently, rebinding of SK is mainly the result of non-specific binding, whereby it is merely adsorbed to the surface of the MIPs without specific recognition of size, shape and functionality of SK. These results support the results obtained for the recovery of the MIPs.

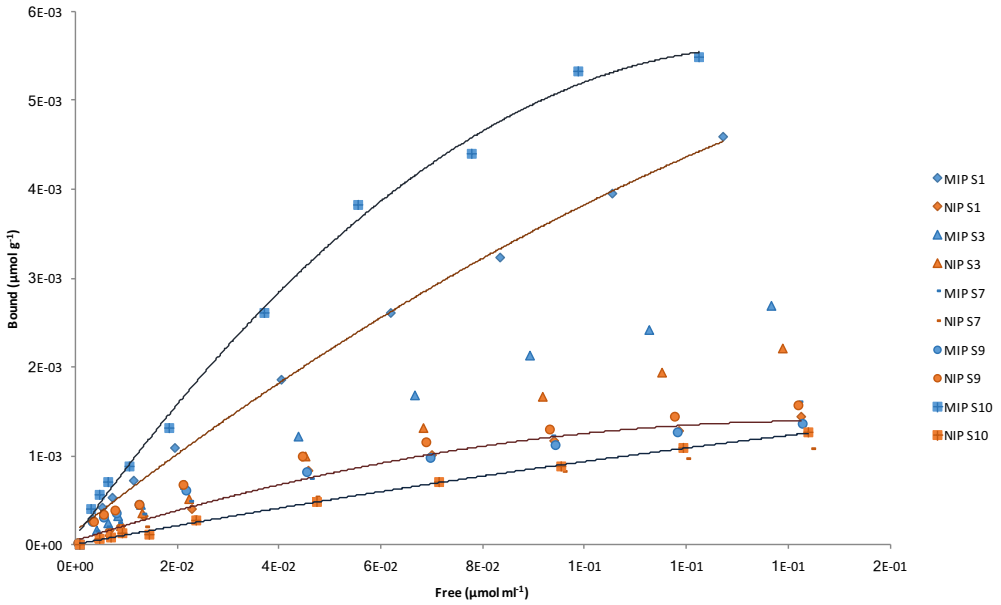

Fig 3 Binding isotherms for the molecularly imprinted polymers (MIPs) and their corresponding non-imprinted polymers (NIPs) obtained in batch-rebinding studies in porogen solvents.

The low specificity was confirmed by the low imprinting factors (0.9-1.6), which were assessed as the mean ratio for all concentration levels of the amount of SK bound to MIP and NIP, respectively (Table 3). Imprinting factors of 2.6 and 5.6 were obtained for MIP S1 and S10, respectively, demonstrating good specificity for SK. Additionally, Scatchard analysis demonstrated that the high affinity rebinding sites obtained for MIP S1 and S10 were only present in very low amounts (Table 3). Consequently, due to fast saturation of the highly specific binding sites, the majority of interactions between SK and these polymers were ascribed to non-specific adsorption to the surface of the polymers. A possible explanation for the higher imprinting effect in MIP S1 and MIP S10 is the ratio of template/monomer/cross-linker that was used, as this greatly influences the imprinting effect [34]. In general, depending on the functionality of the template, an excess of functional monomer and cross-linker is used, often in a ratio of 1/3 and 1/5 for template/monomer and functional monomer/cross-linker ratios, respectively. For MIP S1, however, the functional monomer was present in excess relative to the amount of cross-linker. Consequently, this might result in a decreased rigidity of the polymer network and thus loss of shape and size complementarity of the imprinted sites. However, due to the lower amount of cross-linker used, the imprinted sites might be more accessible for the analyte upon

rebinding [35]. For MIP S10 on the other hand, the template and functional monomer were added in equivalent amounts. As the functional monomer was not added in excess, less strong imprinted sites are expected, which shows in the apparent dissociation constant (Table 3). However, as SK only contains 1 functionality for interaction with the monomer, a 1/1 template/functional monomer ratio seems sufficient for successful imprinting [34].

Table 3 Summary of the maximum amount of binding sites (B_{max}), apparent dissociation constant (K_D) and imprinting factor (IF) obtained by Scatchard analysis and batch-rebinding studies.

		B_{max} (µmol g^{-1})	K_D (µmol l^{-1})	IF
S1	MIP high affinity	2.7	241	
	MIP low affinity	62.9	14285	2.6
	NIP	58.5	9982	
S3	MIP high affinity	6.1	263	
	MIP low affinity	39.6	2922	1.4
	NIP	37.6	3044	
S7	MIP high affinity	10.4	896	
	MIP low affinity	105.6	12650	1.6
	NIP	87.1	12648	
S9	MIP high affinity	6.3	188	
	MIP low affinity	32.4	1760	0.9
	NIP	45.1	3170	
S10	MIP high affinity	2.3	150	
	MIP low affinity	14.2	711	5.6
	NIP	36.1	2976	
A8	MIP high affinity	33.8	1506	1.0
	NIP	25.4	1600	
A10	MIP high affinity	7.3	223	
	MIP low affinity	41.5	2051	1.0
	NIP	168.9	6044	

3.2.3. Selectivity

Evaluation of the binding isotherms and Scatchard analysis indicated poor specificity for all MIPs, with the exception of MIP S1 and MIP S10. For this reason, the cross-reactivity of a selection of MIPs separately and combined was investigated through linear discriminant analysis. When applying each MIP separately, a high degree of cross-reactivity (92-100%) between the different structure analogues (IND, 6-MOID, 5-MID) was observed (data not shown). These results indicate a lack of selectivity of the polymers towards SK, which limits the application of the latter into sensor devices as this will result in a high degree of false positives and negatives. In order to increase selectivity towards SK and its

different structure analogues, a combination of 6 MIPs and 1 NIP (MIP S1, S3, S9, S10, A8, A10 and NIP S7) was applied in an array or fingerprinting approach. The three discriminant functions obtained for the linear discriminant model were associated with moderate to high canonical correlations (0.546-0.989), indicating that each predictor variable had a moderate to large effect on the differentiation of the outcome results between groups. Moreover, the Wilk's Lambda test indicated that all predictor variables could predict the outcome accurately at a statistically significant level ($p < 0.05$). Finally, the standardized canonical discriminant function coefficients indicated that mainly MIPs S1, S10 and S9 were associated with a high prediction capability. MIPs A8, A10 and NIP S7 demonstrated less contribution to the prediction, which can be explained by the absence of imprinted sites for SK as these polymers were imprinted with AEON or not imprinted, respectively.

Table 4 Cross-validation results of the linear discriminant (LDA) model obtained for the MIP array in a cross-reactivity study.

	Accuracy (%)	Precision (%)	Sensitivity (%)	Specificity (%)
Overall	87.5	84.6	91.7	83.4
SK	87.5	71.4	83.3	88.9
IND	100	100	100	100
5-MID	87.5	80.0	66.7	94.4
6-MOID	100	100	100	100

Finally, cross-validation according to a leave-one-out classification demonstrated an overall classification accuracy of 87.5% (Table 4). The highest accuracy was obtained for IND and 6-MOID, whereas for SK and 5-MID, less accuracy was obtained (Table 4). This is most likely due to the fact that the latter compounds are region-isomers, whereby a higher degree of cross-reactivity is expected. However, compared to the use of MIPs separately, a combined approach significantly increased selectivity and enabled to distinguish between very closely related structure analogues [36, 37]. A possible explanation for the lack of selectivity of the MIPs separately lies in the structure of the template SK. Indeed, as SK is a small molecule (131.2 Da) with merely one functionality, imprinting thereof remains challenging [23, 24, 30]. Consequently, the number of complementary interacting functionalities was low, whereby also the position between the template and functional monomer in the pre-polymerization mixture was less defined, which adds to the lack of specificity and selectivity.

Additionally, in case of imprinting with MAA, the formation of intermolecular hydrogen bonds between the functional monomers themselves can further decrease specificity and selectivity of the MIPs as less functionalities remain accessible for interaction between the template and functional monomer [38]. Furthermore, comparison with monoclonal antibodies against SK indicates that monoclonal antibodies demonstrate a higher degree of cross-reactivity with IND and 6-MOID compared to the presented MIP array [39]. Moreover, the MIPs developed in this study also showed a two-fold lower dissociation constant, indicating that the interaction between SK and MIPs is stronger compared to interaction with antibodies [39]. In comparison to other MIPs developed against SK, a higher degree of cross-reactivity (50%) with IND was observed in comparison to the proposed MIP array. Accordingly, by applying a MIP array, discrimination between analytes is feasible, which was previously demonstrated by Xu et al. and Schnee et al., for atrazine analogues and substituted benzene compounds, respectively [36, 37].

3.3. Application of the MIP array on boar samples

In a final experiment, the use of the proposed MIP array for the detection of SK and/or IND in boar neck fat samples was evaluated. Group membership (positive or negative for boar taint) was investigated through linear discriminant analysis.

The discriminant function obtained for this model was associated with a high canonical correlation (0.739). Moreover, the Wilk's Lambda test indicated that all MIPs could predict the outcome (positive or negative for boar taint) accurately at a statistically significant level ($p < 0.05$). Finally, the standardized canonical discriminant function coefficients indicated that MIPs imprinted with SK were associated with an overall high prediction capability. Furthermore, the MIP array showed good classification accuracy (82.7%) for SK/IND after cross-validation according to a leave-one-out classification. Moreover, satisfactory precision (78.8%) and a very high sensitivity (92.9%) were obtained, indicating a relatively low false negative rate (7%). However, a higher false positive rate (29%), associated with a specificity of 70.8% was observed. This can be explained by the fact that

negative boar neck fat samples also contain SK and IND, although in levels below the odour thresholds of 200 and 100 µg kg^{-1}, for SK and IND, respectively [40]. Overall, good performance characteristics were obtained for the MIP array, indicating its use for the analysis of boar neck fat samples. Furthermore, false positive results will not lead to consumer dissatisfaction due to the presence of boar taint in meat. However, they can lead to economic losses in pig husbandry as often penalties are charged for tainted boar carcasses. Moreover, compared to untargeted RAMAN spectroscopy of tainted and untainted boar neck fat samples, similar classification accuracy was obtained. However, RAMAN spectroscopy was associated with a sensitivity and specificity of 72% and 88%, respectively, leading to a higher false negative rate in comparison to the MIP array [10].

4. CONCLUSIONS

In this study, a MIP array with high selectivity (classification accuracy 82.7%) for the recognition of SK and IND was developed. Batch-rebinding studies indicated an increase in recovery in buffer solutions as a rebinding medium compared to ethanol. Furthermore, although very poor specificity and a high degree of cross-reactivity was observed for the MIPs separately, very good selectivity was observed when combining a set of MIPs in an array. Finally, this array was successfully applied on boar neck fat samples negative and positive for the presence of SK. These results emphasize the applicability of the array and its use for the implementation in sensor devices or screening assays for the detection of SK in neck fat.

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CHAPTER VII

RAPID EVAPORATIVE IONISATION MASS SPECTROMETRY FOR HIGH-THROUGHPUT SCREENING IN FOOD ANALYSIS: THE CASE OF BOAR TAINT

Adapted from:

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ABSTRACT

Boar taint is a contemporary off-odour present in meat of uncastrated male pigs. As European Member States intend to abandon surgical castration of pigs by 2018, this off-odour has gained a lot of research interest. In this study, REIMS was explored for the rapid detection of boar taint in neck fat. Untargeted screening of samples (n=150) enabled discrimination between gilt, tainted and untainted boars. The obtained OPLS-DA models showed excellent classification accuracy, i.e. 99% and 100% for gilt and boar samples or solely boar samples, respectively. Furthermore, the obtained models demonstrated excellent validation characteristics ($R^2(Y)$=0.872-0.969; $Q^2(Y)$=0.756-0.917), which were confirmed by CV-ANOVA ($p<0.001$) and permutation testing. In conclusion, in this work for the first time highly accurate and high-throughput (<10 sec) classification of tainted and untainted boar samples was achieved, rendering REIMS a promising technique for predictive modelling in food safety and quality applications.

1. INTRODUCTION

During the past decades, the public awareness of food safety and quality has significantly increased [1]. The organoleptic properties of food play a crucial part in this, as they are reflective of the first impressions consumers will develop [2]. To effectively ensure the food quality and safety, an analytical platform for the fast and accurate detection of quality parameters in food imposes itself [3]. In this study, REIMS was proposed as a new analytical approach for *in-situ* detection of food anomalies and its applicability was demonstrated for an important contemporary off-flavour in meat industry, i.e. boar taint.

Boar taint is an off-odour caused by the accumulation of IND, SK and AEON in adipose tissue [4-6]. IND and SK are two indolic compounds derived from the biological degradation of L-tryptophan in the hindgut and their odour is often described as faecal-like [4, 5]. AEON on the other hand is a pheromone produced in the Leydig cells of the testis and has a urinary- or sweaty-like odour [7]. Initially, the surgical castration of pigs was implemented to prevent boar taint; however, increasing awareness on animal welfare has led to a European intent to voluntarily abandon the surgical castration of piglets by 2018 [8]. Consequently, the rearing of entire male pigs, one of the alternatives to surgical castration could cause adverse consumer reactions due to the re-occurrence of boar taint and thus lead to economic losses in pig husbandry [9, 10]. In order to maximize the marketing potential of meat from entire males, sorting strategies to detect boar taint containing carcasses at the slaughter line are in order [11].

One of the main challenges for the detection of boar taint at the slaughter line is the high rate at which pigs are slaughtered, on average 600 per hour. Over the past years, several candidate methods for at-line detection of boar taint have been proposed, including sensory and analytical methods. However, none of these meet the required performance characteristics needed at the slaughter line [12]. Indeed, sensory methods, e.g. the soldering iron method, whereby neck fat is singed with a soldering iron and the released smell assessed by a trained assessor, are directly applicable at the slaughter line and

provide a fast and holistic detection of boar taint, but they rely on the sensory abilities of one trained assessor [13, 15]. Consequently, sensory methods are subject to inter-individual variation and moreover are associated with habituation and fatigue [14]. Furthermore, various analytical methods show potential for the at-line detection of boar taint but often lack sensitivity, specificity or high-throughput [12]. Indeed, the use of sensor technology, e.g. thickness shear mode resonator sensors and parasitoid biosensors, offers sensitive and fast detection of the boar taint compounds but these sensors often show poor specificity or lack thorough testing and validation [16-20]. The use of a mass spectrometric based electronic nose for targeted screening of the boar taint compounds was promising, however, results were preliminary, lacking thorough validation [21]. The detection of the boar taint compounds by means of high-throughput GC-MS on the other hand offers satisfactory precision (RSD% < 20%) [22, 23]. However, although these methods are fast (run-to-run of 3.5 to 6 min), they do not meet the speed requirements needed at the slaughter line. Moreover, as insufficient sensitivity is obtained on a portable GC-MS instrument, up until now these methods cannot be implemented directly at the slaughter line [22]. More recently, RAMAN spectroscopy was evaluated for the detection of boar taint [24, 25]. Targeted detection of IND, SK and AEON was associated with very large prediction errors: 173 µg kg^{-1} and 1460 µg kg^{-1} for SK and AEON, respectively [24]. More accurate results (88% identification accuracy) were obtained with an untargeted classification approach, enabling identification of aberrant adipose tissue samples. However, data acquisition lasted 20 min per sample, limiting the potential use of RAMAN spectroscopy at the slaughter line [25].

