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High-performance liquid chromatographic method for the determination of 3-methylindole (skatole) and indole in adipose tissue of pigs

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ABSTRACT

A rapid method for the determination of skatole (3-methylindole) and indole in adipose tissue of pigs by reversed-phase high-performance liquid chromatography has been developed. Tissue samples were melted in a microwave oven, and 100 μl of the liquid fat were dissolved in 1 ml of *n*-hexane and extracted with acetonitrile–water (75:25, v/v). Portions of 100 μl of the solution were used for chromatographic analysis. Elution was performed on a reversed-phase column with a mobile phase composed of acetic acid and isopropanol (70:30, v/v). A fluorescence detector was used for quantification. The detection limit was 4 ng/g fat. The mean recoveries of added amounts of skatole and indole were 98.9 and 93.8%, respectively. The mean coefficients of variation were: inter-assay, 6.6% (skatole) and 8.8% (indole); intra-assay, 4.2% (skatole) and 2.9% (indole). Mean skatole concentrations in fat samples from boars (40 ng/g; $n = 349$) were not significantly higher than those from barrows (24 ng/g; $n = 98$).

INTRODUCTION

Skatole and indole are formed by microbial degradation of tryptophan in the intestines of several species by different microbes [1–4]. Skatole has received considerable attention because its toxic effects have been demonstrated [5,6]. Such toxic effects have not yet been investigated in the pig, because interest has focused on the accumulation of these substances in adipose tissue. Skatole and, to a lesser degree, indole, may cause an unpleasant faecal odour in pig meat products [7–9]. The development of accurate methods for the determination of these substances in faeces [10] and blood plasma [11] may yield information on intestinal formation [10,12], resorption [11] and hormonal effects on the formation [12]. However, a sensitive and simple method for tissue determinations, which would also allow analysis in bioptic material, is required.

Several methods for the measurement of skatole and indole in adipose tissue of pigs have been described. The gas chromatographic (GC) methods require tedious sample preparation, based either on volatility [7,13] or extraction with organic solvents combined with purification steps [14]. Skatole was also determined by photometric quantification after derivatization with 4-dimethylaminobenzaldehyde. This method is used for screening large numbers of carcasses at the slaughter line [15] but is not specific. Therefore the method did not even lead to a significant correlation with those concentrations obtained by a GC method [16]. Reversed-phase high-performance liquid chromatography (HPLC) on different resins with UV detection has been used for the

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determination of skatole and indole [17–19], but also required tedious concentration and purification steps.

Therefore, the aim of our study was to develop a rapid and accurate HPLC method for the measurement of both indole and skatole in tissue samples of pigs. The development was based on a method that was described earlier [20] but was further simplified.

EXPERIMENTAL

Equipment

A Merck Hitachi HPLC system (Merck, Darmstadt, Germany) was used consisting of an L-6000 HPLC pump, an AS-2000 autosampler, an L-1050 fluorescence spectrophotometer, an LKB (Bromma, Sweden) column oven (Model 2155) and an ERC-1512 solvent degasser (Erma C.R., Tokyo, Japan). Separation of the analytes was performed on a 65 mm × 4.6 mm I.D. LiChrospher RP-18 column (particle size 5 μm, Bischoff Analysentechnik, Leonberg, Germany) protected by an identically packed pre-column (10 mm × 4.6 mm I.D.). Peaks were recorded and integrated using a Perkin-Elmer Nelson Systems interface (Cupertino, CA, USA), linked to a personal computer.

Materials

All chemicals were analytical grade, and solvents were LiChrosolv quality (Merck). Standard substances (skatole, 2-methylindole, indole) were reagent grade supplied by Serva (Heidelberg, Germany), and were prepared as stock solutions (0.1 mg/ml) in monthly intervals.

Origin of samples

Samples from back fat (10–20 g) were obtained from carcasses of 349 boars and 98 barrows (100–110 kg slaughter weight) from the commercial breeding company PEN-AR-LAN (Maxent, France) and stored at −20°C until assay. All animals were kept indoors and fed standardized diets *ad libitum*.

Preparation of samples and HPLC analysis

All samples were measured in duplicate. The fat samples were heated for 4 min at 180 W in a microwave oven (Siemens, Munich, Germany). Aliquots of 100 μl of the liquid fat were transferred to 10-ml reagent tubes containing 1 ml of *n*-hexane. After addition of 2-methylindole (25 ng) as an internal standard (I.S.) to each tube they were vortex-mixed for 30 s. The latter substance was chosen as the I.S. because it is chemically similar to skatole and indole but does not occur naturally in adipose tissue. After the addition of 1 ml of acetonitrile–water (75:25, v/v), a solvent distribution was carried out by vortex-mixing for another 30 s. After centrifugation (15 min at 3000 *g*) the upper *n*-hexane layer was withdrawn using a vacuum pump. The remaining acetonitrile–water phase was transferred into 1-ml autosampler vials. Aliquots of 100 μl were analysed by HPLC.

