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Meat analysis: Method validation for boar taint analytics by SPME-GC/MS

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Meat analysis: Method validation for boar taint analytics by SPME-GC/MS

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Introduction

The surgical castration of boar piglets without anesthesia is a current method in most european countries since decades. Castration serves to prevent the development of boar taint which is mainly produced in the testicles. Boar taint is usually perceived as unpleasant in smell and taste and is therefore undesirable (1). Responsible for the boar taint odor are the pheromone androstenone formed in the testes and the protein degradation products indole and skatole (2). According to the present protection of animals act the castration is allowed without numbing of piglets up to an age of seven days. This painful intervention is criticized by animal welfare groups and discussed accordingly in terms of animal welfare. A bill of 2012 for the change of protection of animals act stipulates a ban of the surgical boar's piglet's castration without numbing has to be realised till 2017. A subsequent legislative procedures notes alternatives to boar´s castration without anesthesia in such a short time is not feasible so the adoption of the amendment of protection of animals act was shifted to the beginning of 2019. Thus, a ban on castration of piglets without anesthesia will be enacted in 2019 european level. (4); (5); (6)

Consequently different alternatives to surgical castration without numbing are discussed and examined. One of these alternatives is boar fattening. Disadvantages are potentially possible occurrence of boar taint and thus a lower acceptance of boars in products. Regardless of selecting an adequate alternative there is an urgent requirement for an analysis procedure. To check whether an alternative has brought the desired success, a dependable quantification of odour components must be guaranteed, allowing a judgement. In dependence on an existing analysis procedure of Dr. J. Fischer (7), an adequate analysis procedure was implemented and validated for simultaneous quantification of the boar taint compounds skatole, indole and androstenone in boar's fat on the basis of a headspace-solid phase microextraction-GC/MS with the use of deuterated internal standards (HS-SPME-GC/MS).

The aim of this work was the determination of an optimal SPME fiber for extraction of boar taint causing compounds. For this purpose, different SPME fibers were tested and compared with respect to their extraction performance. As a feature for the extraction efficiency, the peak area of each analyte is used for each fiber.

Theoretical Background

This chapter describes the various boar taint causing compounds and also deals with the theory of solid-phase microextraction.

Boar taint causing compounds

The term «boar taint» describes the off-flavor of pork. This so-called off-flavor is an undesirable occurring odor in pigs and is perceived by consumers in general as sweaty masculine, sweet, urine- and / or fecal-like. In general, the compounds androstenone, skatole and indole have been identified as the main compounds causing boar taint. (2); (8)

Androstenone

The steroid androstenone (5α-androst-16-en-3-one) is a pheromone and an important part of boar taint. As visible in Figure 1, the sex hormone has a steroid structure having 19 carbon atoms and possesses similarities with the androgen testosterone.

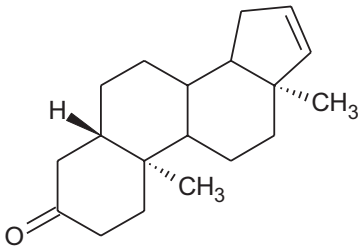

Figure 1: Structure of androstenone

Androstenone (ANON) is formed predominantly after reaching sexual maturity in testicles. The task of the pheromone consists in trigger the sow creating a pairing situation. Due to the lipophilic property androstenone accumulates in fatty tissue primarily. Significant levels of androstenone are therefore finding exclusively in adipose tissue of boars and not in adipose tissue of castrates and sows. (7); (9)

Skatole, indole

Skatole (3-methylindole) belongs to the group of indoles. Skatole (SK) and indole (IND) are main components of boar taint due to their intensive fecal-like odor. These substances have a nitrogen-containing heterocycle (Figure 2 and 3) and indicate a volatile character.