To overcome these bottlenecks, in this study, REIMS is proposed as a new emerging technique that circumvents long analysis times by enabling direct ionisation from the sample combined with mass spectrometric analysis. As such, REIMS analysis takes only a few seconds and guarantees point-of-control analysis [26-30]. Additionally, REIMS offers highly accurate histological identification of tissues and demonstrated a correct classification performance of 90-98% [26]. Originally it was intended for *in-vivo* identification of tissues during medical interventions, but recently also found its application niche in food analysis as its feasibility was successfully demonstrated for the identification of the

species of origin in meat products [31]. In this study, REIMS was explored to develop a predictive model for accurate high-throughput identification of boar taint in pig adipose tissue, a first of its kind step towards achieving at-line classification of boar carcasses.

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

The reference standards IND or 2,3-benzopyrrole (CAS 120-72-9) and SK or 3-methylindole (CAS 83-34-1) were obtained from Sigma Aldrich (St. Louis, MO, USA). The reference standard 5α-androst-16-ene-3-one (AEON, CAS 18339-16-7) was obtained from Steraloids (Newport, RI, USA). For each compound, standard solutions were prepared in isopropyl alcohol at a concentration of 20 µg ml^{-1}. Also a mixture of IND, SK and AEON was prepared in isopropyl alcohol at a concentration of 20 µg ml^{-1}.

2.2. Samples

Both gilt (blank) samples and boar neck fat samples were collected at the slaughter line. In order to select boar samples negative and positive for boar taint, boar carcasses were screened for boar taint at the slaughter line by means of the soldering iron method optimized by Bekaert et al. [14]. All samples were cooled during transport to the lab and were immediately stored upon arrival at -80°C until analysis. The presence or absence of boar taint in the samples was confirmed by an in-house validated UHPLC-HR-Orbitrap-MS analysis method [32]. Samples containing levels of IND, SK and/or AEON above and below the applied odour thresholds in neck fat (IND: 100 µg kg^{-1}, SK: 200 µg kg^{-1}, AEON: 500 µg kg^{-1}) were considered as positive and negative for boar taint, respectively. Of the samples included in the positive group according to sensory analysis, the boar taint compound levels did not exceed these thresholds according to chemical analysis. Of the negative group, 3 samples had boar taint compound levels exceeding these thresholds. In total, 50 samples for each group were collected (Fig 1 & 2).

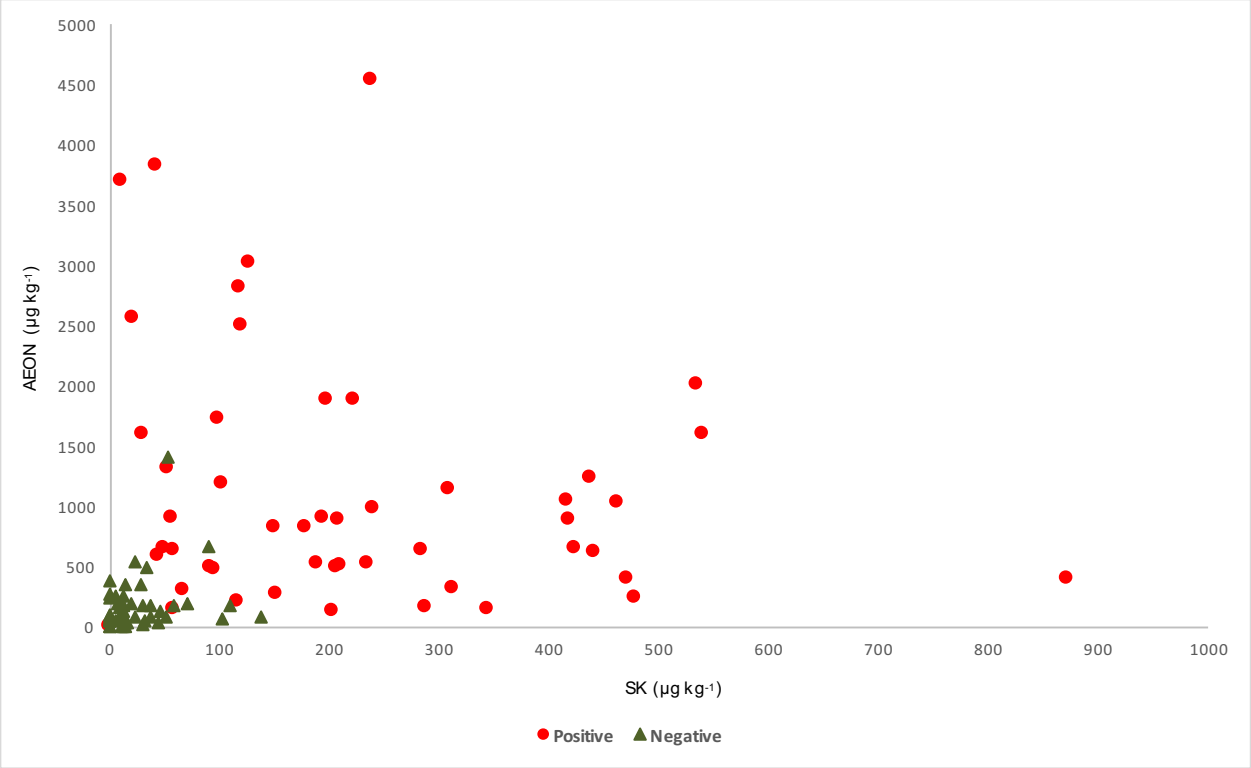

Fig 1 Scatter plot of the androstenone (AEON) and skatole (SK) neck fat levels of the selected boar samples for the positive and negative group.

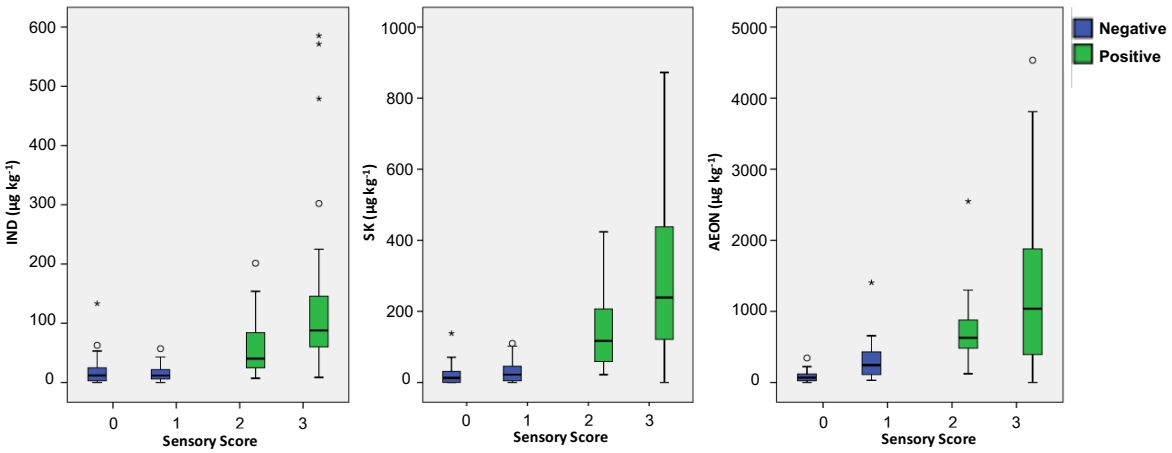

Fig 2 Boxplots of the indole (IND), skatole (SK) and androstenone (AEON) levels and sensory scores for the selected boar samples for the positive and negative group.

2.3. Instrumentation

The iKnife hand-held sampling device (Waters, Wilmslow, UK) was used to apply a localized high frequency electric current to the surface of each sample, which instantly vaporizes molecules from the

latter. It consisted of a monopolar cutting device with a shortened knife blade of approximately 6 mm and was applied in dry cut mode in combination with a diathermy electrosurgical generator at 45 W. Sampling was carried out for 3 to 5 seconds and for each sample, 2 technical replicates were analysed, thus taking into account repeatability of the analysis. Isopropyl alcohol was used as a dopant to stimulate ionisation of the boar taint compounds and lipids. Mass spectrometric analysis was carried out on a Xevo G2-XS Q-TOF instrument equipped with a helical coiled ribbon collision surface supplied with a constant current power supply set to 4.5 A (Kanthal D 1.0 x 0.1 mm) (Waters, Wilmslow, UK). All analysis occurred in REIMS TOF MS sensitivity mode with continuum data acquisition. Isopropyl alcohol was infused directly into the REIMS source at a constant flow rate of 100 µl min^{-1} to promote the ionisation of lipid (fatty acid and phospholipid) species. The mass resolution was typically set at 18750 and 19195 for *m/z* 281.2537 and 773.5432, respectively. The cone voltage was set at 100 V. Mass spectrometric analysis was performed in negative ionisation mode with a mass range of 50 – 1200 *m/z* and scan speed of 1 s/scan. For quality control purposes, the instrument was calibrated using sodium formate to check for mass shifts, ensure mass accuracy and sensitivity. Furthermore, the endogenous matrix ion PE (34:1) $[M\text{-}NH_4]^-$ $C_{39}H_{76}NO_8P$ with *m/z* 699.497 was used as a lock-mass compound to monitor mass accuracy and instrumental drift. Additional quality control occurred by including replicate burns of a QC sample (bovine muscle) that were collected between every 10 pig neck fat samples. The intensity of the base peak ion at *m/z* 699.497 was recorded and plotted for quality control monitoring. In order to prevent carry-over, the iKnife, transfer tubing and venturi device required regular cleaning with methanol between every 10 samples. Cleanliness of the REIMS source was performed after approximately 1500 burns. Cleaning of the Xevo G2-XS Q-TOF instrument occurred once during the study (after 1 week).

2.4. Untargeted identification approach of neck fat samples

In preliminary experiments, targeted detection of the boar taint compounds on the REIMS-Xevo G2-XS Q-TOF instrument was evaluated (data not shown). Reference standards of the boar taint

compounds could easily be detected. However, analysis of fortified neck fat resulted in ion suppression due to matrix effects, whereby the boar taint compounds could not be detected. For this reason, an untargeted mass spectrometric analysis approach was evaluated for the discrimination between boar taint positive and negative carcasses. Untargeted analysis was performed by profiling both boar (negative and positive) and gilt (blank) samples and thus effectively providing a mass spectral fingerprint for the latter. Samples were analysed in duplicate on 3 consecutive days in order to take into account reproducibility. Each sample was cut 3 to 5 times, with each cut lasting for 3 to 5s. This enabled the analysis of multiple locations of the sample. This experiment was repeated on 3 additional days, with different cone voltage settings (60 or 100 V) of the ionisation source, in order to check the robustness of the measurements. Afterwards, the mass spectrometric fingerprints were used to construct predictive models for the classification of gilt and boar samples into blank (gilt) and boar taint positive and negative groups.

2.5. Chemometric data analysis

Post-acquisition, all data files were pre-processed using the bridge conversion tool (Waters, Wilmslow, UK) that used standard Masslynx pre-processing algorithms. For each sample, recorded raw mass spectrometric scans resulting from each burn were combined into one average spectrum. The resulting data were lock-mass corrected using the endogenous matrix ion with *m/z* 699.497, background substracted to remove noise and TIC normalised using a TIC replicate threshold setting of 100,000. All models were built using a mass range selection of *m/z* 50-1200 Da, spectral intensity threshold of $2e^6$ counts and data bin width of 0.1 Da. In total, 1817 features were retained for model building. Furthermore, all data were log-transformed and pareto scaled to generate normally distributed data and reduce noise, respectively, prior to model building. Next, multivariate regression analysis was performed in SIMCA 14 (Umetrics, Umea, Sweden). Principal component analysis (PCA) was used for unsupervised data analysis to reveal outliers, groups and trends. Afterwards, orthogonal partial least-square discriminant analysis (OPLS-DA) was used to construct prediction models able to predict the Y-

variable (classification of samples in groups) from the X-matrix (mass spectrometric fingerprint). In order to avoid over-fitting of the data, the quality of the OPLS-DA models was evaluated through the goodness of fit ($R^2(Y)$) and the predictive ability of the models ($Q^2(Y)$). Permutation testing (20 permutations) was performed to assess the risk that the model is spurious, i.e. that the model fits the training set but does not predict Y well for new observations. Additionally, CV-ANOVA (cross-validated analysis of variance) and cross-validation, according to a leave 1/7 out classification, were performed to confirm the validity of the models. In parallel, OMB version 1.1.29.0 (Waters Corporation, Wilmslow, UK) was used as a model builder recognition software tool. To this end, a linear discriminant analysis (LDA) model including 80% of randomly selected samples of each group was built. The remaining 20% was used as a test set for external validation of the model and run through the recognition software, whereby the observed classifications (based on two burns) were recorded in post-acquisition mode. Each sample was classified to the closest class. If a sample was located outside the 3σ standard deviation range, it was considered as an outlier.

3. RESULTS AND DISCUSSION

3.1. Discrimination between boars (tainted and untainted) and gilts

To demonstrate the classification potential of REIMS, 50 blank (gilt), 50 boar taint positive (tainted) and 50 negative (untainted) samples were analysed. In a first experiment (data not shown), both negative and positive ionisation mode were taken into account, to increase the range of detected metabolites, and were considered separately. In negative ionisation mode, better classification accuracy (98%) was observed compared to positive ionisation mode (94%). For this reason, it was decided to continue all analysis in negative ionisation mode for model building. In a first model, the classification potential for discrimination between gilts, tainted and untainted boars was investigated. The PCA plot revealed 17 potential outliers (Fig 3A); however, only 5 of the latter were identified as true suspected outliers using the Hotelling's T2 plot. Four outliers originated from the blank group and 1 outlier from the boar taint positive group. Since the values of these outliers were located between

the 95% and 99% confidence limit, they were omitted from further data analysis. The validity of the supervised OPLS-DA model was evaluated through $R^2(Y)$ and $Q^2(Y)$, CV-ANOVA testing and permutation tests. Generally, $Q^2(Y)$ values > 0.5 are regarded as good for biological models [33]. In this study, values obtained for $R^2(Y)$ and $Q^2(Y)$ were 0.872 and 0.756, respectively, indicating an excellent fit and predictive abilities. Moreover, CV-ANOVA analysis ($p < 0.001$) demonstrated that the obtained OPLS-DA model was highly significant. Finally, permutation testing demonstrated that the predictive abilities of the original model ($R^2(Y)$ and $Q^2(Y)$) were higher in comparison to the permutated models (Fig 3B).

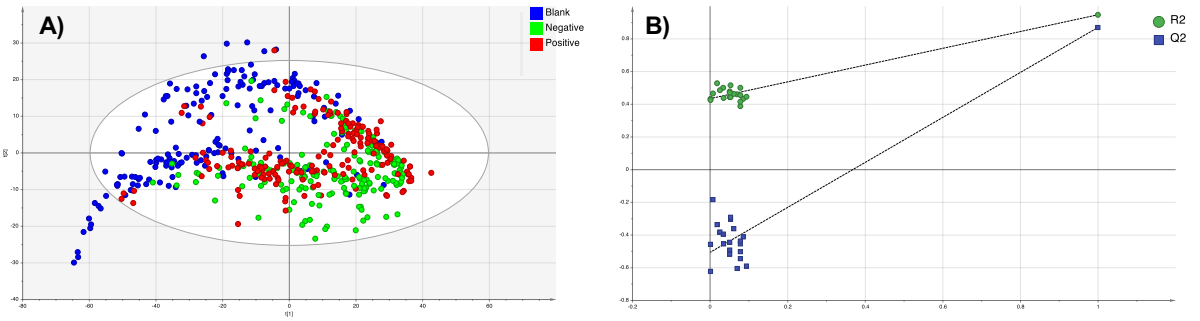

Fig 3 A) Score plot of a principal component analysis model, B) Permutation plot as a validation criterion for an orthogonal partial least-squares discriminant analysis model for a dataset containing blank (gilt), negative (untainted boar) and positive (tainted boar) neck fat samples in negative ionisation mode (n = 150).