The mobile phase was 0.02 *M* acetic acid–2-propanol (70:30, v/v), and the flow-rate was 1.1 ml/min. The column was operated at 50°C. The excitation wavelength was 275 nm, and the emission wavelength 345 nm (signal range at 20 mV full scale).

Quantification

For preparing calibration curves, various amounts of skatole and indole (10–750 ng) were added to 1 g of pork fat that contained no detectable amounts of endogenous skatole and indole. The calibration samples in fat and the biological samples were treated identically. The peak areas of the calibration samples and the I.S. were determined, and the ratio was used to evaluate the biological samples.

Determination of the quality characteristics

The extraction yield was determined by adding known amounts (Table I) of indole and skatole to 100 μl of liquid fat. After the sample treatment, the peak areas of these substances were compared with the same amounts added to 1 ml of acetonitrile–water (100% value).

The lower limit of detection for both skatole and indole was defined as that concentration of

TABLE I

ACCURACY AND INTRA- AND INTER-ASSAY COEFFICIENTS OF VARIATION OF SKATOLE AND INDOLE DETERMINATIONS

Known amounts of skatole and indole were added to pork fat and measured by HPLC after extraction with *n*-hexane and solvent distribution against water–acetonitrile.

Concentration (ng/g)		Coefficient of variation	
Added	Measured (mean ± S.D., n = 7)	Intra-assay (n = 7)	Inter-assay (n = 7)
Skatole			
100	100 ± 8	4.5	8.0
200	200 ± 13	7.6	6.5
400	350 ± 20	1.7	5.7
600	640 ± 39	3.0	6.1
Indole			
100	100 ± 8	3.9	8.9
200	180 ± 19	3.2	10.6
400	350 ± 30	1.6	8.6
600	640 ± 45	3.0	7.0

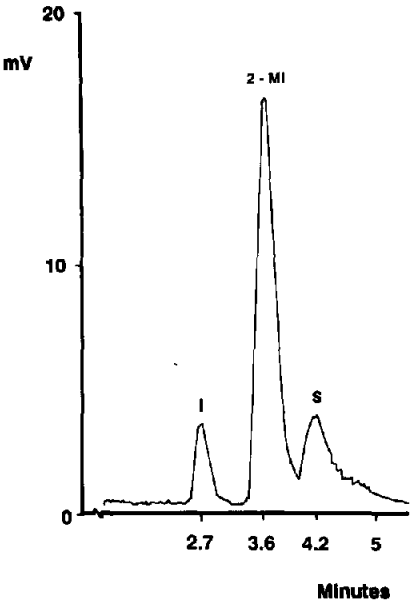

Fig. 1. Chromatogram of a sample from a boar. The concentrations were 0.25 μg/g for the I.S. 2-methylindole (2-MI) and 0.047 μg/g both for indole (I) and skatole (S).

the relevant indole peak that exceeded the maximum baseline noise by a factor of 2. The accuracy of the measurements was determined by adding known amounts of skatole and indole (100, 200, 400 and 600 ng) to 1 g of pork fat. The reproducibility was determined by repeated measurements of the same samples either on the same day (intra-assay variation, n = 7) or on consecutive days (inter-assay variation, n = 7). Practicability was defined as the mean number of samples that could be handled by one person per day.

RESULTS

Quality criteria

The procedure for extraction and the chromatographic conditions allowed a satisfactory separation between 2-methylindole (I.S.), indole and skatole without interference from other substances (Fig. 1). The retention times were 2.7 min for indole, 3.6 min for 2-methylindole and 4.2 min for skatole. Quantitative evaluation was carried out using calibration standards in pork fat.

TABLE II

CONCENTRATIONS OF SKATOLE AND INDOLE IN BACK FAT OF BOARS AND BARROWS

Zero was below the detection limit.