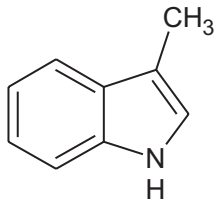

Figure 2: Structure of skatole

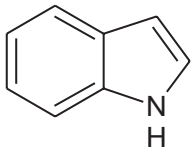

Figure 3: Structure of indole

Both skatole and indole are bacterial degradation products of the amino acid l-tryptophan in the intestinal tract. Skatole is metabolized in a two-stage forming process. Thereby bacterias such as Escherichia coli convert l-tryptophan to indoleacetic acid in the intestine. Subsequently, this compound is metabolized by specific lactobacilli to skatole. (10) Such as androstenone, skatole and indole accumulate in adipose tissue due to lipophilic properties. However, both substances are also weakly hydrophilic because of the amino group. Hence they are also in the meat juice and thus to be found in meat. The n-octanol-water partition coefficient K_{OW}, which describes the ratio of a substance between a polar and a nonpolar phase, is for skatole at K_{OW} = 400:1. The K_{OW} value for androstenone, however, is higher by a factor of 1000, whereby the skatole has a lower lipid affinity (7). Skatole and indole occur in male and female pigs.

Further boar taint causing compounds

It seems that sensory conspicious boars do not show expected increased androstenone-, skatole- or indole levels. This concludes that maybe other materials have influence on the boar's smell (11). A substance with potential influence is ortho-aminoacetophenone (2-AAP). This compound is a metabolite of skatole and has also been identified as atypical aging, an off-flavor of white wine. 2-aminoacetophenone is described as foxy (wet dog), sour or racemose. The structure is similar to indole and shown in Figure 4. (11); (12)

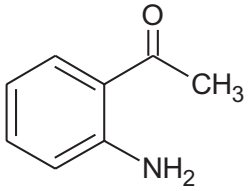

Figure 4: Structure of ortho-aminoacetophenone

Another possible pig odorant is para-cresol (CR). P-cresol is a product of bacterial degradation in the intestine, wherein the starting substrate is the amino acid tyrosine. The smell of this phenolic compound is perceptible even in very low concentrations and is described as the typical stable smell. (13)

Figure 5: Structure of para-cresol

Solid-phase microextraction

The solid-phase microextraction (SPME) is a method for sample preparation and is applied in environmental analysis, food analysis, aroma analysis and forensics. The technique involves the sampling, extraction and sample enrichment in one step and leads the injection into the measuring principle. SPME was developed in 1990 by J. Pawliszyn and is due to the continuous rapid development in automation (autosampler) and isotopic labeling (deuterated internal standards) used in routine laboratory. (14)

For SPME technique a sampler is used. In Figure 6, the sampler is shown. This sampler includes the most important section, the fiber (adsorbent) for extraction. The sampler is attached to a plunger and consists of a stainless steel needle. Around this needle a quartz fiber is fixed, which is coated with an adsorbent. Since the adsorbent has a low mechanical stability, it is shielded in a protection cannula against mechanical influence.

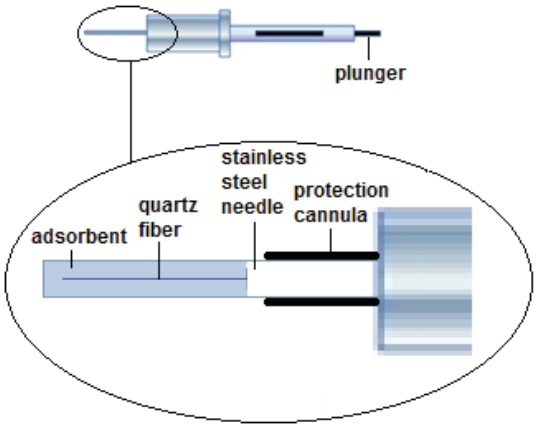

Figure 6: Schematic construction of a SPME sampler (15)

The sampling is divided into three phases which are shown in figure 7. In the first phase a so-called preequilibration takes place (A). In this phase the sample stays in a constant tempered heating block (agitator) at defined temperature, time and agitation. Thus, the analytes between the sample matrix and gas phase are distributed to a constant balance. In the next phase, the extraction takes place at a constant temperature, time and agitation (B). First the protective needle pierces the septum of the sample vessel. The plunger is pressed down, whereby the fiber is extended in the gas phase. From that moment the extraction begins. The sample

molecules from the gas phase are deposited and enriched on the fiber whereas interfering components are not enriched in the best case. After a constant equilibrium distribution of the analytes has set between the gas phase and the fiber, the fiber is withdrawn into the protective cannula, whereby the extraction is terminated. Only thereafter the protective cannula is withdrawn from the septum of the sample vessel. In the last phase desorption takes place (C). The sampler pierces the septum injector and pushes the fiber in the liner from the gas chromatograph. Due to the high injector temperature, the analytes are desorbed from the fiber and reach the GC column. (16)