The obtained OPLS-DA model showed separation between the gilt and boar groups (Fig 4). The two boar groups on the other hand showed some overlap, nevertheless, cross-validation demonstrated that the obtained model had a total correct classification rate of 99% according to an internal cross-validation (leave 1/7-out). All blank and negative samples were correctly classified, whereas of the boar taint positive samples, 98% was correctly classified. The remaining 2% was classified as negative. The classification results obtained by chemical and sensory analysis, which were used as Y-information for model building, could form the basis of this misclassification. Indeed, based on the sensory scores of the neck fat samples, these samples were severely tainted. However, chemical analysis by means of UHPLC-HRMS revealed boar taint levels of SK and AEON barely exceeding the proposed odour thresholds of 200 and 500 µg kg^{-1}, respectively. Since previous studies also report a discrepancy between the presence of SK and AEON on the one hand and the sensory evaluation of boar samples

on the other, this could potentially lead to biased class information in the Y-axis, causing misclassification in the OPLS-DA model [34, 35]. In parallel to the OPLS-DA model, an LDA model including 80% randomly selected samples was built and loaded into a model builder recognition tool. The remaining 20% of samples were run through the real-time recognition software, which resulted in a 95% correct classification rate for the tainted boar group and thus false negative rate (β error) of $\leq$ 5%. Additionally, for the gilt group also a correct classification rate of 95% was observed. For the untainted boar group on the other hand, a correct classification rate of 65% was observed due to the presence of 1 outlier and allocation of 5 and 1 samples as tainted and blank (gilt), respectively. Despite the high percentage of false positive results, a false positive rate (α-error) of $\leq$ 5% was observed for the gilt and untainted boar samples combined. For the untainted group separately, a false positive rate of 26% was observed. In contrast to false negatives, false positive classifications will not result in a loss of consumers' confidence in pork industry. However, since tainted boar meat is often subject to penalty fees, the number of false positives should be minimized [12, 36]. For this reason, further optimisation and inclusion of more samples in the model is necessary.

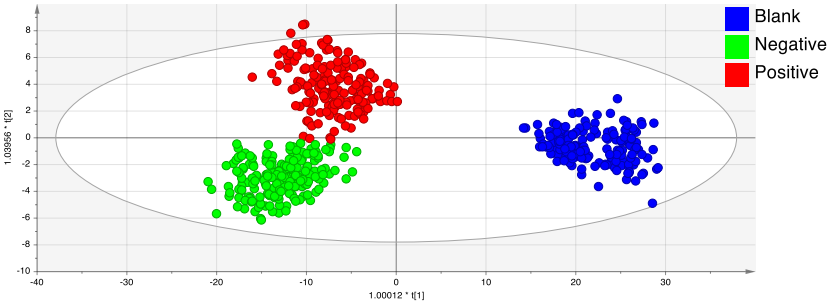

Fig 4 Score plot of a partial least-squares discriminant analysis model for a dataset containing blank (gilt) (n=50), negative (untainted) (n=50) and positive (tainted) (n=50) boar neck fat samples in negative ionisation mode.

3.2. Discrimination between tainted and untainted boars

Despite the fact that the risk of boar taint is limited to carcasses of uncastrated pigs, the indolic compounds are also present, although to a lesser extent in sows, barrows and gilts. Nevertheless, only boar carcasses should be screened at the slaughter line. Therefore, the gilt group was omitted in a

second model, and an OPLS-DA model was constructed to evaluate the classification potential for tainted and untainted boars.

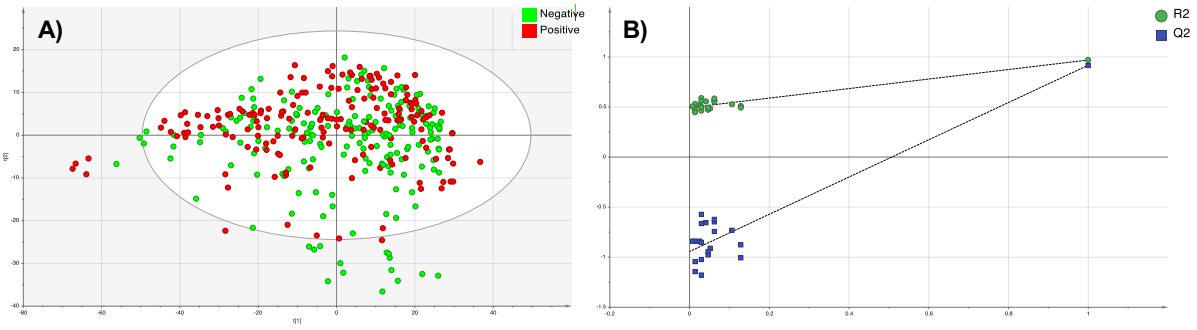

Fig 5 A) Score plot of a principal component analysis model, B) permutation plot as a validation criterion for an orthogonal partial least-squares discriminant analysis model for a dataset containing negative (untainted boar) and positive (tainted boar) neck fat samples in negative ionisation mode (n = 100).

The obtained model was significant ($p < 0.001$) and demonstrated excellent predictive properties ($R^2(Y) = 0.969$, $Q^2(Y) = 0.917$). Moreover, a permutation test showed that the predictive abilities of the latter model were higher than those obtained for the permutated models (Fig 5). Finally, cross-validation (leave 1/7 out and in-silico validation) revealed that all samples were correctly allocated to the boar taint positive of negative group, thus indicating 100% accuracy, specificity and sensitivity of the obtained OPLS-DA model (Fig 6). The obtained results indicate that the untargeted REIMS analysis technique is promising for implementation at the slaughter line. As this technique involves the use of highly expensive, lab based equipment (Xevo G2-XS Q-TOF instrument), implementation in a harsh environment such as the slaughter line is unconventional and remains a challenge. However, as the iKnife is connected through a 3m long tubing to the Xevo G2-XS Q-TOF instrument, the instrument itself could be placed in a separate room next to the slaughter line where humidity and temperature are controlled. As such, the at-line application of boar taint screening remains ensured and the need for sampling is excluded. Apart from the practical challenges, implementation also involves a high investment cost for abattoirs. However, because of the high number of pigs slaughtered in an average abattoir, i.e. 600/h in Belgium and in light of the increasing number of entire male pigs that will need to be slaughtered, costs per analysis per carcass are estimated to remain below 1 euro. This estimate

was based on an annual slaughter of 20-50% entire male pigs, whereby 10-25% of all slaughtered carcasses should be screened for boar taint, starting from a 50/50 distribution between male and female carcasses. This indicates that it is practically and economically feasible to implement the REIMS technique at the slaughter line for routine boar taint screening.

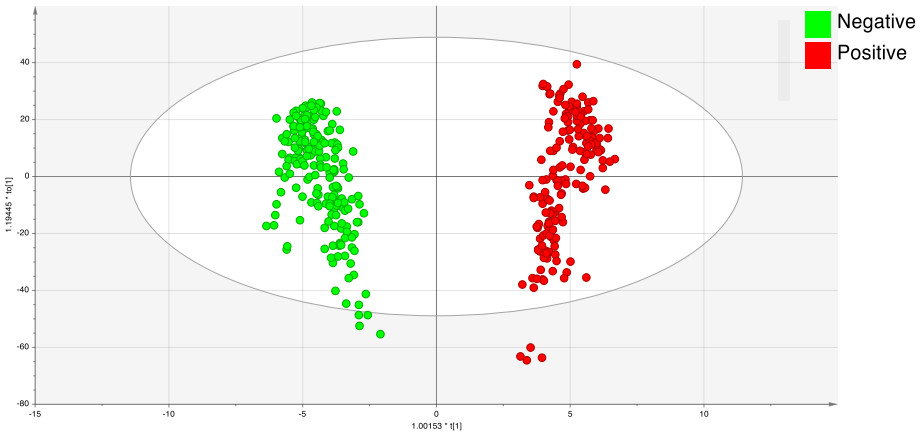

Fig 6 Score plot of a partial least-squares discriminant analysis model for a dataset containing negative (untainted) (n=50) and positive (tainted) (n=50) boar neck fat samples in negative ionisation mode.

Compared to previously reported studies, much higher classification accuracy for tainted and untainted boar carcasses was obtained by REIMS. Sensitivity and specificity of sensory methods ranged between 36-88% and 11-85%, respectively, and fluctuated greatly, depending on the trained assessor [37, 38]. Recently, a classification accuracy between tainted and untainted boar samples of 81% was obtained using a portable RAMAN device. However, it should be noted that only true positive and negative samples were taken into account, whereby a cut-off of 1500 µg kg^{-1} was chosen for AEON, while in this study, a cut-off value of 500 µg kg^{-1} was considered. Moreover, an uncertainty range of ± 20% of the threshold level was considered for chemical analysis for sample inclusion in the RAMAN experiment [25]. Furthermore, compared to the targeted detection of IND, SK and AEON, applying an untargeted approach could benefit the true identification of aberrant carcasses. Indeed, up until now, the presence of SK and AEON in neck fat samples of boars only accounts for 76% of the explained variance between the presence of the latter compounds and the intensity of boar taint assessed by trained experts, indicating that also other unknown compounds attribute to the presence of boar taint

[34, 35]. This was confirmed by a second OPLS-DA model ($R^2(Y)$ = 0.582; $Q^2(Y)$ = 0.529) using quantitative UHPLC-HR-Orbitrap-MS data of IND, SK and AEON as predictive information to classify the samples under investigation as tainted or untainted, as a decrease in accuracy (89%), specificity (82%) and sensitivity (97%) was observed in comparison to the applied untargeted approach (100%) (Fig 7).

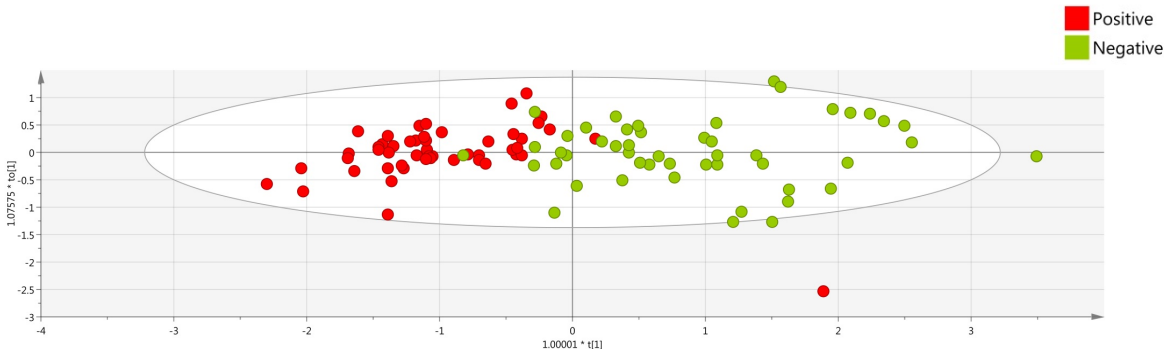

Fig 7 Score plot of an orthogonal partial-least squared discriminant analysis model for negative (untainted boar) and positive (tainted boar) neck fat samples (n = 100) with quantitative UHPLC-HRMS data for indole, skatole and androstenone as predictive information.

3.3. Candidate biomarkers

After model building, S-plots were constructed in order to reveal significant ions responsible for sample allocation (Fig 8). In the S-plot, the x-axis corresponds to the contribution (covariance (p)) of the ion to the variance of the observations, e.g. absence or presence of boar taint. The y-axis on the other hand represents the correlation (p(corr)) between samples and the reliability of the results. In order for an ion or a combination of ions to be relevant, cut-off values of $|p| \geq 0.03$ and $|p(corr)| \geq 0.5$ are advised in metabolomics studies [40, 41]. In total, 60 ions demonstrated a high contribution to the presence of boar taint in neck fat. However, none of the latter or a combination of the 4 most relevant compounds were reliable ($|p(corr)| < 0.5$) to allocate samples in the boar taint negative or positive group. Consequently, in order to correctly classify between tainted and untainted boar carcasses, the complete mass spectrum should be taken into account. A possible explanation for the lack of reliable candidate biomarkers could be the observed matrix effect. Indeed, analysis of the boar taint compounds in adipose tissue resulted in ion suppression effects whereby IND, SK and AEON could not be detected above the background matrix ions. Although a different ionisation technique was applied,

ion suppression of the boar taint compounds was also observed using GC-MS analysis in full scan mode [22]. Consequently, the ion suppression effects led to insufficient sensitivity to apply a targeted approach to identify aberrant boar carcasses.

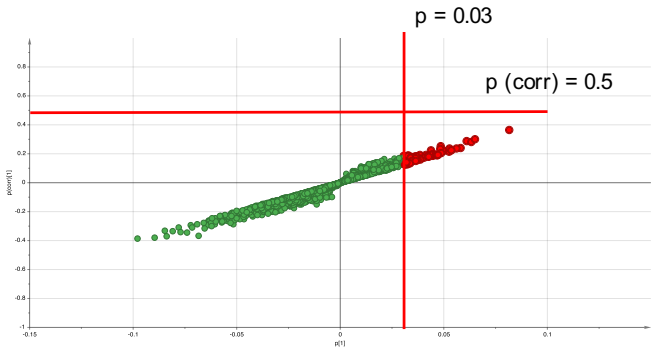

Fig 8 Loading S-plot representing the contribution ions obtained in negative ionisation mode towards the presence of boar taint. Cut-off values of $|p(corr)| \geq 0.5$ and $|p| \geq 0.03$ were applied.

3.4. Validation

To monitor and guarantee the repeatability of the measurements, a QC sample was analysed after every 10 pig neck fat samples. Repeatability was then plotted as the intensity of the base peak ion of the endogenous lock-mass compound. In total, 5% and only 0.57% of the measurements exceeded the 2SD and 3SD warning limits, respectively. As over 94% of the measurements lay within these limits, good repeatability may be concluded. In a final experiment, also the robustness of REIMS for sample classification was evaluated. To this end, all data were re-acquired on different days but with a change in heater power settings of the collision surface. When taking into account the three sample groups (blank, boar taint negative and positive), a decreased classification accuracy (89%) was observed when working with a lower heater power, whereby an even percentage of false positive and negative results was obtained (16%). Since ionisation of compounds is enhanced by higher energy and thus a higher heater power, the decrease in accuracy was most likely due to a loss of sensitivity in ion intensity. When omitting the blank group from the model and considering only the two boar groups, excellent classification accuracy (100%) was achieved. This indicates that despite the change in heater power settings, the REIMS spectra are very reproducible. However, it should be noted that when applying a

lower heater power, the obtained OPLS-DA model showed less reliable predictive abilities as $Q^2(Y)$ and $R^2(Y)$ were 0.291 and 0.939, respectively, most likely originating from the decrease in sensitivity. This was confirmed in a permutation test, which indicated that the model fits the data well but cannot be used to accurately predict new observations (Fig 9). Consequently, careful consideration should be given to the MS settings in order to ensure the validity of each model.

