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Short communication

Rapid determination of skatole and indole in pig back fat by normal-phase liquid chromatography

J.A. Garcia Regueiro*, M.A. Rius

IRTA. Unitat Química Alimentària, Centre Tecnologia de la Carn, Granja Camps i Armet, 17121 Monells, Spain

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Abstract

A rapid normal-phase high-performance liquid chromatographic method for the quantitative determination of indole and skatole in pig back fat samples has been developed. The compounds were extracted by shaking the samples at ambient temperature in hexane–2-propanol (92:8, v/v). The sample preparation procedure was simple because it was not necessary to remove the fat from the samples. The compounds were separated on a 250×4.6 mm I.D., 5 μm Hypersil aminopropylsilica column. Fluorescence (excitation at 280 nm and emission at 360 nm) was used for selective detection. Recoveries for skatole and indole, relative to the internal standard, were 100.3±0.9% and 99.6±4.4%, respectively. Linearity determined in fat samples was in the range of 0.05–1 μg/g and the correlations observed were R^2=0.9914 for indole and R^2=0.9916 for skatole.

Keywords: Fats; Food analysis; Skatole; Indoles

1. Introduction

At present, the use of entire male pigs for meat production has some advantages compared with castrated pigs [1,2]. Uncastrated male pigs grow faster, the feed conversion efficiencies are better, the meat is leaner and the animal welfare is better [3,4]. However, the castration of male pigs has been practised in order to avoid the occurrence of boar taint, a male odour present in 5–10% of carcasses.

The major compounds responsible for the boar taint are associated with the fatty tissue and more precisely with the nonsaponifiable fraction. Sink and Beery [5,6] postulated that the odour was due to C_{19}-Δ^{16}-steroids, compounds that function as sex pheromones in pigs. Patterson [7] isolated the hormone androstenone (5α-androst-16-en-3-one) as the principal component responsible for boar taint in the fat of noncastrated pigs. Androstenone has been described as having an intense urine/perspiration like odour. The biosynthesis of androstenone and its contribution to boar taint has been described [8–12].

Skatole and indole, which can be formed as a result of the breakdown of tryptophan by bacteria in the large intestine, have also been shown to be implicated in boar taint [13–15]. Fat samples containing high levels of skatole produce a fecal-like odour on heating. Skatole cannot explain the total off-odour or boar taint. Correlation between skatole and boar taint scores determined by sensory evalua-

*Corresponding author.

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tion is of the order of 0.7, explaining only 50% of the score [17]. If androstenone content was included, about 66% of the variation in odour score could be explained. The magnitude of those correlations does not exclude the contribution of other unidentified compounds. So which of the two off-odour compounds is the main contributor to boar taint is still a controversial issue [18]. Studies from different countries have led to different conclusions. This may be due to variation in slaughter weight, genotype and differences between laboratories in the organoleptic assessment of the meat.

Several chromatographic methods for the determination of skatole in back fat using normal-phase [22] and reversed-phase HPLC [19–21,23,25] or GC [24,26] have been described. However, the critical step in the determination of skatole in adipose tissue is the sample preparation due to the fact that skatole is a very lipophilic compound and it is necessary to remove the fat from the sample. The sample preparation procedures include liquid–liquid extraction [25], steam distillation [16] and solid-phase extraction in the normal-phase [19–22,28] or reversed-phase modes [23]. These procedures are time consuming and they present difficulties in achieving high absolute recoveries.

Mortensen et al. [18] developed a routine spectrophotometric on-line method for the determination of skatole in back fat. Skatole was extracted from fat with Tris–acetone followed by measurement of absorbance at 580 nm after derivatization with 4-dimethylamino benzaldehyde in ethanol–sulphuric acid. This method is not specific for skatole because it is possible that other indolic compounds interfere with the skatole determination leading to overestimation of the content of this compound when its concentration is low. Moreover, a high correlation exists ($r=0.973$) between the results of this method (expressed as a skatole equivalent) and the skatole concentrations found by HPLC [23].

In order to simplify the HPLC methods for the determination of skatole and indole in back fat, a new isocratic fast HPLC method was developed. The extract was analyzed directly, without any previous clean-up procedure. The methodology was applied to the study of the concentration of skatole and indole in 16 samples of back fat randomly selected from a local slaughterhouse.