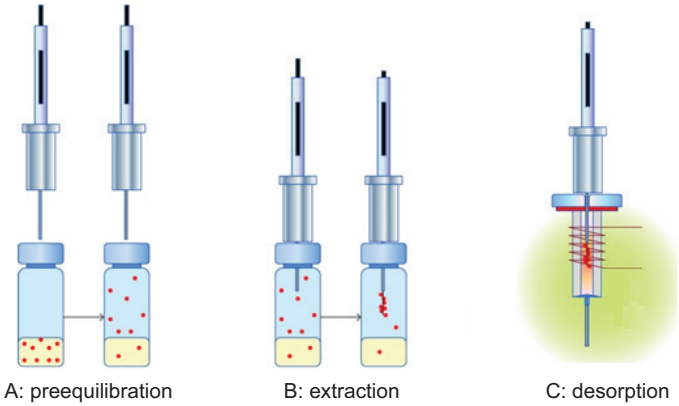

Figure 7: SPME Sampling (15)

During the extraction, there will be two different equilibria. On one hand the analytes and interfering components are in equilibrium between sample matrix and gas chamber, on the other hand, there is a balance of these substances between gas space and fiber. As soon as the fiber is in the gas phase, the dissolved substances in the gaseous phase are attached to the fiber, followed by indirect extraction of the substances from sample matrix. Thereby the substances are in solution equilibrium between sample matrix and gas phase. Henry's law constant describes this balance, thus the tendency of a substance is described to leave a phase (fugacity). Figure 8 shows this behaviour. If there is a substance with a high Henry constant, the transition from sample matrix in gas phase is accomplished faster. This results in a faster extraction (A).
A low Henry constant is followed by a low fugacity, extending the extraction time (B). (17)
During extraction the analytes and interfering components

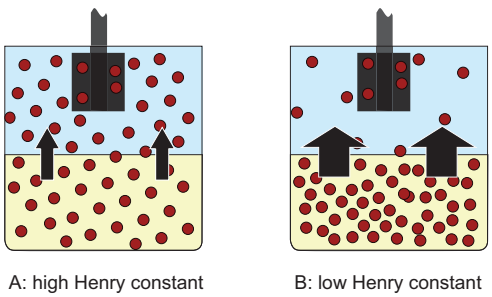

Figure 8: Headspace-SPME of substances with high and low Henry constants (17)

attach at the fiber until equilibrium has been established. It is also to distinguish between the adsorption and absorption equilibrium. The extraction starts with adsorption, the analytes and interfering components deposit on the surface of the fiber. If the diffusion coefficient of the respective substance is low only adsorption takes place. However a high diffusion coefficient leads to absorption. The substance is deposited in the inside of the fiber. This means a higher capacity of the fiber. (17)

In case of SPME technology the fiber is of great importance. Commercially different fiber materials are available in different combinations and layer-thick. Basically polar fiber materials are used for the extraction of polar substances and non-polar substances fiber materials are used for the extraction of non-polar substances. In table 1 the commonly materials are listed in descending polarity: (16); (14)

Fiber material	Polarity
Polyacrylate (PA)	
Carbowax (CW)	
Polydivenylbenzole (PDVB)	
Carboxen (C)	
Polydimethylsiloxane (PDMS)	

Table 1: Fiber materials in descending polarity

Advantages of SPME technique are rapid sample preparation and automation. Sampling, extraction and enrichment can be integrated in one step. In particular, the solvent-free extraction eliminates the need for complicated purification steps, whereby consequently sources of error and the risk of cross-contamination are minimized.

Materials and methods

In this chapter, the materials used, the methods of the analysis principle and the experimental study are presented.

Materials

In table 2 to table 4, the hardware of the GC/MS-system is shown.