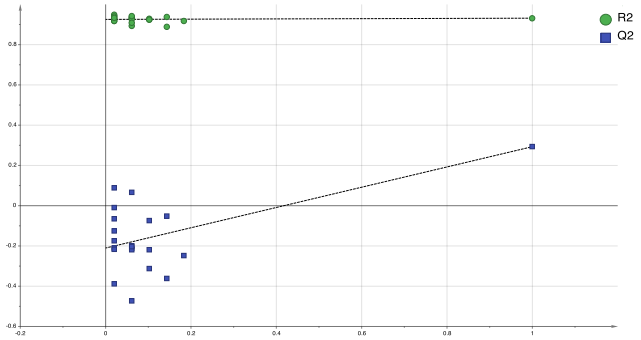

Fig 9 Validation experiment: Permutation plot as a validation criterion for an orthogonal partial least-squares discriminant analysis model for a dataset containing negative (untainted boar) and positive (tainted boar) neck fat samples in negative ionisation mode (n = 100) with a cone voltage setting of 60 V.

3.5. Mass spectral content

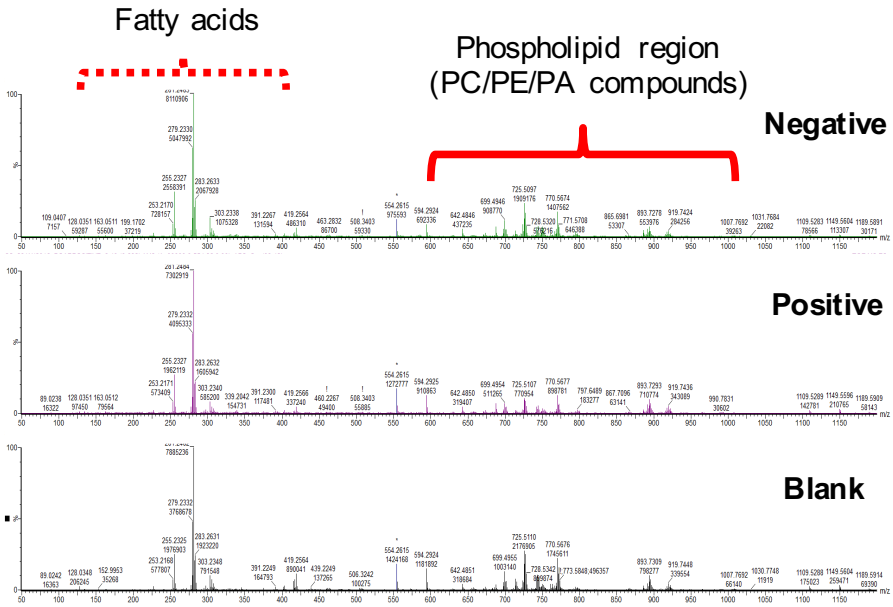

Fig 10 Mass spectral fingerprint for negative (untainted (n=50), positive (tainted) (n=50) boar and blank (gilt) (n=50) neck fat samples obtained in negative ionisation mode.

Untargeted profiling of neck fat samples revealed differences in spectra between gilts, tainted and untainted boars (Fig 10). In order to situate the spectral differences between the latter groups, putative identification was performed of the most abundant ions in the fatty acid and phospholipid region, providing class information of the compounds. To this end, the selected ions were cross-referenced to the LipidMaps (www.lipidmaps.org) and Lipidblast database (http://fiehnlab.ucdavis.edu/projects/LipidBlast). This search was based on the obtained accurate masses and a mass tolerance window of ± 0.01 Da was applied. In most cases, the *m/z* value could not be assigned to one single compound. Nevertheless, the lipid classes could be revealed. The spectral differences in negative ionisation mode between the gilt, boar taint positive and negative group were mainly situated in the fatty acid and phospholipid region of the obtained mass spectra. Moreover, primary clustering between the spectra of the boar taint positive and negative group was observed (Fig 11).

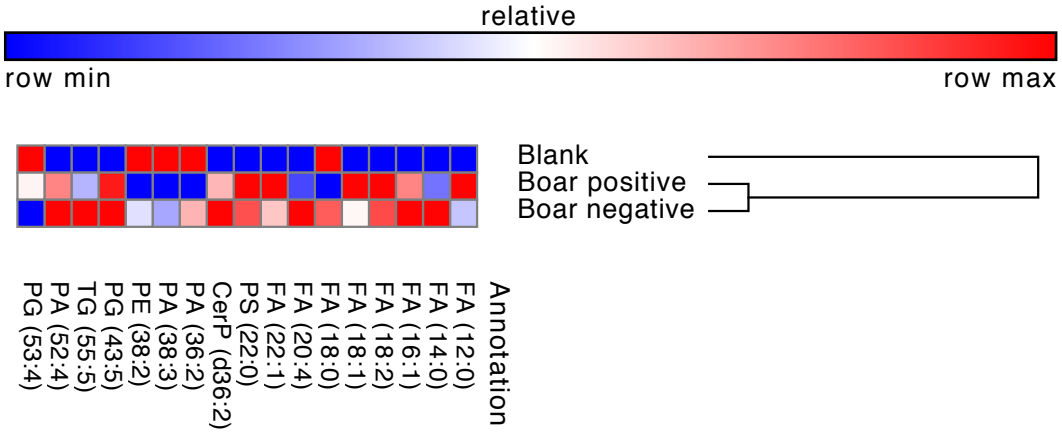

Fig 11 Heat map (GEN-E software, http://www.broadinstitute.org/cancer/software/GEN-E/index.html) visualizing a selected number of putatively identified compounds in blank (gilt) (n=50), positive (tainted) (n=50) and negative (untainted) (n=50) neck fat samples, with hierarchical clustering of the different samples.

In general, monounsaturated fatty acids (MUFAs) (16:1, 18:1, 22:1), tentatively identified as palmitoleic acid, oleic acid and erucic acid, respectively, were predominantly present in the boar taint positive group. Intermediate levels were observed in the boar taint negative group and the lowest levels in the blank or gilt group (Fig 11). Similarly, also polyunsaturated fatty acids (PUFAs) (18:2 &

20:4), tentatively identified as linoleic acid and arachidonic acid, were mostly abundant in the boar taint positive and negative groups. The saturated fatty acids (SFAs) lauric acid (12:0) and myristic acid (14:0) on the other hand were predominantly present in the boar taint positive and negative groups, respectively. Furthermore, stearic acid (18:0) was mainly present in the blank or gilt group in comparison to the two boar groups. These differences are most likely associated with the differences found in the phospholipid region of the mass spectra as the majority of the signal intensity in the fatty acid region originates from fragmentation of these phospholipids (Fig 11). Recent studies demonstrated similar trends in fatty acid composition in boars, gilts and surgically castrated pigs [25, 39, 42]. Pauly et al. [39] found a significantly lower amount of SFAs and higher amount of PUFAs in entire males in comparison to surgically castrated pigs and immunocastrates. Similar trends were reported by Mackay et al. [43], who observed a decrease of 21% in n-6-PUFAs in castrates in comparison to boars. Furthermore, in a recent study, significantly higher amounts of total PUFAs were observed in boar carcasses with low levels of AEON (23.4%) in comparison to boar carcasses with high levels of AEON (19.7%). This was due to increased levels of linoleic acid and alpha-linolenic acid [42]. However, these results were not conclusive as higher PUFA and MUFA levels were observed in highly tainted fat samples by Liu et al. [25]. The mechanism behind these differences in fatty acid composition lies in the regulation of fat deposition and differences in lipid synthesis and metabolism. Indeed, recently an increased expression of stearoyl-CoA desaturase and delta-6-desaturase, two enzymes involved in lipid synthesis, was demonstrated in boar adipose tissue in comparison to castrates [43]. The latter enzymes are responsible for the formation of unsaturated fatty acids, explaining the higher amount of PUFA found in boars. Not only differences in lipid composition between boars and gilts were observed but also significant reciprocal differences between boars with high and low boar taint levels [25, 42, 44]. Although the mechanisms behind the influence of high SK and AEON levels on lipid synthesis and metabolism are not completely unravelled, it has been reported that high SK levels induce CYP2E1 activity, an enzyme involved in lipid peroxidation, consequently lowering PUFA levels in adipose tissue. High levels of AEON on the other hand inhibit gene expression of CYP2E1 and block

induction of the latter by SK [45]. Since phospholipids are partly composed out of fatty acids, alterations in fatty acid composition can also manifest itself in the phospholipid region [42]. Based on the differences of the latter between gilts and boars but more importantly, boars with high and low boar taint levels, lipid profiles could explain the observed discrimination between these three groups and classify carcasses as tainted or untainted.

4. CONCLUSIONS

The results obtained in this study demonstrated tainted carcasses could be correctly classified by an untargeted approach. This makes REIMS suitable not only for discrimination between gender samples (gilt versus boar) but also for discrimination within gender (tainted versus untainted boars). This discrimination originated from alterations in lipid profiles, mainly situated in the fatty acid and phospholipid region. However, to this end, a fingerprinting approach was necessary as no reliable candidate biomarkers could be identified. Moreover, as REIMS eliminates extensive sample pre-treatment procedures, analysis takes under 10 seconds, which makes it the first technique that enables *in-situ* detection of boar taint combined with highly accurate classification. Finally, in view of implementing this untargeted approach in an at-line environment, the MVA software further empowers the applied technology as it enables real time recognition of unknown samples through screening against a known database. For this reason, this new analytical *in-situ* monitoring platform is very promising for other applications in food safety or quality, whereby rapid characterization of food products is requisite.

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CHAPTER VIII

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

1. MAIN RESEARCH FINDINGS AND SCIENTIFIC CONTRIBUTIONS

The general aim of this research was to study the valorisation of tainted boar meat and develop a fast and reliable detection method for boar taint at the slaughter line. To achieve these goals, different objectives were defined. The accomplishment of these objectives has been extensively described in chapter II to VII and the main findings are summarized in Fig 1. A detailed discussion on each of these chapters and integrated vision on the entire thesis and future perspectives will be provided in this chapter (VIII).

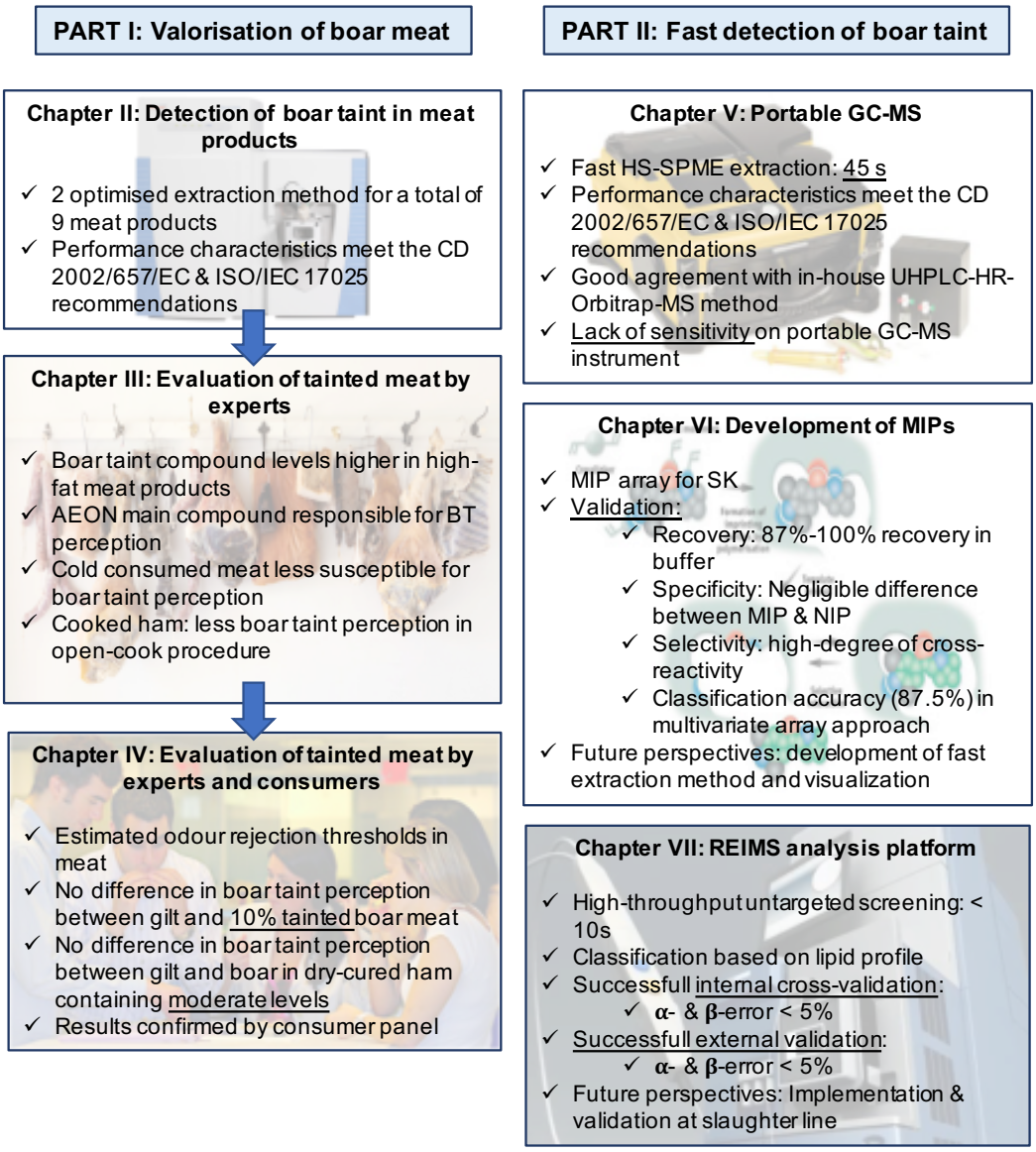

Fig 1 Schematic overview of the main research findings of this PhD study.

1.1. PART I: Valorisation of tainted boar meat

1.1.1. Detection of boar taint compounds in meat

Studies evaluating the influence of boar taint on the acceptance of boar meat mostly report on the levels of AEON and SK in adipose tissue. However, little is known about the boar taint compound levels in fresh meat cuts and processed meat products. In order to estimate the effects of tainted boar meat on pig industry and in particular meat industry, it is mandatory to gain more insight in the acceptable levels of the boar taint compounds in meat and meat products. To this extent, during this work, a UHPLC-HR-Orbitrap-MS method was developed for the quantification of boar taint in 9 different meat products (Chapter II). Due to the diverse properties of the meat products included in the study, 2 sample preparation methods were optimized; one method for the leaner meat cuts and products (cooked ham, minced meat, tenderloin, bacon, cutlets, blade loin, dry-cured ham) and a second protocol for meat products with a higher fat content (dry-fermented sausage, liver paste). Three existing protocols were used as a basis and were further optimized to fit the different sample matrices included in this study [1-3].