2. Material and methods

2.1. Chemicals

Indole, skatole (3-methylindole), 7-ethylindole, 2-indolecarboxilic acid, 3-indolebutyric acid, 3-indolecarboxylic acid, 2-methylindole and 5-methylindole were obtained from Sigma (St. Louis, MO, USA). Hexane and 2-propanol were of HPLC grade from Panreac (Spain). All other chemicals were of analytical-reagent grade.

2.2. Back fat samples

Back fat samples for the validation of the method were obtained from a local butcher. Samples that did not exhibit measurable concentrations of indole and skatole were used for fortification procedure and to evaluate the linearity and the repeatability of the method. The samples were kept at −20°C until analysis. Sixteen samples of back fat from different carcasses were taken from a local slaughterhouse. They were analyzed by the presented method.

2.3. High-performance liquid chromatography

For HPLC, a LKB system was used consisting of a Rheodyne injector with a 100-μl loop and a 2150 HPLC pump. The detector used was a Kontron SFM 25 fluorometer and the data were collected with a Carlo Erba integrator. The column used was an Hypersil aminopropylsilica (APS 2) (5 μm) (250× 4.6 mm I.D.) operated at ambient temperature. The mobile phase consisted of hexane–2-propanol (92:8, v/v) and the flow-rate was 1.5 ml/min. The detection was carried out by the measurement of the fluorescence with the following wavelengths: excitation at 280 nm and emission at 360 nm. The volume of sample injected was 50 μl.

2.4. Linearity, repeatability and factor response

Linearity and repeatability of the method were evaluated with standard solutions of indole, skatole

and 7-ethylindole (I.S.) and with fat samples. The linearity determined with standard solutions was in the range of 0.5–4 ng for indole and skatole and 2–16 ng for 7-ethylindole, and in the range of 0.05–1 μg/g in back fat samples. The repeatability was evaluated injecting seven times the same sample at a concentration of 0.1 μg/g of skatole and indole. 7-Ethylindole (0.5 μg/g) as internal standard was added to all the samples prior to the analytical procedure. The factor response was evaluated varying the concentrations of skatole and indole (0.01, 0.02, 0.03, 0.04, 0.05, 0.08 and 0.1 ng/μl) versus a fixed concentration of the internal standard 7-ethylindole (0.1 ng/μl). A 50-μl volume of each solution was injected. The efficiency of recovery was evaluated by spiking five samples of back fat. A 1-g amount of fat was spiked to obtain concentrations of 0.05, 0.1, 0.2, 0.4 and 0.5 μg/g of skatole and indole, and 0.5 μg/g of 7-ethylindole as internal standard. The samples were prepared by duplicate and analyzed by normal-phase HPLC.

2.5. Sample treatment

A 500-ng amount of 7-ethylindole dissolved in hexane–2-propanol (92:8, v/v) as internal standard was added to 1 g of back fat. The mixture was extracted with 10 ml of hexane–2-propanol (92:8, v/v) in a 50-ml vessel. The samples were shaken in a magnetic stirrer (SBS A-13 Serie D, Spain) for 30 min at room temperature to achieve the extraction of indolic compounds. The solution was filtered in a PTFE filters (13 mm×0.45 μm, Teknokroma, Spain). A 50-μl volume of the filtered extract were injected into the HPLC system.

3. Results and discussion

3.1. HPLC analysis

The aim of this study was to develop a fast and easy method for the determination of skatole and indole in fat back samples. Other methods that have been described are very time consuming because it is necessary to remove the fat from the samples. For this purpose some procedures have been applied: solid-phase extraction with columns of silica gel or freezing the samples at −20°C [19–21,25]. Nevertheless, it was observed the coprecipitation of the compounds studied and sometimes the method was not sensitive or reproducible.

The extraction of skatole and indole was achieved with a mixture of hexane–2-propanol (92:8, v/v). The samples were shaken during different intervals of time (15, 30, 45, 60, 120 min and 12 h) to evaluate the influence on the extraction. At 30 min the recovery of skatole and indole was complete and it became independent of further increases in time. The mixture obtained could be analyzed by HPLC directly, without removal the fat from the samples. This fact allowed a very simple procedure to extract skatole and indole from back fat samples.

The analysis of the samples was performed by normal-phase HPLC because it provided a more effective separation of more hydrophobic interfering substances than the reversed-phase mode (Fig. 1). Triglycerides were eluted with lower retention times than skatole and indole; since, they were not retained in the column as was observed when the reversed phase was applied. The chromatographic analysis of indole and skatole was achieved in 5 min. Fluorescence detection was selected because it has a better sensitivity and selectivity than UV detection [22,23].