Gas chromatograph	Shimadzu GC for Mass Spectrometer GC-2010 Plus
Mass Spectrometer	Shimadzu GCMS-QP 2010 Ultra
Injector	Shimadzu AOC-5000, PAL-xt
Evaluation unit	Workstation, Shimadzu GCMS solution 2.7, extension AOC-5000

Table 2: Instrumental hardware of the GC/MS system

Septum	Supelco Thermogreen LB2; No. 20633
Liner	Restek Split/Splitless Liner; 3,5 mm x 5.0 x 95; No. 20956

Table 3: Hardware injector

Column	Macherey-Nagel Optima 5 HT
	0,1 µm film thickness, 30 m x 0,32 mm id; No. 32401-32
Carrier gas	Helium 5.0

Table 4: Hardware gas chromatograph

Chemicals	CAS	Manufacturer	Purity	Order no.	Batch no.
Methanol	67-56-1	Th. Geyer	≥ 99,95%	1428.2500	D3M009253M
Indole	120-72-9	Sigma Aldrich	≥ 99%	I3408-2G	MKBL8993V
2-aminoaceto-phenone	551-93-9	Sigma Aldrich	98%	A37804-56	MKBN5864V
Skatole	83-34-1	Sigma Aldrich	98%	M51458-56	STBB0062V
Androstenone	18339-16-7	Sigma Aldrich	≥ 98%	A8008-25MG	012M4008V
Para-cresol	106-44-5	Sigma Aldrich	99%	C85751-5G	STBC4479V
d_6-indole	-	ELFI Analytik GbR	-	-	-
d_3-skatole	-	ELFI Analytik GbR	-	-	-
d_3-androstenone	-	ELFI Analytik GbR	-	-	-

Table 5: Used chemicals

Fiber no.	1	2	3	4	5	6
Brand	SUPELCO	PAL System	PAL System	PAL System	PAL System	PAL System
Material	PDMS/DVB	PDMS	PDMS	PDMS	Polyacrylate	Carbo WR
Film thickness	65 µm	7 µm	30 µm	100 µm	85 µm	95 µm
Film length	10 mm	10 mm	10 mm	10 mm	10 mm	10 mm
Batch no.	55690	1301011	1302011	1303011	1304011	1305011
Order no.	57327-U	FIB-P-7/10	FIB-P-30/10	FIB-P-100/10	FIB-A-85/10	FIB-C-WR-95/10
Package ordern no.	57327-U	FIB-SEL5-S1				
Color	Pink	Green	Yellow	Red	Silver	Blue

Table 6: Examined SPME fibers

Methods of measuring principle

In the following tables the parameters of the measuring principle are shown.

Solvent cut time	3,5 min
Temperature transfer line	250 °C
Temperature ion source	200 °C
Tuning	Autotuning m/z 264

Table 7: Parameters of the measuring principle

The temperature program of the column oven, the purge-flow- and split program are shown in table 8 to table 10. Further gas chromatographic and SPME parameters are listed in table 11, table 12 shows the SIM characteristics of the mass spectrometer.

Rate [°C/min.]	Final temperature [°C]	Hold time [min]
-	35	3
250	80	0
80	320	3
-80	260	1,07

Table 8: Temperature program of the column oven

Rate [mL/min.]	Final flow [mL/min.]	Hold time [min]
-	0,5	3
400	3.0	17

Table 9: Purgeflow program

Split	Hold Time [min]
Splitless	3
1:100	17

Table 10: Split program

Linear velocity	45 cm/sec.
Temperature of injection	270°C
Duration of SPME equilbration	5 min.
Temperature ofSPME equilibration	100°C
Duration of SPME extraction	30 min.
Temperature of SPME extraction	100°C
Duration of SPME desorption	20 min.

Table 11: Further gas chromatographic and SPME parameters

Start [min.]	End [min.]	Mass number							
		Ch. 1	Ch. 2	Ch. 3	Ch. 4	Ch. 5	Ch. 6	Ch. 7	Ch. 8
5.00	6.00	77.05	107.10	108.10	-	-	-	-	-
6.00	7.05	89.10	90.10	92.10	117.15	120.10	122.15	123.15	135.15
7.05	8.50	130.15	131.15	132.15	134.15	-	-	-	-
8.50	15.00	239.15	242.20	257.20	260.20	272.25	275.25	-	-