For all sample matrices and all three compounds, excellent validation results were obtained. The method showed a very high sensitivity for the detection of the boar taint compounds (LOQ: 5 – 10 µg kg^{-1}). Consequently, this method was considered fit-for-purpose for the quantification of the boar taint compounds in meat products. In addition, to illustrate its applicability, cooked ham and dry fermented sausage samples were analysed. The boar taint compound levels obtained in the meat fraction of the products were significantly lower in comparison to the analysis of neck fat samples of the same carcasses. This is most likely due to the distribution and accumulation of the boar taint compounds in adipose tissue. Moreover, these compounds demonstrate relatively high partition coefficients (LogP IND: 2.14; LogP SK: 2.60; LogP AEON: 4.9), rendering them lipophilic [4, 5]. In general, higher levels are observed in meat products with a high intrinsic fat content (dry fermented sausage, minced meat, blade loin, bacon), in comparison to leaner meat products (ham, cutlets, tenderloin) [6]. Furthermore,

next to the intrinsic fat content of meat products, reduction of the boar taint compound levels in meat may also be influenced by production processes. For example, Coker et al. demonstrated that AEON levels decrease in the *longissimus dorsi* muscle after curing [7]. Also different *Lactobacillus species*, typically added during fermentation of meat products, have been identified to catabolise SK and thus lower SK levels in the final product [8]. Despite the pronounced differences in boar taint compound levels between the lean part of the meat product and neck fat of the carcass, overall strong correlations ($r > 0.80$) were observed between neck fat and the lean meat part of the products. Only for cooked ham, rather low correlations for AEON ($r = 0.55$) were reported by Wauters et al. [6].

1.1.2. Evaluation of boar meat by trained expert panels and consumers

The question to use either trained assessors or consumers for sensory evaluation has been the subject of an on-going debate. Trained assessors are traditionally selected based on their sensory acuity for basic tastes, odours and textures, e.g. boar taint. After selection, these assessors are trained to describe the sensory characteristics of a product in a reliable and repeatable manner [9, 10]. For this purpose, a standardized training protocol is of particular importance [11]. Consumers on the other hand are not capable to describe specific sensory characteristics, as they review a product as a whole. Moreover, traditionally, consumers have not been regarded to evaluate sensory attributes reliably. However, the use of consumers for sensory evaluation can be useful to estimate the performance of a product in the market, as they are representative of a target population. Consequently, the choice for either a trained expert or consumer panel for sensory evaluation must be well considered [9, 10].

In the specific case of boar taint, trained experts are indispensable to describe boar taint related attributes in meat products. In order to achieve this, the determining factor for expert selection is their AEON sensitivity, as part of the population shows anosmia to AEON [11, 12]. In this context, trained experts with high sensitivity to AEON may be considered as representative for the most sensitive consumers in a population. For this reason, based on the sensory outcome of a trained expert panel, a first assessment of the influence of boar taint on the appreciation of a meat product was made in

chapter III and IV. Afterwards, additional consumer tests were conducted in chapter IV to estimate the potential of meat products, derived from tainted boar meat, on the market.

In chapter III, evaluation of meat products by trained experts demonstrated the negative impact of both AEON and SK on the sensory perception of meat. Furthermore, experts were able to distinguish between the presence of AEON and SK, whereby mostly the presence of AEON was associated with a negative sensory experience. These results indicated that AEON and SK have a different relative contribution to the sensory perception of boar taint in meat products. Also in literature, the presence of AEON was found to be of relatively higher importance in comparison to SK; however, only in case panellists or consumers sensitive to AEON were included in the study [13, 14]. Therefore, the effect of AEON on the sensory perception of boar meat may be overestimated compared to real-life. However, in another study using trained experts, a stronger effect of SK was observed [15]. Different reasons for these inconsistent results between studies can be put forward. First, the difference in sensitivity of trained panels may be attributed to differences in outcomes between studies. This stipulates the need for harmonized training protocols [12, 16]. Second, differences in boar taint compound levels and their relative contribution to samples may add to the inconsistency in results. Indeed, it has been shown that the presence of AEON or SK in meat has a negative influence on the sensory perception, depending on the levels of the latter compounds. Furthermore, in case of simultaneous prevalence (if both AEON and SK are high), boar taint compounds may even enhance the perception of one another [17]. Accordingly, it should be encouraged to consistently report the boar taint compound levels measured in samples included in the study design. Finally, in an ideal situation, the number of samples should be increased in order to improve the power of the study design and gain a full understanding of the influence of both boar taint contributing compounds and their concentrations on the sensory perception of meat products. However, due to budgetary reasons, this can often not be achieved.

The difference in boar taint perception between meat products was already reported [18]. However, most studies investigated the influence of boar taint on 1 or 2 products. Due to the many differences

in applied methodology between studies (sensitivity of panellists, training of panel, boar taint compound levels in carcasses, type of evaluation scale, etc.), comparison is difficult and may lead to biased results [19]. For this reason, in chapter III, 8 different meat products (minced meat, cutlets, bacon, blade loin, tenderloin, cooked ham, dry-cured ham, dry fermented sausage), for which ingredients were derived from the same boars, were evaluated. Overall, meat products that were consumed cold (cooked ham, dry-cured ham, dry fermented sausage) experienced less influence of boar taint on their sensory perception. For minced meat and tenderloin, an intermediate influence of the presence of boar taint was observed. Finally, the effect of boar taint on the sensory perception was most pronounced in cutlets, bacon and blade loin. The results obtained in chapter III indicated that the perception of boar taint is affected to a certain extent by processing, but cannot be completely eliminated. A first factor was the temperature at which meat products were consumed. This can be explained by the release of the (semi)-volatile boar taint compounds AEON and SK when meat and/or fat of boars is heated [18, 20, 21]. Also, results indicated that the higher the fat content, the more boar taint is perceived in meat products, as the boar taint compounds are relatively lipophilic and accumulate in adipose tissue [6]. Finally, also production processes affected boar taint perception. For example, in dry fermented sausage, fermentation by acidification through addition of cultures of *S. xylosus and S. carnosus* has proven to effectively reduce boar taint perception [22]. In dry-cured ham, this effect may be explained by oxidation of the boar taint compounds in the fat fraction during the drying process and under the influence of the pro-oxidative effect of salt [23]. Finally, in cooked ham, the boar taint compound levels may decrease through evaporation during the cooking process [18, 20, 21]. This effect was most pronounced when using a cooked-in as compared to an open-cook procedure. The results obtained in chapter III indicated that there were differences in boar taint perception between different meat products. More research is necessary to identify process specific parameters for meat products able to reduce boar taint compound levels or mask boar taint perception.

In order to investigate the effect of production processes on the reduction of the boar taint compound levels in meat and their relation to consumer acceptance, it is advisable to determine rejection threshold levels for the boar taint compounds in different meat cuts and products. Based on the results in Chapter III, 3 meat products (minced meat, dry fermented sausage and dry-cured ham) with high potential for masking boar taint were selected, and their acceptability was further investigated (Chapter IV). First, for each of the latter meat products, rejection thresholds for the boar taint compounds in meat were estimated by ROC analysis, based on the sensory evaluation by a trained expert panel. The obtained rejection thresholds were found to vary between meat products (AEON: 121 – 342 µg kg^{-1}; SK: 44 – 83 µg kg^{-1}). Furthermore, the estimated thresholds revealed good sensitivity (AUC > 0.753); however, in some cases, low specificity (AUC < 0.500) was observed, indicating the need for a larger dataset in order to make a more accurate estimation of rejection thresholds for meat products. Second, as the results obtained in chapter III indicated that boar taint perception could not be completely masked by different production processes, the effect of diluting tainted meat was investigated. For minced meat and dry fermented sausage, tainted meat was mixed with blank (gilt) meat in a 1 over 9 ratio, as a maximal mixing percentage of 10% may be anticipated based on the natural occurrence of taint boars in Belgian and Dutch farms as assessed by the human nose method [24, 25]. For dry-cured ham, moderately tainted carcasses (sensory score 2) were selected, and the effect of dry-curing in relation to the estimated rejection thresholds was evaluated. Results showed that no significant differences in sensory performance characteristics were observed by a trained expert panel between minced meat and dry fermented sausage containing 10% tainted meat and blank meat (gilts), indicating the effectiveness of diluting tainted meat. Evaluation by consumers confirmed the beneficial effects of this strategy on the perception of boar taint, as no preference was observed for blank (gilt) meat. As a result, no impairment of likeability is expected when minced meat and dry fermented sausage containing 10% tainted meat were to enter the market. However, even though diluting tainted meat may be effective to eliminate boar taint perception, the question remains on how to valorise clean meat cuts and more expensive parts of the carcass such as hams, loins, etc.,

which cannot be used as raw material for the production of processed meat products. In that sense, it would be of interest to determine rejection thresholds for each meat cut separately, in order to valorise the complete boar carcass. Another strategy that may be applied, but remains to be investigated for boar taint, involves the addition of odour-absorbing materials to packaged foods. Indeed, commercialized odour-absorbing sachets, e.g. MINIPAX® and STRIPPAX® (Multisorb technologies, USA) have been used to absorb the off-odours mercaptane and H_2S in packaged foods during distribution. Also the molecular-sieve technology, a material consisting of a crystalline zeolite known as 'Smellrite/Abscents', has effective odour absorbing properties, as it traps odours within its molecular structure [26].

1.2. PART II: Development of fast and reliable detection methods for boar taint at the slaughter line

1.2.1. Fast HS-SPME-GC/MS

To accommodate the encountered issues of at-line boar taint detection, the use of portable GC-MS was pursued in chapter V, as these instruments are especially designed to operate under extreme and harsh environmental conditions, e.g. the slaughter line. Furthermore, fast sampling and extraction of boar taint compounds, which is often regarded as laborious and rate-limiting, was achieved by using the soldering iron to effectively release the boar taint compounds from neck fat and SPME fibres to extract the boar taint compounds. As such, remarkably fast extraction was achieved (45 s). Despite the fact that extraction of boar taint compounds occurred as controlled as possible by placing the SPME fibre in close proximity of the soldering iron (1 cm) and working under a fume hood to allow constant air flow during extraction, high RSD% values (35 – 50%) were obtained without the use of internal standards. These results emphasized the need for standardization of the analysis conditions to minimize variation of the results, especially when working in pre-equilibrium circumstances during extraction. This could possibly be improved by for example using a custom-made extraction device to reproducibly extract boar taint compounds from a fat matrix. The favourable results that were

obtained using such device were already demonstrated by Ruiz et al. for the extraction of aroma compounds from dry-cured ham [27].

After development of a fast extraction procedure and successful validation on a benchtop GC-MS instrument, the method was transferred to a portable GC-MS instrument. A very fast method with run-to-run time of 3.5 min was developed, which allows high-throughput of samples providing a first result after 4 min 15 s, followed by sequential results every 3.5 min. However, due to matrix interference and ion suppression effects, sensitivity of the portable GC-MS instrument lacked, as IND and SK could only be detected (S/N > 3) from 1000 and 2000 µg kg^{-1}, respectively. Moreover, AEON could not be detected, even at a fortification level of 10,000 µg kg^{-1}. The construction of the narrow bore column, characterised by a very small diameter (0.1 µm) contributed to this lack of sensitivity as this is associated with a small sample capacity [28]. To increase sensitivity, several adaptations to the method could be implemented, such as derivatisation and purge-and-trap [29, 30]. However, given the practical limitations encountered at the slaughter line, the latter adaptations would overreach the point-of-control analysis goal. In addition, apart from the lack of sensitivity, one analysis still took 3.5 min, which is too long for the at-line detection of boar taint, as on average 600 carcasses are slaughtered each hour, which leaves approximately 6 seconds per analysis.

1.2.2. Molecularly imprinted polymers

Since the beginning of research on boar taint, numerous methods for its detection have been developed. Among these methods, a number of immunological methods for measuring AEON have been reported [31-36]. However, efforts on the detection of SK by means of an immunoassay remain limited, which is most likely due to its small size and limited functionality, rendering it a poor epitope [37, 38]. The use of these immunoassays was mainly intended for research purposes. However, similar assays have been successfully used for different food screening applications, requiring a rapid result. In this light, the development of a fast screening method seemed promising.

Immunoassays rely on the use of antibodies directed against specific antigens or analytes of interest. However, they are characterised by some drawbacks such as poor stability in harsh environmental conditions and can only be developed against target molecules that represent antigenic properties. Molecular imprinting is an emerging technique that focusses on synthesizing artificial recognition elements. The main advantages of MIPs are their superior thermal and chemical resistance in comparison to antibodies. Moreover, although challenging, MIPs can be developed against any target molecule [39, 40]. This was of particular interest for boar taint, as boar taint compounds are relatively small and present limited functionalities. The development of a MIP based array for the detection of boar taint was investigated in chapter VI.

One of the difficulties that were encountered during the development of MIPs against SK, was their poor specificity. This was mainly due to the lack of imprinting effect because of the small size and low functionality of SK. Indeed, SK only contains one secondary amine function that can interact with the functional monomers during polymerization, which hampers the formation of a strong pre-polymerization mixture. In addition, the use of organic solvents as a porogen for the development of MIPs favours the formation of H-bonds between SK and the functional monomers, which are generally regarded as relatively weak non-covalent interactions [41, 42]. Consequently, mostly non-specific interactions and a high degree of cross-reactivity were observed in batch-rebinding experiments. To improve the intrinsic specificity and selectivity of the MIPs against SK, the pre-polymerization complex should be better defined. This could possibly be achieved by applying a grafting technique. As such, the template molecule is linked to a carrier and has a fixed 3D orientation, whereby interactions during polymerization occur less randomized in comparison to the precipitation polymerization approach that was premised in this study [43]. Another strategy that may be followed is the sacrificial-spacer approach. This approach implies the formation of a covalent bound between the template and functional monomer, which is by definition stronger and more specific than the formation of H-bounds when using a non-covalent approach. Afterwards, the covalent bound is hydrolysed and rebinding of the target molecule to the MIP occurs in a non-covalent manner. This approach, by means of the

formation of silyl ether and carboxylic esters, has been successfully applied to improve the specificity of MIPs towards poorly functionalized N-heterocycles such as pyridine [44-46].

In this study, the lack of selectivity was counteracted by combining different MIPs in a fingerprinting approach, which made it possible to discriminate between different closely related structure analogues. As such, good selectivity and specificity could be obtained. Application of the MIPs on extracts of fat samples demonstrated the potential of the MIP array to detect SK in a biological matrix. However, to make this array applicable outside a laboratory environment, more in-depth research on the development of an adequate detection method is required. In this study, UHPLC-MS was used to evaluate the amount of analytes bound to the MIPs. However, on-site, other possibilities for the detection should be pursued, as for example the use of colloidal gold or quantum dots linked to the MIP particles [47]. Besides, also the extraction method for the boar taint compounds in adipose tissue should be further optimized and simplified. Although the extraction method used in this study was straight-forward (melting in microwave and extraction with methanol), it would not be applicable at the slaughter line.

Finally, although the development of MIPs for a screening assay was an interesting approach to follow, it proved inadequate for the intended purpose of the fast detection of boar taint at the slaughter line. In terms of specificity and selectivity, superior techniques are available, e.g. GC-MS. Likewise, also MIPs developed against AEON (data not included in this PhD study) did not render the desired results in terms of recovery, specificity and selectivity, which further hampers the use of MIPs in a fast screening method for boar taint. Added to this, a fast screening method would still lack high-throughput for implementation at the slaughter line, but may be valuable for pre-mortem detection of boar taint, e.g. at farm level, in for example saliva.

1.2.3. Rapid evaporative ionisation mass spectrometry

The majority of analytical methods developed for the detection of boar taint focus on measuring the three known boar taint compounds AEON, SK and IND. However, it is questioned whether the measurements of these three compounds is sufficient for detecting boar taint. Indeed, research showed that the correlation between chemical and sensory analysis of boar taint is rather low, namely 0.30, 0.36 and 0.17 for AEON, SK and IND, respectively [48]. Optimisation and validation of both sensory and analytical methodologies increased these correlations to 0.51, 0.42 and 0.40 for AEON, SK and IND, respectively [49]. Still, it is hypothesized that other unknown compounds may play a role in the occurrence of boar taint. In addition, the lack of mutually recognized odour thresholds in neck fat complicate the development of standardised methods for the detection of boar taint [50]. Furthermore, the divergent physicochemical properties of AEON on the one hand and the indolic compounds on the other, hamper the development of a fast method for the simultaneous detection of these compounds [50].