Animal	n	Skatole (ng/g)		Indole (ng/g)	
		Mean ± S.D.	Range	Mean ± S.D.	Range
Boar	349	40 ± 8.3	0–1005	20 ± 5.2	0–736
Barrow	98	24 ± 1.9	0–111	18 ± 3.9	0–195

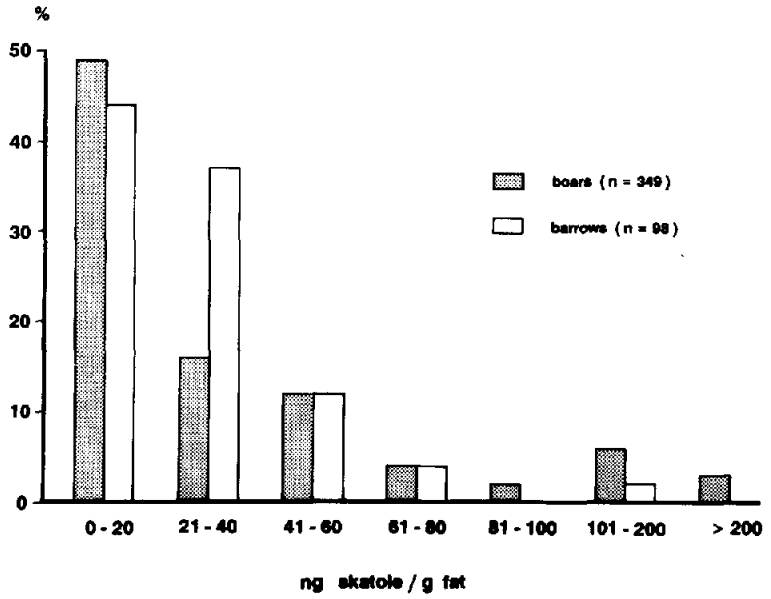

Fig. 2. Relative distribution of skatole concentrations in fat samples from boars (349) and barrows (98).

The calibration curves were linear up to 750 ng/g (7.5 ng per injection). For all three indoles the mean extraction yield from pork fat was 86%. The lower limit of detection was 40 pg per injection (100 μl) for skatole and indole, corresponding to 4 ng/g fat.

The accuracy values are given in Table I. The recovery of skatole ranged from 88 to 107% (mean 98.9%), depending on the amount added. For indole the recovery was only slightly lower (mean 93.8%, range 88–107%). The mean intra-assay coefficients of variation (C.V.) were 4.2% for skatole (range 1.7–7.6%) and 2.9% for indole (range 1.6–3.9%). The mean inter-assay C.V. was 6.6% for skatole (range 5.7–8.0%) and 8.8% for indole (range 7.0–10.6%).

One person could measure about 100 fat samples per day in duplicate, including evaluation of the chromatograms.

Concentrations in fat samples

The mean concentrations of skatole and indole in fat samples from 349 boars and 98 barrows are given in Table II. Compared with indole, the mean concentrations of skatole were 2-fold higher in the boars and 1.3-fold in barrows. Neither the range nor the mean of skatole concentrations differed significantly between boars and barrows. Statistical evaluation revealed a positive correlation between the concentrations of skatole and indole (boars: $r = 0.44, p \leq 0.001$; barrows: $r = 0.36, p \leq 0.001$), demonstrating that high concentrations of skatole are accompanied by high indole values.

Fig. 2 shows that the distribution of skatole concentrations is uneven, both in the boars and the barrows. Thus the highest number of samples is found in the category of low concentrations, up to 40 ng/g. Higher concentrations are found both in boars and in barrows, with the exception of concentrations above 200 ng/g, which in this study were detected in only a few boars but not in barrows.

DISCUSSION

A rapid and sensitive method for the determination of skatole and indole in adipose tissue of pigs was developed. Compared with other methods [14,21], only small volumes of organic solvents are required for sample preparation.The evaporation of solvent is also avoided, thus im-

proving both the practicability and the precision for determination of volatile substances.

The extraction yield of skatole and indole from fat was 86%. This procedural loss is automatically corrected by the I.S., so that the recovery of added skatole and indole was 99%.

The lower limit of detection was *ca.* 40 pg (4 ng/g fat) for both indoles, which is typical for fluorimetric detection in general [22,23] and similar to those methods based on capillary GC [21]. Such a high sensitivity also allows accurate determination when the amount of adipose tissue is limited, *e.g.* in bioptic material.

The skatole and indole concentrations found in the present study were in the range of those reported by other authors [13,21]. Skatole and indole were highly significantly correlated, demonstrating the competition of microbes for tryptophan as the common precursor. The same statistical relationship still exists in samples from barrows, even though skatole concentrations are slightly lower than those in boars.

This minor difference is in accordance with previous results [13], and suggests an influence of the anabolic status on the formation of these substances. Additionally, the lower mean value of skatole in barrows does not reflect the broad distribution of skatole concentrations covering a range from 0 to 111 ng/g fat, which is of nearly equal heterogeneity in boars and in barrows.

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