Table 12: SIM characteristics of the mass spectrometer

Substance	Ret. Time [min.]	Mass number	Relative Intensity	Function in analysis
p-cresol	5,32	107,10	100,0	Quantifier
		108,10	89,1	Qualifier
		77,05	36,7	Quantifier
d_6-indole	6,68	122,15	100,0	Quantifier
		123,15	57,1	Qualifier
Indole	6,69	117,15	100,0	Quantifier
		90,10	45,8	Qualifier
		89,10	31,8	Qualifier
2-AAP	6,74	120,10	100,0	Quantifier
		135,15	75,6	Qualifier
		92,10	54,1	Qualifier
d_3-skatole	7,26	132,15	100,0	Quantifier
		134,15	77,0	Qualifier
Skatole	7,28	130,15	100,0	Quantifier
		131,15	63,1	Qualifier
d_3-androstenone	11,92	260,20	100,0	Quantifier
		275,25	38,5	Qualifier
		242,20	11,5	Qualifier
Androstenone	11,93	257,20	100,0	Quantifier
		272,25	69,2	Qualifier
		239,15	23,0	Qualifier

Table 13: Analysis parameters of the analytes

Methods of experimental investigation

For each fiber 10 µl standard solution is added in five headspace vials. The standard solution which is dissolved in methanol contains analytes with following concentration:

- c (CR, 2-AAP, SK, IND) = 0,5 ng/µL
- c (ANON) = 2,5 ng/µL

In the first vial 1 µL internal standard solution is added. This solution includes the internal standard in following concentrations:

- c (d_3-SK, d_6-IND) = 2,5 ng/µL
- c (d_3-ANON) = 20 ng/µL

In Figure 9 the procedure of the experimental study is shown schematically.

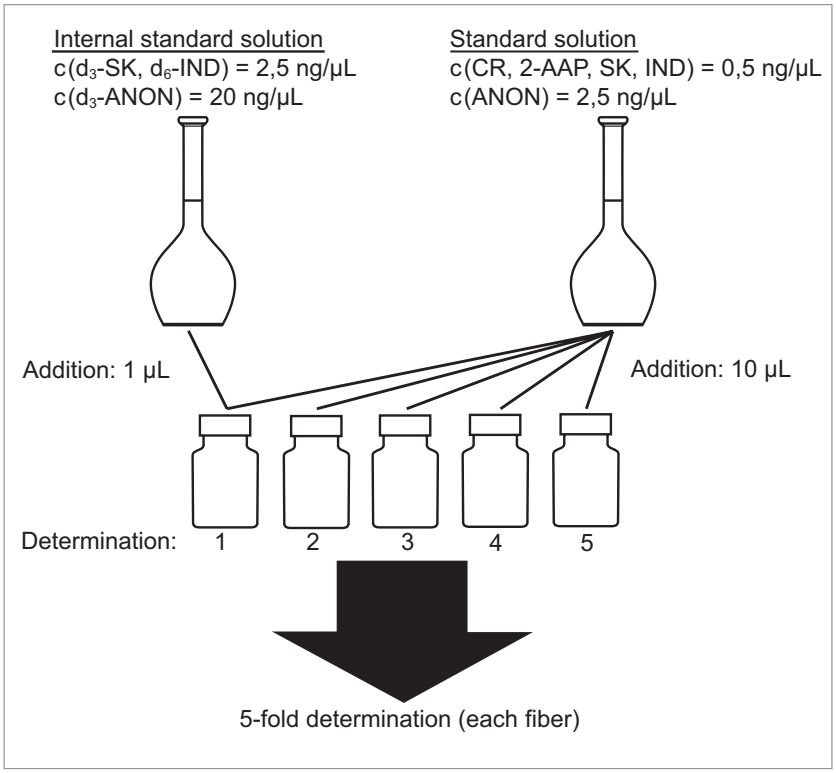

Figure 9: Procedure of the experimental study

Results

In the following the results of the investigation are presented. Figure 10 shows an example chromatogram with the respective qualifier of the analytes.