Among the instrumental methods, different sensor techniques have been applied for detecting boar taint. The working principle of these sensors was based on detecting the difference in profiles between tainted and untainted samples, which was correlated with their AEON and/or SK content [51-55]. Recently, untargeted RAMAN spectroscopy was applied for the fast detection of boar taint, and demonstrated a performance accuracy of 81% [56]. As a result, untargeted detection approaches seem to be promising for the fast detection of boar taint. Although RAMAN analysis only took a couple of seconds, data acquisition lasted for 20 minutes, hampering high-throughput detection [56]. Furthermore, the BOARCHECK report regarding the study on rapid methods for boar taint at the slaughter line stipulates a maximum relative uncertainty of 10%. Consequently, RAMAN still needs further optimization to improve its performance accuracy [57].

Recently, REIMS surfaced as a promising technique for point-of-control monitoring of samples. It is an AMS technique enabling direct ionisation and instantaneous mass spectrometric analysis from the

sample, thus circumventing sample clean-up, extraction, and chromatographic separation. Originally, REIMS was conceptualised for *in-vivo* identification of tissues during medical interventions, but has also found applications in bacterial identification and detection of meat and fish fraud [58-61]. In chapter VII, the use REIMS was explored for the fast detection of boar taint.

Chemometric data analysis demonstrated that it was feasible to discriminate between tainted, untainted and gilt neck fat samples based on the mass spectrometric fingerprints. The discrimination potential was hypothesized to rely on differences in lipid profiles between the three groups of samples. Database screening indicated that the unsaturated fatty acids were more abundant in (un)tainted boar samples in comparison to gilt samples. MUFAs were predominantly present in the tainted sample group. The obtained chemometric models were successfully validated and revealed excellent predictive properties (classification accuracy > 99%) of the REIMS technique for the detection of boar taint in neck fat tissue. Compared to other studies applying REIMS for the detection of fish and meat fraud, similar accuracy (97-100%) was obtained [58, 59].

The results obtained in chapter VII demonstrated that the REIMS technique is the most promising route for the fast detection of boar taint as very accurate results can be acquired within seconds. The AMS principle combined with software for *in-situ* classification of new samples render it an extremely promising platform to monitor the presence of boar taint in carcasses. However, in view of implementing this technique at the slaughter line, some bottlenecks should be considered. The Xevo G2-XS Q-TOF MS instrument (Waters, Wilmslow, UK), used in this study, is intended for in-laboratory use. As this instrument is highly sensitive to fluctuations in environmental conditions, in terms of humidity and temperature, installing this instrument at the slaughter line will be a challenge. Other bottlenecks that should be overcome rely in the monopolar character of the iKnife hand-held device that was used in this study. Indeed, for safety issues, the slaughter line is equipped with an electrical grounding. As the iKnife device consists of a monopolar cutting device, its use at the slaughter line would cause an electrical short circuit. Consequently, the iKnife device should be redesigned to a

bipolar cutting device, or the use of an infrared (IR) laser may be opted for at-line use. Finally, next to these practical considerations, the developed models should also be validated and trained at the slaughter line to guarantee the validity of the obtained results in this study.

2. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

2.1. Alternatives to the surgical castration of pigs

In 2010, European Member States voluntarily intended to abandon the surgical castration without use of analgesia and/or anaesthesia by 2012, and by 2018 to completely convert to alternatives for surgical castration. However, putting this declaration of intent into practice appears difficult. Indeed, transition to alternatives such as the production of immunocastrated and entire male pigs remains limited. Despite this intention agreement, only in a few Member States (Ireland, The Netherlands, Portugal, Spain and The UK) less than 20% of the pigs are surgically castrated. In Belgium, approximately 67% of pigs is still surgically castrated [62]. In the remaining Member States, surgical castration is performed on over 80% of pigs, with or without anaesthesia and/or analgesia. This lack of initiative to switch to alternatives for castration can mainly be explained by an insufficient market acceptance for immunocastrated and entire male pigs. In exporting countries, including Belgium, transition to immunocastration or rearing entire male pigs is hampered due to the risk on boar taint in entire male pigs and a sceptical attitude towards immunocastration as it is often regarded as a chemical intervention that could pose a potential risk to public health. Since the international trade does not allow any tainted boar meat and some Member States refuse to import carcasses from immunocastrated pigs, no national initiatives are taken by individual Member States to impose a complete prohibition on surgical castration. This emphasizes the need for a legal framework on European level in order to prevent compromising the competitiveness in pig industry as a consequence of unilateral legislation [63].

In order to increase market acceptance for both immunocastrated and entire male pigs, various difficulties should be addressed. In the case of immunocastration, some optimisation is required in

terms of achieving the best time between the first and second vaccination, and carcass meat quality. To raise entire male pigs, difficulties that need to be addressed include reduction of boar taint prevalence, valorisation of tainted boar meat and implementation of fast detection methods for boar taint at the slaughter line. During the past years, several research initiatives have been taken in Europe to meet the current bottlenecks that are encountered to transition to the production of immunocastrated and entire male pigs. Research projects were mainly focussed on the current status on the surgical castration of pigs (PIGCAS EU study, DG SANTE CASTRUM EU study), optimisation of alternatives to surgical castration on farm level (VLEVACIM demo project, CASPRAK ILVO, VLEVAGEWICHT ILVO), reduction of boar taint prevalence in pig carcasses (CASSEL ILVO-KULeuven, TAINTLESS UGent-ILVO), the development of detection methods for boar taint (DG Sanco BOARCHECK EU study, AT-LINE UGent-VLAIO 131420, BOARLINE UGent LNE, the Danish meat research Institute) and valorisation of boar meat (VLEVAVLEES ILVO, FF BOARVAL UGent-ILVO, FF REDBOAR UGent-ILVO-KULeuven, DG Sanco CAMPIG EU study). As such, progress has been made to render immunocastration and production of entire male pigs as sustainable alternatives for surgical castration and to put these alternatives into practice. Moreover, recently two EU projects, COST: IPEMA and SUSI, were initiated to facilitate the development of integrated solutions for raising entire males and immunocastrated pigs. The specific aims of these projects are to coordinate different research initiatives, fill current knowledge gaps and encourage consultation among actors in pig industry.

Research within this PhD study allowed to gain new insights regarding acceptance of tainted boar meat (FF BOARVAL) and the development of candidate at-line methods that would allow at-line screening of boar carcasses (AT-LINE UGent VLAIO SB 131420). As such, the deliverables of this PhD study contributed to important research gaps, i.e. the lack of understanding of acceptance of boar meat and fast screening methods for boar taint. However, still some future points of attention can be listed. First, the detection of aberrant boar carcasses is hampered by a lack of understanding boar taint. To this end, general consensus on a definition for boar taint and mutually recognized thresholds for the boar taint compounds is necessary to develop and validate accurate analytical methods.

Furthermore, different analytical techniques have surfaced as highly promising for the fast screening of boar taint; however, implementation at the slaughter line still needs to be evaluated. Finally, in this study, a better understanding in acceptance of boar meat was obtained and some masking strategies for boar taint in meat products were identified. Nonetheless, more insights in specific masking strategies are required to allow integral carcass valorisation. A schematic overview of a proposed workflow for identifying, processing and marketing boar carcasses is depicted in Fig 2.

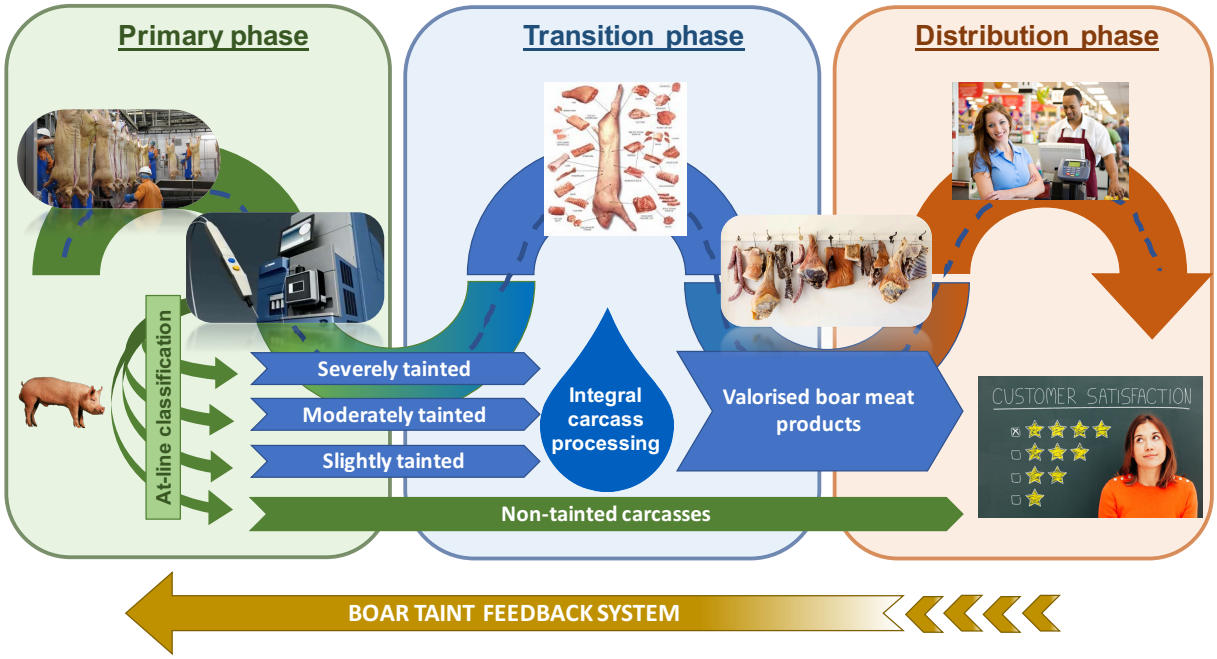

Fig 2 Schematic overview of a proposed workflow for identifying, classifying, processing and marketing of entire male pig carcasses.

2.2. At-line monitoring of tainted carcasses

2.2.1. Mutually recognized sensory thresholds for boar taint perception

Currently, agreement on the sensory thresholds for boar taint compounds is still lacking [50]. This is mostly due to the differences in sensory and chemical methodology applied for boar taint research. Furthermore, also differences in sensory perception between countries, sexes and individuals, and the interaction effect between AEON and SK contribute to this lack of consensus. In literature, several thresholds have been reported, ranging from 500 to 3,500 µg kg^{-1} and 150 to 250 µg kg^{-1} for AEON and SK, respectively [12, 49].

International consensus on these sensory thresholds would lead, not only to an unambiguous understanding of consumer acceptance of tainted boar meat, but is also important for the fast detection of boar taint at the slaughter line. In scientific studies, thresholds are often based on levels of AEON and SK resulting from chemical analysis as the gold standard. However, these thresholds are contradictory to the lower boar taint prevalence that is observed in the industry based on sensory evaluation [49, 63]. Therefore, international consensus on these odour thresholds should be reached in order to accurately select tainted carcasses at the slaughter line. Indeed, a recent study of Mörlein et al. evaluating the 'safe box' approach indicated that 17% of boar samples was at risk for consumer rejection using tentative rejection thresholds of 1,500 and 200 µg kg^{-1} for AEON and SK, respectively. Lowering the applied thresholds to 1,000 and 150 µg kg^{-1} for AEON and SK, respectively, increases the percentage of samples at risk for boar taint to 29%. Consequently, a high number of carcasses would be sorted out at the slaughter line, even though their sensory scores did not always exceed 2, i.e. slightly deviant smell [17]. Extension of this 'safe box' approach taking into account the interaction between AEON and SK in the sensory perception of boar taint may increase the accuracy of the applied model and could be used to develop sorting strategies for at-line boar taint monitoring. As such, defining thresholds for the boar taint compounds would allow categorisation of carcasses into different categories, e.g. slightly, moderately and strongly tainted. Carcasses of each category could then be passed to specific processing routes (Fig 2).

2.2.2. Implementation and validation of rapid detection methods for boar taint at the slaughter line

Fattening entire male pigs is only considered a full alternative to the surgical castration of pigs if tainted carcasses can be sorted out at the slaughter line. To this end, fast analysis methods are requisite as on average 600, but up to 1,200 carcasses can be slaughtered per hour. Up until now, only sensory analysis methods could be applied at the slaughter line due to their high operating speed. However, there was a demand for the development of more reliable methods to detect boar taint at the slaughter line. In

this PhD study, several analytical techniques have been evaluated for the fast detection of boar taint; among them portable GC-MS, the use of MIPs and REIMS.

The use of REIMS showed the most promising for at-line implementation as a high accuracy and in-situ characterization of new samples was achieved. However, prior to the practical use of this technique at the slaughter line, additional research is necessary to overcome some practical issues regarding at-line installation of such delicate technology. First, the apparatus would benefit from adaptations to allow more easy implementation in locations with very high relative humidity (>90%) and low temperatures that can be encountered at the slaughter line. Also, miniaturization and automation of the apparatus may effectuate more easy implementation at the slaughter line. Second, also user safety should be taken into account. As the slaughter line has an electrical grounding and the iKnife cutting device consists of a monopolar electrosurgical knife, a short circuit may occur. Consequently, adaptations to the sampling device are necessary. As such, the development of a bipolar cutting device or the use of laser technology may be preferred. Finally, easy-to-use software and straightforward handling of the apparatus may allow non-analytically trained personnel to operate the instrument and perform the analysis. Apart from technical adaptations to the instrument, the methodology should be validated at the slaughter line to confirm the results obtained in this study. To this end, several factors should be evaluated, e.g. robustness and predictive accuracy of the applied model, biological variance (breed, feed), instrument variance (different operators, sampling methods), etc. In addition, extension of the developed model to classify tainted carcasses in subcategories, e.g. according to boar taint compound levels (semi-quantitative) or according to boar taint compound class (AEON, SK or both compounds), would enable the development of more specific sorting approaches of carcasses upon targeted distribution and processing of these tainted carcasses. Finally, as REIMS provides untargeted analysis, the inclusion of additional quality parameters, e.g. PSE, DFD, may allow the development of an integrated quality monitoring platform for pig meat. These research aims will be further elaborated in the follow-up BOARLINE project (UGent-LCA, Waters Corporation).

2.3. Valorisation of tainted boar meat

In view of the impending ban on surgical castration of pigs, it is expected that the fattening of entire male pigs will gain an increased market share. As a result, also the number of tainted carcasses with which the meat processing industry is confronted will increase. In this study, several strategies were identified that were able to mask boar taint perception. However, apart from diluting tainted meat, complete elimination of boar taint perception was not feasible. Therefore, further research is necessary to completely unravel the influence of different processing techniques on reducing the boar taint compound levels in meat. To this end, the boar taint compound levels should be determined prior and after processing. Also, additional research is requisite in order to enable integral valorisation of tainted carcasses, including more noble parts of the carcass such as loins and hams. As such, detailed workflows and recipes may be suggested for the processing of tainted meat and distribution of each carcass part according to predetermined quality standards, i.e. without odour deviations. This research aim will be elaborated in the follow-up REDBOAR project (UGent-LCA, ILVO, KULeuven). Finally, also specific marketing strategies may cater to the sensitization of retailers to market boar meat as for example ‘purely natural’.