Several mobile phases were evaluated. The elution of the studied compounds by hexane presented a low resolution. Hexane–2-propanol (92:8, v/v) was selected because it allowed a good separation of the compounds and did not affect the measurement of fluorescence at the wavelength used. García Regueiro and Díaz [21] discussed the use of hexane–2-propanol (94:6, v/v) as mobile phase to separate skatole and indole. However, using these conditions the internal standard used (7-ethylindole) coeluted with skatole. The determination of other skatole related compounds (2-indolecarboxylic acid, 3-indolebutyric acid, 3-indolecarboxylic acid) was not possible with the chromatographic conditions applied.

During the development of the method other indolic compounds such as 2-methylindole and 5-methylindole were investigated as internal standards. Those compounds showed a better response to fluorescence than 7-ethylindole but they could not be used as internal standards because were not well separated from skatole.

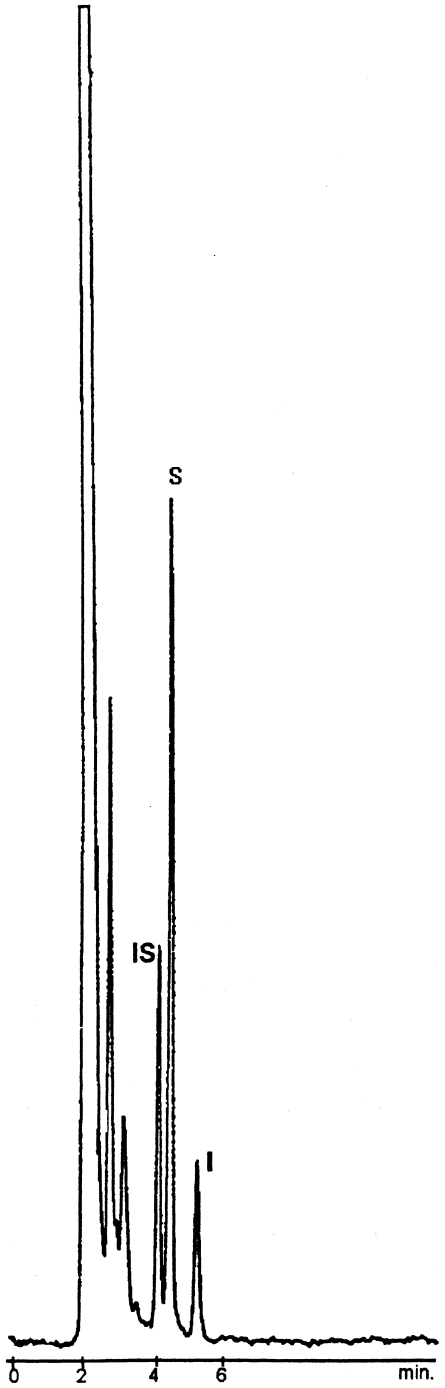

Fig. 1. Normal-phase HPLC analysis of a back fat sample. Peaks: I.S., 7-ethylindole; S, skatole; I, indole. Column: Hypersil aminopropylsilica (5 μm; 250×4.6 mm I.D.). The mobile phase consisted of hexane–2-propanol (92:8, v/v) at a flow-rate of 1.5 ml/min; fluorescence detection (excitation at 280 nm and emission at 360 nm).

3.2. Recovery

The recovery of the analytical method was assessed by spiking samples with a low concentration of skatole and indole (<0.05 μg/g) and with a known concentration of skatole and indole to obtain concentrations of 0.05, 0.1, 0.2, 0.4 and 0.5 μg/g. The recoveries relative to the internal standard for skatole and indole were 100.3±0.9% and 99.6±4.4%, respectively. This recovery of skatole was similar to the recovery obtained by Mortensen et al. using the spectrophotometric method, who reported a recovery of 95–105% [18]. Nevertheless, the analytical procedure of the spectrophotometric method was not specific and other compounds with similar chemical characteristics could interfere with the analysis and overestimate the content of skatole at low concentrations. Hansen-Møller [22,23] found a good correlation (r=0.973) between the HPLC method and the colorimetric method and they obtained a recovery of skatole close to 100%. The recovery obtained by García-Regueiro and Díaz using a normal-phase HPLC method and a Florisil clean-up procedure was 90% [21]. Whereas Hanson et al. [16] using a GC method reported a recovery of 47%.