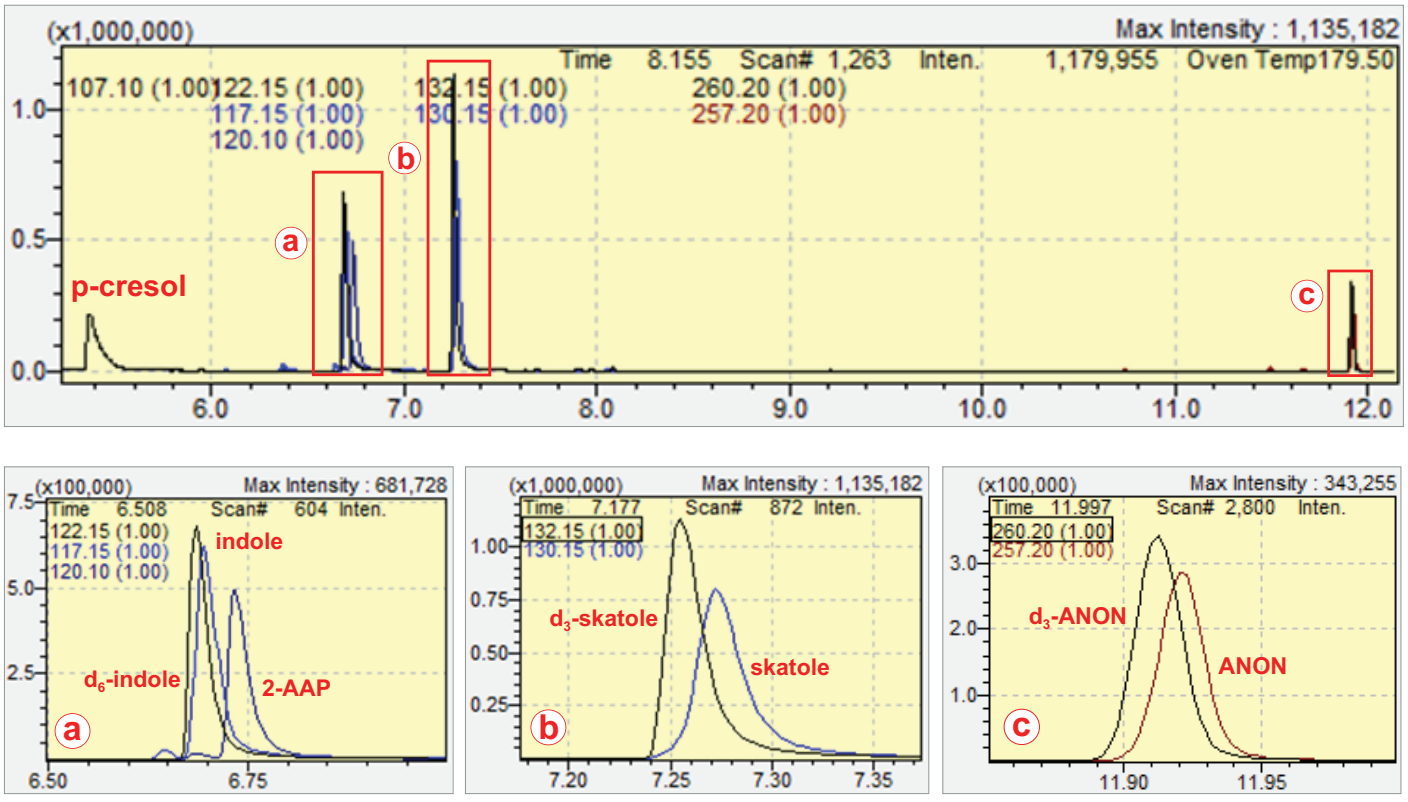

Figure 10: example chromatogram; fiber C-WR-95 PAL System

In table 14, the peak areas of analytes are displayed as mean values of 5-fold determinations.

Fiber	Peak area							
	CR	IND	2-AAP	SK	ANON	d_6-IND	d_3-SK	d_3-ANON
SUPELCO PDMS/DVB 65/10	225541 ± 45289	659435 ± 141754	463472 ± 94601	903137 ± 179311	462189 ± 68019	725547	1663217	809633
PAL System PDMS 7/10	5785 ± 1266	16126 ± 1924	11273 ± 1525	30752 ± 3123	220430 ± 53165	5006	28522	258464
PAL System PDMS 30/10	10456 ± 3129	36964 ± 7310	26647 ± 5603	73749 ± 13582	421127 ± 107752	6452	41133	231254
PAL System PDMS 100/10	38796 ± 8501	146460 ± 24667	111487 ± 17519	204636 ± 99321	613270 ± 142885	19236	130555	358611
PAL System PA 85/10	492834 ± 61937	1101223 ± 141465	646165 ± 66438	1216326 ± 114267	519987 ± 106797	129438	644905	383115
PAL System CWR 95/10	1122148 ± 77921	1404500 ± 79121	1109305 ± 79141	1492964 ± 43536	306579 ± 28250	1237269	1624586	425367