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SUMMARY

Summary

The surgical castration of male piglets has been widely practiced for centuries. The main reason for this practice is the prevention of boar taint, i.e. an off-odour that can be released by heating meat or fat of non-castrated boars. However, since research showed that surgical castration causes pain, even in very young animals, societal pressure against this practice has risen. For this reason, in 2010 a European declaration on alternatives to the surgical castration of pigs was issued, in which participating Member States voluntarily engaged to no longer perform surgical castration without the use of anaesthesia and/or prolonged analgesia by 2012, and in the long run to ban any form of surgical castration by 2018. Two valuable long-term alternatives are immunocastration or the fattening of entire male pigs. However, the main issue associated with the latter alternative is the possible occurrence of boar taint.

In order to prevent an impairment of consumer acceptance due to the presence of boar taint, some measurements have to be taken in order to render the fattening of entire male pigs profitable and sustainable. First, boar taint prevalence should be reduced through adaptations of pig management. Second, alternative processing routes should be mapped to valorise tainted carcasses. Finally, there is an urgent need for fast and reliable detection methods for boar taint at the slaughter line to sort out tainted carcasses. In this regard, the objectives of this PhD study were the evaluation of the sensory acceptability of boar meat (Part I) and the development of a fast and reliable detection method for boar taint (Part II).

Research on the sensory evaluation of boar meat already indicated that the perception of boar taint is more pronounced in case of warm consumed meat or meat products. Moreover, different strategies including smoking, seasoning, fermenting, dry-curing and cooking have been proposed to reduce boar taint compound levels and/or mask the perception of boar taint. However, results between studies vary as the sensory evaluation greatly depends on the sensitivity of trained experts or consumers, the boar taint compound levels in the meat under investigation and other study conditions (evaluation scale, CLT/HUT test, etc.). Consequently, comparison of results is hampered and conclusions remain

inconclusive. For this reason, part I of this PhD study focussed on the sensory acceptance of a wide variety of meat products (chapters II-IV).

The aim of **chapter II** was to develop and validate an accurate ultrahigh performance liquid chromatography (UHPLC) analysis method coupled to high-resolution mass spectrometry (HRMS) for the quantification of boar taint compounds in 9 different meat products (cutlets, bacon, blade loin, tenderloin, dry fermented sausage, cooked ham, dry-cured ham, minced meat and liver paste). This should allow to gain a better understanding of the variance and acceptability of AEON, SK and IND levels in these meat products. Two extraction methods were optimized for the more lean and fatty meat products, respectively. Succesful validation of both optimized methods was achieved for all meat products according to CD 2002/657/EC and ISO/IEC17025 guidelines.

In **chapter III**, research focussed on the sensory evaluation of those different meat products by trained experts. Results demonstrated that in case of a trained expert panel, sensory perception was most affected by high levels of AEON. Furthermore, also differences in boar taint perception were observed between the different meat products, whereby the cold consumed meat products showed the most potential for masking boar taint. Apart from the temperature at which these products were consumed, also production related processes may enable the reduction of boar taint compound levels, e.g. cooking, smoking, fermenting, dry-curing. However, although some promising results were obtained, the perception of boar taint could not be completely masked.

Based on the results obtained in chapter III, three meat products (minced meat, dry fermented sausage, dry-cured ham) with a high potential for processing tainted meat were selected for evaluation by experts and consumers (**chapter IV**). First, sensory thresholds for the individual boar taint compounds were estimated for each meat product, which varied between 121-342, 44-89 and 24-65 µg kg^{-1}, for AEON, SK and IND, respectively. Afterwards, taking into account a natural boar taint prevalence of 0-14% observed on individual pig farms, the dilutive effect of tainted boar meat in a 1/9 ratio on the sensory perception of minced meat and dry fermented sausage was evaluated. Also,

moderately tainted (sensory score 2) carcasses were selected for the production of dry-cured ham. The beneficial effect of diluting tainted meat was demonstrated as no significant difference in general acceptability was observed between gilts and 10% tainted meat by experts as well as consumers. Also dry-curing proved effective for masking boar taint in moderately tainted carcasses, and thus preventing consumer dissatisfaction. Furthermore, the results obtained in chapter IV demonstrated the applicability of the estimated thresholds in meat as a tool for identifying masking and reducing strategies for the perception of boar taint.

In order to systematically direct tainted carcasses to alternative processing circuits, there is an urgent need for a fast and reliable detection method for boar taint at the slaughter line. Over the passed years, several attempts have been made to develop fast detection methods for boar taint for at-line application. However, apart from sensory approaches (soldering iron method) and the Danish colourimetric method, which only takes into account SK and IND content, limited succes was achieved. In this regard, the objective of part II (chapters V-VII) of this PhD study was the evaluation of different candidate techniques for the high-throughput detection of boar taint.

In **chapter V**, headspace solid phase microextraction (HS-SPME) hyphenated to gas chromatography and mass spectrometry (GC-MS) was applied in the development and optimization of a candidate method for the fast and accurate quantification of the boar taint compounds. Remarkably fast extraction (45 s) of the boar taint compounds was achieved by singeing neck fat with a soldering iron in the direct presence of the SPME fibre, which adsorbed the released volatiles. Afterwards, the SPME fibre was introduced to the GC-MS instrument for separation and detection of the extracted volatiles. Validation according to CD 2002/657/EC and ISO/IEC 17025 guidelines demonstrated overall good performance characteristics of the developed method. Moreover, cross-validation with an in-house UHPLC-HR-Orbitrap-MS method showed good agreement, emphasizing the accuracy of the developed HS-SPME-GC-MS method. However, the lack of sensitivity obtained on the portable GC-MS instrument hampered application of the latter method for the high-throughput detection of boar taint.

As an alternative to SPME-GC-MS, also the possibility to apply MIPs in a fast screening assay for boar taint was evaluated. In **chapter VI**, different molecularly imprinted polymers (MIPs) for SK were developed by means of a non-covalent precipitation polymerization approach. Characterisation of the developed MIPs demonstrated very good recovery (> 87%) in buffer solutions with a pH ranging from 3 to 10. Batch-rebinding studies and Scatchard analysis on the other hand revealed the low specificity of the MIPs, indicative of a lack of imprinting effect. Consequently, the MIPs showed a high degree of cross-reactivity towards different SK structure analogues. However, selectivity of the latter MIPs was significantly increased by combining them in an array and applying a fingerprinting approach (classification accuracy 87.5%). This array was succesfully applied on boar neck fat samples (classification accuracy 82.7%), demonstrating the potential application of this array for boar taint screening based on SK content. However, prior to usage, an adequate detection system should be developed and validated. Furthermore, also the extraction method (melting and methanol extraction) should be re-vised in terms of time-management prior to at-line use.

As the use of portable GC-MS and the development of MIPs targeted against SK did not render the desired results for the fast detection of boar taint, in **chapter VII** the application of rapid evaporative ionisation mass spectrometry (REIMS) was explored for this purpose. This new emerging technique circumvents long analysis times by enabling direct ionisation from the sample, combined with mass spectrometric analysis. As such, REIMS only takes a few seconds and guarantees point-of-control monitoring. Untargeted mass spectrometric profiling of pig neck fat samples with the latter technique revealed alterations in lipid profiles between gilts, tainted and untainted boars. These spectral differences were mainly situated in the fatty acid and phospholipid region, whereby monounsaturated fatty acids were predominantly present in the tainted boar group. Polyunsaturated fatty acids on the other hand were most abundant in the boar groups. The obtained mass spectral fingerprints were used to construct classification models (OPLS-DA) for boar taint, which enabled discrimination between gilts, tainted and untainted boars. The obtained OPLS-DA models showed excellent classification accuracy, i.e. 99% and 100% for gilt and boar samples or solely boar samples, respectively.

Furthermore, the obtained models demonstrated outstanding validation characteristics (R^2(Y)=0.872-0.969; Q^2(Y)=0.756-0.917), which were confirmed by CV-ANOVA ($p<0.001$) and permutation testing. External validation confirmed the highly accurate classification potential of the REIMS technique for boar taint (alpha and beta-error ≤ 5%). Consequently, the results obtained in this study make REIMS the first technique enabling highly accurate and fast (< 10s) detection of aberrant boar carcasses, rendering it very promising for at-line implementation.

Finally, in **chapter VIII**, the general discussion and future perspectives of this PhD study were represented. Future research should focus on laying down internationally recognized sensory thresholds for the boar taint compounds, in order to gain a full understanding of consumer acceptance of boar meat and enable at-line classification of carcasses according to valid sensory thresholds. Besides, research should also aim at an integral valorisation of tainted carcasses, including the more noble parts. Therefore, detailed workflows and recipes should be identified for the processing of tainted meat. Finally, in this study, REIMS was identified as a highly promising technique for in-situ classification of boar carcasses. However, prior to at-line implementation, some practical considerations should be overcome. Also, at-line validation of the mehod and applied model should be performed to guarantee the validity of the obtained results. In addition, extension of the developed classification model may enable more specific sorting of carcasses into subcategories. To close, binding legislation and consideration within the pig sector, going from pig farmers to retailers, must ensure the sustainability of fattening entire male pigs as an alternative to surgical castration, both in terms of animal welfare, practical and economical considerations.

SAMENVATTING

De chirurgische castratie van mannelijke biggen wordt al eeuwenlang op grote schaal toegepast. De belangrijkste reden hiervoor is het voorkomen van berengeur, i.e. een storende geur die vrijkomt wanneer vlees of vet van niet gecastreerde varkens verhit wordt. Uit onderzoek bleek dat chirurgische castratie pijn veroorzaakt, zelfs bij zeer jonge dieren, waardoor de maatschappelijke druk tegen deze praktijk sterk is toegenomen. Om deze reden werd in 2010 een Europese intentieverklaring opgesteld, waarin de deelnemende lidstaten zich engageerden om vrijwillig te stoppen met chirurgische castratie zonder verdoving en/of pijnstilling vanaf 2012, en om volledig af te stappen van chirurgische castratie vanaf 2018. Twee waardevolle alternatieven op lange termijn zijn immunocastratie en het afmesten van intacte beren. De grootste bekommernis geassocieerd met het laatstgenoemde alternatief is echter het mogelijk voorkomen van berengeur.

Om negatieve consumentenreacties tengevolge van berengeur te vermijden, moeten enkele maatregelen genomen worden om van het afmesten van intacte beren een duurzaam en winstgevend alternatief te maken voor de chirurgische castratie van varkens. Ten eerste moet de prevalentie van berengeur verlaagd worden door middel van toepassen van verschillende managementstrategieën. Ten tweede moeten alternatieve verwerkingsroutes in kaart gebracht worden om karkassen met berengeur te valoriseren. Ten slotte is er een dringende vraag naar snelle en betrouwbare detectiemethodes voor berengeur aan de slachtlijn om berengeurhoudende karkassen tijdig uit te sorteren. In dit opzicht waren de doelstellingen van dit doctoraatsonderzoek enerzijds gericht op de sensorische evaluatie van berenvlees (Deel I), en anderzijds de ontwikkeling van een snelle en betrouwbare detectiemethode voor berengeur (Deel II).

Onderzoek naar de sensorische evaluatie van berenvlees toonde reeds aan dat de perceptie van berengeur sterk verminderd was in koud geconsumeerde vleesproducten. Bovendien werden verschillende productieprocessen voorgesteld, waaronder roken, kruiden, fermenteren, drogen en koken, om de concentraties van de berengeurcomponenten te verminderen en/of de perceptie van berengeur te maskeren. Omdat sensorische evaluatie echter sterk afhankelijk is van de gevoeligheid

van getrainde experten of consumenten, de concentraties van berengeurcomponenten in het vlees, en andere studieomstandigheden (evaluatieschaal, CLT/HUT test, enz.), kunnen resultaten tussen studies sterk variëren. Bovendien zijn er sterke geografische verschillen in de perceptie van berengeur. Bijgevolg is het vergelijken van resultaten uit verschillende studies moeilijk en blijken conclusies niet eenduidig. Om deze reden richte deel I van dit doctoraatsonderzoek zich op de evaluatie van de sensorische acceptatie van een breed scala aan vleesproducten in Vlaanderen (hoofdstukken II-IV).

Hoofdstuk II beoogde de ontwikkeling en validatie van een accurate ultrahoge performante vloeistofchromatografie (UHPLC), gekoppeld aan hoge resolutie massaspectrometrische (HRMS) analysemethode voor de kwantificatie van de drie gekende berengeurcomponenten in 9 verschillende vleesproducten (kotelet, spek, spiering, varkenshaas, salami, kookham, droog-gezouten ham, gehakt en paté). Dergelijke methode moet toelaten om een beter inzicht te verwerven in de variatie en aanvaardbaarheid van de AEON, SK en IND concentraties in de vleesproducten opgenomen in deze studie. Twee extractiemethodes werden geoptimaliseerd voor de respectievelijk relatief magere en vetrijke vleesproducten. Beide methodes werden succesvol gevalideerd voor alle vleesproducten volgens de CD 2002/657/EC en ISO/IEC 17025 richtlijnen.

De onderzoeksfocus in **hoofdstuk III** richtte zich op de sensorische evaluatie van verschillende vleesproducten (kotelet, spek, spiering, varkenshaas, salami, kookham, droog-gezouten ham en gehakt) door getrainde experten. De resultaten toonden aan dat in het geval van een expertenpanel, de waarneming van berengeur het meest werd beïnvloed door hoge AEON concentraties. Verder werden ook verschillen waargenomen in de sensorische perceptie van berengeur tussen verschillende vleesproducten, waarbij de koud geconsumeerde vleesproducten het meeste potentieel toonden voor het maskeren van berengeur. Afgezien van de temperatuur waarbij deze producten werden geconsumeerd, konden ook verschillende productieprocessen geïdentificeerd worden verantwoordelijk voor de reductie van berengeur in vlees, waaronder koken, roken, fermentatie en

drogen. Hoewel veelbelovende resultaten verkregen werden, kon de perceptie van berengeur slechts gedeeltelijk gemaskeerd worden.

Op basis van de resultaten verkregen in hoodstuk III, werden drie vleesproducten (gehakt, salami, droog-gezouten ham) met het meeste potentieel voor het maskeren van berengeur geselecteerd voor evaluatie door experten en consumenten (**hoofdstuk IV**). Eerst werd een inschatting gemaakt van de sensorische drempelwaarden voor de berengeurcomponenten in elk van deze vleesproducten. Deze waarden varieerden tussen 121-342, 44-89 and 24-65 µg kg^{-1}, voor respectievelijk AEON, SK en IND. Daarna werd, rekening houdende met een natuurlijke berengeurprevalentie van 0-14% waargenomen op individuele varkensbedrijven, het effect van inmenging van berengeurhoudend vlees in een 1/9 verhouding op de sensorische perceptie van gehakt en salami geëvalueerd. Daarnaast werden matige stinkers (sensorische score 2) geselecteerd aan de slachtlijn voor de productie van droog-gezouten ham. Het gunstige effect van het verdunnen van vlees met berengeur kon worden aangetoond, gezien er geen significant verschil werd waargenomen in algemene acceptatie tussen gelten en vlees met 10% inmenging van berenvlees, dit zowel voor getrainde experten als consumenten. Ook het drogen en zouten van ham van matige stinkers bleek effectief voor het maskeren van berengeur en bijgevolg het voorkomen van consumentenontevredenheid. Bovendien toonden de bekomen resultaten de toepasbaarheid aan van de geschatte geurdrempelwaarden in vlees als hulpmiddel voor het identificeren van productiestrategieën voor het maskeren van berengeur.