3.3. Linearity and response factor

The linearity and repeatability of the method were evaluated with samples that exhibited a low content of skatole and indole and which were used for the fortification procedure. The linearity obtained in standard solution for the studied compounds was: indole (y=34.926 x+0.877, R^2=0.9994), skatole (y=105.13x−0.843, R^2=0.9995) and 7-ethylindole (y=18.857x−0.3149, R^2=0.9997). Also, the linearity was evaluated in back fat samples in the range of 0.05–1 μg/g and the linear plots determined respect to the 7-ethylindole were: indole (y=63 440x− 63.143, R^2=0.9914) and skatole (y=157 192x+ 2815.9, R^2=0.9916). Since, the determination and quantification of skatole and indole were possible in the range of concentrations in which they can be found in fat samples.

The relative response of skatole and indole with reference to internal standard was evaluated. The

linear equations obtained were: indole, $y=2.204x-0.0603$ ($R^2=0.9911$) and skatole, $y=5.996x-0.061$ ($R^2=0.9984$). The relative responses were: skatole/internal standard (5.996) and indole/internal standard (2.204). 7-Ethylindole showed a lower response than either compounds. The main problem associated with the use of 7-ethylindole as internal standard was the complete separation of skatole. However in the conditions applied, 7-ethylindole did not interfere in the determination of skatole in standards solutions as in fat samples. Another aspect to be considered is that 7-ethylindole had to be added in higher concentrations than skatole and indole. However, this fact did not affect the linearity of fat samples.

3.4. Intra-assay variability

The within-day ($n=7$) R.S.D.s for skatole, indole and the internal standard 7-ethylindole in back fat samples were 1.1%, 2.5% and 3.4% respectively for absolute areas and 1.4%, 2.2% and 5.7% for relative areas. Retention times repeatability showed an R.S.D. value below 0.4% for the three compounds.

3.5. Analysis of skatole and indole in back fat samples

The method was applied to 16 samples of back fat to determine their skatole and indole concentrations. Indole and skatole concentrations obtained in the analysis of back fat samples from different pigs showed great variation (Table 1). Skatole and indole concentrations are influenced by factors such as animal nutrition, sex and age [28–30] and this can produce the large differences observed in the results. The maximum values were: skatole 1.04 μg/g and indole 0.68 μg/g. Considering the concentration sum of skatole+indole: 6 out of 16 samples showed a total concentration >0.40 μg/g, 8 out of 16 a concentration between 0.10 and 0.40 μg/g and 2 out of 16 showed a concentration <0.10 μg/g. In most of the samples (11 out of 16) the concentration of skatole was higher than the concentration of indole. Skatole concentration was higher than 0.250 μg/g in six samples which could present boar taint on the basis the threshold value reported by Bonneau et al. [31]. Nevertheless, the concentration of indole was higher in some samples (5 out of 16) and could therefore interfere with the estimation of skatole and its effect on boar taint when non-specific methods of analysis were used. High levels of indole could be found in pigs kept in pens with high temperatures and a high stocking rate [27]. There was no statistically significant correlation between the concentration of skatole and indole. The value obtained for R^2 was 0.348.

Table 1
Skatole and indole concentrations (μg/g) in boar fat determined by normal-phase HPLC (for conditions see text)

No.	Indole	Skatole	Skatole+indole	Skatole/indole
1	0.016	0.029	0.045	1.81
2	0.056	0.081	0.137	1.45
3	0.043	0.073	0.116	1.70
4	0.031	0.046	0.077	1.48
5	0.588	0.581	1.169	0.99
6	0.088	0.041	0.129	0.46
7	0.078	0.050	0.128	0.64
8	0.069	0.394	0.463	5.71
9	0.379	0.152	0.531	0.40
10	0.033	0.083	0.116	2.51
11	0.044	0.075	0.119	1.70
12	0.104	0.365	0.469	3.51
13	0.677	0.273	0.950	0.40
14	0.454	1.041	1.495	2.29
15	0.074	0.279	0.353	3.77
16	0.071	0.248	0.319	3.49
$x\pm$ S.D	0.175±0.218	0.238±0.267	0.413±0.433	2.02±1.48

4. Conclusions

A rapid, reproducible and simple method for the determination of indole and skatole in back fat samples from pigs was developed. Compared with other methods sample preparation was simple because no clean-up of the samples was necessary. The use of organic solvents was limited and there were no time-consuming and solvent evaporation steps. The method could be suitable for rapid analysis of skatole and indole in fat samples.

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