Table 14: Peak area of analytes as mean values of 5-fold determination

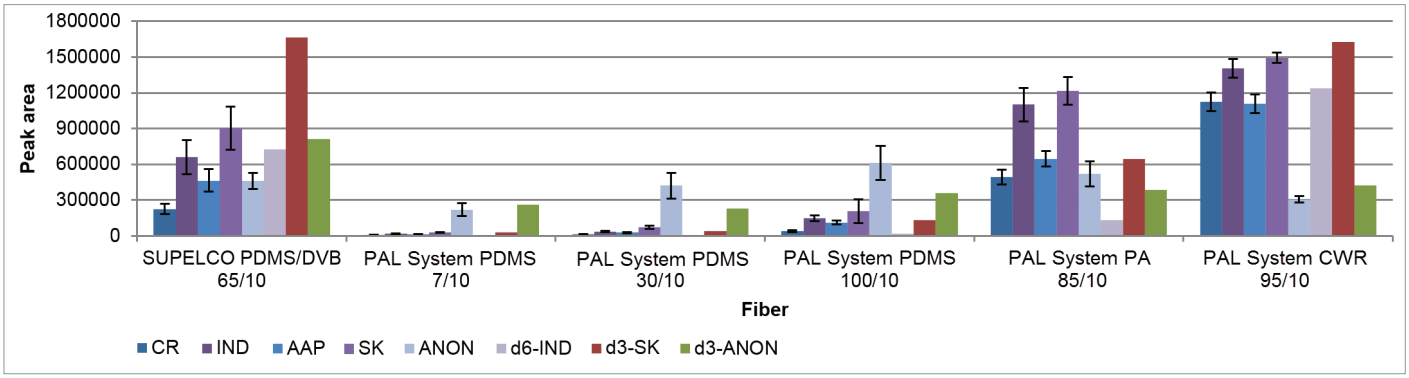

Figure 11: Peak areas of analytes as mean values of 5-fold determination using standard deviation as error bars

Discussion

The extraction behavior of all fibers was investigated with equal conditions during preequlibration, extraction and desorption. Only in fiber material differences existed. In this manner influences due to various temperature, duration or agitation can be eliminated and all peak area changes are attributed to fiber material.

Decisive for the choice of the fiber material is the polarity of the analyte. A polar analyte adsorbs particularly well to polar fiber materials, by contrast, nonpolar analytes better bind to nonpolar fiber materials. Basically, there is the problem that a rather non-polar analyte as androstenone has to be detected with polar analytes as indole and skatole simultaneously. Furthermore, fiber thickness seems to have influence on extraction. A comparison of analyte peak areas of the fiber «PAL System PDMS» with layer thicknesses of 7 µm, 30 µm and 100 µm show increasing peak areas when layer thickness is rising (Figure 11). The low peak areas of the polar analytes para-cresol, indole, 2-aminoacetophenone and skatole can be explained by the non-polar fiber material polydimethylsiloxane. In contrast, the surface for the non-polar an-drostenone is significantly higher. Due to the overall low peak areas polydimethylsiloxane is, regardless of the fiber layer thickness, not recommended as the only material. The fiber «PAL System PA 85/10» with a film thickness of 85 µm is highly polar, which is reflected in higher peak areas of indole and skatole. The fiber «SUPELCO PDMS/DVB 65/10» is a combination of polydivenylbenzole and polydimethylsiloxane. This is a non-polar material and a less polar material. Both fibers have average high peak areas of the analytes. The fiber «PAL System CWR 95/10» has a relatively thick layer with 95 µm. The material carbowax is polar. This polarity and the

high layer thickness apparently lead to very high peak areas of the polar analyte. However, the peak area of androstenone is only a little lower compared to «PDMS/DVB» and «PA» fiber. Peak areas of the internal standards are very high (d_3-skatole and d_6-indole) and acceptable (d_3-androstenone). For this reason, it is useful to use the fiber «PAL System CWR 95/10» for study analysis of boar taint causing compounds.

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