Om berengeurhoudende karkassen systematisch naar alternatieve verwerkingscircuits te leiden, is er dringend behoefte aan een snelle en betrouwbare detectiemethode voor berengeur aan de slachtlijn. Tijdens de afgelopen jaren werden verschillende pogingen ondernomen om dergelijke methodes te ontwikkelen. Afgezien van de sensorische benaderingen (soldeerboutmethode) en de Deense colorimetrische methode, die enkel rekening houdt met de gehaltes aan SK en IND, werden slechts beperkte successen geboekt. In dit opzicht was het doel van deel II van dit doctoraatsonderzoek de

evaluatie van verschillende kandidaat-technieken voor de high-throughput detectie van berengeur (hoofdstukken V-VII).

In **hoofdstuk V** werd headspace vaste fase microextractie (HS-SPME) en gaschromatografie gekoppeld aan massaspectrometrie (GC-MS) toegepast voor de ontwikkeling en optimalisatie van een methode voor de snelle en accurate kwantificatie van de berengeurcomponenten. Een opmerkelijk snelle extractie (45 s) van de berengeurcomponenten werd bereikt door het nekvet te verhitten met een soldeerbout in de directe aanwezigheid van de SPME vezel, die de vrijgekomen vluchtige componenten adsorbeerde. Nadien werd de SPME vezel geïntroduceerd in het GC-MS toestel waarna scheiding en detectie van de berengeurcomponenten plaatsvond. Validatie volgens de CD 2002/657/EC en ISO/IEC 17025 richtlijnen toonde de globaal goede prestatiekenmerken van de ontwikkelde methode aan. Bovendien kon door cross-validatie een goede overeenstemming worden aangetoond met een interne UHPLC-HR-Orbitrap-MS methode voor de detectie van berengeur. Dit benadrukte de nauwkeurigheid van de nieuw ontwikkelde HS-SPME-GC-MS methode. Het gebrek aan gevoeligheid dat echter verkregen werd op het draagbare GC-MS toestel stond de toepasbaarheid van de methode voor de snelle detectie van berengeur aan de slachtlijn in de weg.

Als alternatief voor SPME-GC-MS werd de mogelijkheid geëvalueerd om moleculair geïnprinte polymeren (MIP's) toe te passen in een snelle screeningstest voor berengeur. In **hoofdstuk VI** werden verschillende MIP's voor SK ontwikkeld met behulp van een niet-covalente precipitatiepolymerisatietechniek. Karakterisering van de ontwikkelde MIP's toonde een zeer goede terugvinding (> 87%) aan in bufferoplossingen met een pH variërend tussen 3 en 10. Herbindingsexperimenten en Scatchard analyse toonden daarentegen de lage specificiteit van de MIP's aan, wat wijst op een gebrek aan inprintingeffect. Bijgevolg vertoonden de MIP's een hoge mate aan crossreactiviteit ten opzichte van verschillende SK-structuuranalogen. De selectiviteit van de MIP's kon echter aanzienlijk verhoogd worden door ze te combineren in een array (classificatienauwkeurigheid 87,5%). Deze array werd succesvol toegepast op nekvetstalen

(classificatienauwkeurigheid 82,7%), wat het potentieel van de array voor de detectie van berengeur aantoont. Voorafgaand aan toepassing van deze array moet echter een adequaat detectiesysteem ontwikkeld en gevalideerd worden. Verder moet ook de extractiemethode (smelten en methanolextractie) worden herzien op vlak van tijdsbesteding teneinde voldoende snelle detectie toe te laten.

Omdat het gebruik van draagbare GC-MS en de ontwikkeling van MIP's gericht tegen SK niet de gewenste resultaten opleverden voor de snelle detectie van berengeur, werd in **hoofdstuk VII** de toepassing van snelle verdampingsionisatie gekoppeld aan massaspectrometrie (REIMS) voor dit doeleinde onderzocht. Deze nieuwe opkomende techniek omzeilt lange analysetijden door directe ionisatie van stalen toe te laten, gecombineerd met massaspectrometrische analyse. Een REIMS analyse neemt hierdoor slechts enkele seconden in beslag waardoor het 'point-of-control' bewaking van stalen mogelijk maakt. Niet-gerichte massaspectrometrische profilering van nekvetstalen onthulde verschillen in lipidenprofielen tussen gelten, stinkers en niet-stinkers. Deze spectrale verschillen waren voornamelijk gelokaliseerd in het vetzuur- en fosfolipidengebied, waarbij mono-onverzadigde vetzuren voornamelijk aanwezig waren in de berengeurpositieve groep. Polyonverzadigde vetzuren waren daarentegen het meest abundant in beide beergroepen ten opzichte van gelten. De verkregen massaspectrometrische vingerafdrukken werden vervolgens aangewend om voorspellende statistische modellen (OPLS-DA) op te stellen voor berengeur, waarbij een onderscheid kon gemaakt worden tussen gelten, stinkers en niet-stinkers. De verkregen OPLS-DA modellen vertoonden een uitstekende classificatienauwkeurigheid, nl. 99% en 100% voor gelten en beren, en enkel beren, respectievelijk. Verder vertoonden de modellen uitstekende validatiekenmerken ($R^2(Y)$=0,872-0,969; $Q^2(Y)$=0,756-0,917), die werden bevestigd door CV-ANOVA ($p < 0,001$) en permutatietesten. Externe validatie bevestigde het nauwkeurige classificatiepotentieel van de REIMS techniek voor berengeur (alfa- en beta-error ≤ 5%). De resultaten die tijdens deze studie verkregen werden maakten REIMS de eerste techniek die uiterst nauwkeurige en snelle (< 10s)

detectie van afwijkende karkassen toelaat, waardoor deze veelbelovend is voor implementatie aan de slachtlijn.

In **hoofdstuk VIII** werden tot slot de algemene discussie en toekomstperspectieven van dit doctoraatsonderzoek weergegeven. Kortom moet toekomstig onderzoek zich eerst en vooral richten op het vastleggen van internationaal erkende geurdrempelwaarden voor de berengeurcomponenten. Dit teneinde een volledig inzicht te krijgen in de consumentenacceptatie van berengeur. Bovendien moeten deze drempelwaarden nauwkeurige classificatie van berenkarkassen aan de slachtlijn toelaten. Hoewel tijdens dit onderzoek reeds veelbelovende resultaten verkregen werden voor het maskeren van berengeur, moet toekomstig onderzoek gericht zijn op de integrale valorisatie van berengeurhoudende karkassen, inclusief de meer edele karkasonderdelen. Het opstellen van gedetailleerde draaiboeken en recepten voor de verwerking van vlees met berengeur moet hieraan tegemoet komen. Tijdens dit doctoraatsonderzoek werd REIMS geïdentificeerd als een veelbelovende techniek voor in-situ classificatie van karkassen. Voorafgaand aan implementatie van deze techniek aan de slachtlijn moeten echter nog enkele praktische belemmeringen overwonnen worden. Daarnaast moeten de ontwikkelde methode en het daarop gebaseerde model ook gevalideerd worden aan de slachtlijn om de kwaliteit van de resultaten verkregen tijdens deze studie te garanderen. Bovendien zou uitbreiding van het bestaande classificatiemodel een meer specifieke sortering van karkassen in subcategorieën mogelijk maken. Tenslotte moeten sluitende wetgeving en overleg binnen de gehele varkenssector, gaande van varkenshouders tot retailers, de duurzaamheid van het afmesten van intacte beren als alternatief voor chirurgische castratie waarborgen. Hierbij moet zowel dierenwelzijn gegarandeerd worden als praktische en economische overwegingen in beschouwing genomen worden.

CURRICULUM VITAE

PERSONALIA

Name	Kaat Verplanken
Address	Blauwesteenstraat 16A, 9070 Heusden
Email	kaat.verplanken@hotmail.com
Mobile	+32(0)484 72 22 69
Date of birth	25th of October 1990
Place of birth	Ghent, Belgium
Nationality	Belgian

EDUCATION

Higher education

Master in Pharmaceutical Care (2011-2013)

Cum Laude

Ghent University

Master Thesis:

Development of a GC-MS based metabolomics approach for gastrointestinal digestion products

Internship: COOPFARMA, Gentbrugge

Bachelor in Pharmaceutical Sciences (2008-2011)

Ghent University

Secondary education

Latin-Sciences (2002-2008)

Sint-Franciscusinstituut, Melle

SCIENTIFIC TRAINING

Doctoral schools in Life Sciences

Transferable skills

Leadership Foundation Course (November 2016)

Project Management (February 2014)

Advanced Academic English Writing Skills (February – April 2014)

Specialist courses

Applied Linear Regression (IPVW: March – April 2015)

Analysis of Variance (IPVW: January – March 2015)

Introductory Statistics – Basics of Statistical Inference (IPVW: November – December 2014)

PROFESSIONAL ACTIVITIES

January 2014 – Current

Laboratory of chemical analysis, Department of veterinary public health and food safety, Faculty of veterinary medicine – Ghent University

VLAIO Scholarship (SB131420):

PhD research: "Development and validation of alternative detection methods for boar taint at the slaughter line"

Funding of the department of environment, nature and energy Flanders (LNE):

BOARLINE: "Development of a fast detection method for boar taint at the slaughter line"

Assistance in project follow-up:

- REDBOAR (FF): Reduction of boar taint in meat products: alternative production processes

- BOARVAL (FF): Impact of tainted boar meat on the perception of meat and meat products in Flanders

Tutor of students:

- Master student Pharmaceutical Care: "Precipitation polymerization in the development of molecularly imprinted polymers for androstenone based on a statistical design"
- Bachelor student Pharmaceutical and Biological Laboratory Techniques: "Untargeted metabolomics as a tool to identify biomarkers for boar taint based on UHPLC-Q-Orbitrap-HRMS"
- Honours program student Veterinary Sciences: "Development of an alternative method for the detection of boar taint at the slaughter line by means of molecularly imprinted polymers"
- Master students Veterinary Sciences:
    - "Comparative study of different options for the surgical castration of piglets"
    - "The detection of boar taint: Current status"

October 2013 – December 2013

Laboratory of chemical analysis, Department of veterinary public health and food safety, Faculty of veterinary medicine – Ghent University

Basic training for scientific research

SCIENTIFIC PUBLICATIONS

Verplanken, K., De Middeleer, G., Dubruel, P., De Saeger, S., Wauters, J., Vanhaecke, L. Molecularly imprinted polymer array for the detection of boar taint compounds, with special emphasis on skatole and indole. *Submitted*.

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*Shared first authors

ABSTRACTS

De Paepe, E., Van Meulebroek, L., Rombouts, C., Huysman, S., **Verplanken, K.**, Hemeryck, L., Wauters, J., Lapauw, B., Vanhaecke, L. (2017). A validated ultra-high performance liquid chromatography coupled to high resolution mass spectrometry multi-matrix polar metabolomics fingerprinting platform. Belgian Metabolomics Day, poster presentation.

Verplanken, K., Wauters, J., Vanhaecke, L. (2017). Betrouwbaar stinkers opsporen aan de slachtlijn. ILVO studiedag castratie, ILVO mededeling, 234, oral presentation.

Verplanken, K., Stead, S., Jandova, R., Van Poucke, C., Claereboudt, J., Vanden Bussche, J., De Saeger, S., Takats, Z., Wauters, J., Vanhaecke, L. (2017). Rapid evaporative ionization mass spectrometry for high throughput screening in food analysis: the case of boar taint. Trends in Food Analysis, oral presentation.

Verplanken, K., Stead, S., Jandova, R., Van Poucke, C., Claereboudt, J., Vanden Bussche, J., De Saeger, S., Takats, Z., Wauters, J., Vanhaecke, L. (2016). Rapid evaporative ionization mass spectrometry for high throughput screening in food analysis: the case of boar taint. BAMST, Meet the Belgian meat researchers, oral presentation.

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Verplanken, K., De Middeleer, G., Dubruel, P., De Saeger, S., Vanhaecke, L. (2016). Synthesis and characterization of MIPs for the capture of the malodorous compound skatole. 9th International Congress on Molecular Imprinting, poster presentation.

Verplanken, K., Wauters, J., Van Durme, J., Claus, D., Vercammen, J., De Saeger, S., Vanhaecke, L. (2016). Development and validation of a rapid detection method for boar taint by means of solid phase microextraction and a person-portable GC-MS. 14th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology, oral presentation.

Verplanken, K., Wauters, J., Van Durme, J., Claus, D., Vercammen, J., De Saeger, S., Vanhaecke, L. (2015). Development and validation of a rapid detection method for boar taint by means of solid phase microextraction and a person-portable GC-MS. BAMST, Meet the Belgian meat researchers, oral presentation.

Verplanken, K., Lenain, P., De Middeleer, G., Dubruel, P., De Saeger, S. (2015). Development and Characterization of artificial receptors for boar taint by means of molecular imprinting. 6th Graduate Student Symposium on Molecular Imprinting, oral presentation.

Verplanken, K., Wauters, J., Vercruysse, V., Aluwé, M., Vanhaecke, L. (2015). Development and validation of a UHPLC-HR-Orbitrap-MS method for the simultaneous determination of boar taint compounds in porcine meat

and meat products. 61st International Congress of Meat Science and Technology (ICOMST), poster presentation.

Wauters, J., Aluwé, M., Vercruysse, V., **Verplanken, K.**, Vanhaecke, L. (2015). Boar taint compound levels in back fat versus meat products: do they correlate?. 61st International Congress of Meat Science and Technology (ICOMST), poster presentation.

Verplanken, K., Wauters, J., Vercruysse, V., Aluwé, M., Vanhaecke, L. (2015). Development and validation of a UHPLC-HR-Orbitrap-MS method for the simultaneous determination of boar taint compounds in porcine meat and meat products. 2nd Symposium on Mass Spectrometry in Food and Feed, oral presentation.

Wauters, J., Aluwé, M., Vercruysse, V., **Verplanken, K.**, Vanhaecke, L. (2015). Boar taint compound levels in back fat versus meat products: do they correlate?. 2nd Symposium on Mass Spectrometry in Food and Feed, poster presentation.

CONFERENCES, WORKSHOPS AND SEMINARS

IPVS Belgian branch, studiedag darmgezondheid. Gent, Belgium, 19th November 2015.

Waters MS Technology days. Brussel, Belgium, 24th September 2015.

Thermo Fischer - Compound Discoverer training course. May 2015.

16th International Symposium on Advances in Extraction Technologies – ExTech 2014. Chania, Crete, Greece, 25th-28th May 2014.

13th International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analyzers (HTC-13) and 3rd International Symposium on Hyphenated Techniques for Sample Preparation (HTSP-3). Brugge, Belgium, 28th-31st January 2014.

Symposium “Meet the Belgian meat researchers” - BAMST 2013. Kortrijk, Belgium, 10th December 2013.

Symposium Trends in Food Analysis VII. Gent, Belgium, 19th September 2013.

DANKWOORD

Dankwoord

Little Alice fell

D

O

W

N

The rabbit hole,

Bumped her head,

And decided to start a PhD

Zo gezegd ving ik na mijn studies een doctoraat aan - *"I almost wish I hadn't go down this rabbit hole – and yet – and yet – it's rather curious, you know, this sort of life!"*. Want inderdaad, doctoreren was een droom, die rooskleurig was maar af en toe dreigde uit te draaien op een nachtmerrie. Aan het einde te zijn gekomen van dit parcours, is het tijd om terug te blikken op de afgelopen 4 jaar. Tijd dus om iedereen te bedanken die me bijstond gedurende dit traject.

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