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Short Communication

Gas chromatographic determination of indole and 3-methylindole (skatole) in bacterial culture media, intestinal contents and faeces

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Abstract

A simple, rapid and inexpensive gas chromatographic method was developed for the determination of indole and 3-methylindole (skatole) in faeces, intestinal contents and bacterial cultures. It involves a simple homogenization and extraction with chloroform. The extract is injected onto a gas chromatograph equipped with a 12.5-m fused-silica capillary column coated with BP20 and a film thickness of 0.5 μm. To simplify the chromatograms and to get a higher sensitivity a nitrogen–phosphorus-sensitive detector is applied. The detection limit for indole and 3-methylindole under the conditions employed is 20 μg/kg, which is well below the values typically found in intestinal contents (up to 100 mg/kg). Recovery for both compounds was close to 100%, and the mean coefficients of variation were 3.5% for indole and 3.0% for 3-methylindole. The method has demonstrated its practical value in the analysis of more than 50 000 samples in our laboratory. More than 100 samples can be analyzed per day.

1. Introduction

Skatole (3-methylindole) is thought to be the main cause of unpleasant smell and taste of meat from uncastrated male pigs, which are of increasing importance in meat production [1]. The compound is produced in the intestine by the microbial degradation of tryptophan [2,3]. To investigate the microbial degradation of tryptophan to indole and 3-methylindole in faeces, intestinal content and microbial cultures it was necessary to develop a rapid and simple method to measure these two compounds.

Several methods for determining 3-methylindole and indole in various types of samples have been reported. Most work has been done on the analysis of 3-methylindole in pig backfat by gas chromatography (GC) [4,5], high-performance liquid chromatography (HPLC) [6], or both in combination [7]. A spectrophotometric method is currently used in Denmark to determine 3-methylindole in carcasses at the slaughterline in abattoirs [8]. Gas chromatography has also been applied to measure 3-methylindole in faeces [9]. Several papers have appeared employing HPLC for measurements of 3-methylindole in faeces from various animal species [10,11]. 3-Methylindole has been determined in gut content by GC [12] and by thin-layer chromatography (TLC)

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[13]. Bacterial cultures have been analyzed by both GC and TLC [14,15].

Here we describe a rapid, inexpensive, and reliable method for determining 3-methylindole and indole in faeces, intestinal contents, and bacterial cultures.

2. Experimental

2.1. Materials

All chemicals used were of analytical grade. Chloroform and methanol were of HPLC-grade (Rathburn Chemicals Limited, Walkerburn, UK). Indole and 5-methylindole (internal standard) were from Sigma (St. Louis, MO, USA) and 3-methylindole was from Merck (Darmstadt, Germany). Kits for recoating the active elements of the nitrogen–phosphorus detector (NPD) were from Hewlett-Packard (Rockville, MD, USA).

2.2. Extraction procedure for bacterial cultures

Culture medium (1 ml) was transferred to a centrifuge tube with a conical bottom, diluted with 1.0 ml distilled water, and made alkaline with 2 drops of 4 *M* NaOH [12]. The internal standard 5-methylindole was added (50 μl of a 100 mg/ml solution in methanol), followed by 1.0 ml chloroform. The mixture was vortex-mixed until an emulsion was formed. To break the emulsion the sample was centrifuged in a bench centrifuge (2700 *g*, 10 min). The chloroform phase was then transferred to autosampler vials for gas chromatography.

2.3. Extraction procedure for gut content and faeces

Contents of gut segments [3] or faeces (10 g) were weighed into stomacher bags (Seward Medical, London, UK) and 90 ml medium containing 0.9% NaCl and 0.1% Tween 80 were added [9], followed by 250 μl of the internal standard 5-methylindole (1 mg/ml in methanol). The sample was homogenized for 2 min in a stomacher (Lab-blender 400, Seward Medical). The homogenate (2.0 ml) was transferred to a centrifuge tube and 2 drops of 4 *M* NaOH and 1.0 ml chloroform were added for extraction. The mixture was vortex-mixed until an emulsion was formed and the sample centrifuged as described above. If the phases did not separate, the emulsion could often be broken by placing the sample in a freezer at −21°C followed by a new centrifugation. This is often necessary with samples from the first two segments of the small intestine because of the high content of bile acids which function as emulsifiers.

2.4. GC system and column

Analyses were performed on a Hewlett-Packard gas chromatograph (Model 5890 Series 2), equipped with a NPD, a Hewlett-Packard automatic sampler (Model 7673), and a Hewlett-Packard integrator (Model 3396 Series 2). A 25-m fused-silica capillary column from Scientific Glass Engineering (Melbourne, Australia), coated with BP20 with a film thickness of 0.5 μm [5], was divided into two columns of 12.5 m. Baseline separation of the components of interest could be obtained with this short column. The carrier gas was helium at a column flow-rate of 2.5 ml/min. The injector and detector temperatures were set to 300°C. The temperature gradient was from 120°C to 250°C at 20°C/min and then isothermally at 250°C for 0–5 min depending on the content of high-boiling impurities. Total analysis time was normally 10 min, including the time for cooling the oven. The sample (3 μl) was injected in the splitless mode. Six to eight thousand analytical runs can be made on the same column until coelution of 3-methylindole and the internal standard make replacement necessary. The instrument was routinely calibrated with a mixture of indole, 3-methylindole and 5-methylindole (5 mg/l of each in methanol).

2.5. Validation of the method

The accuracy of the measurements was determined by adding known amounts of indole and 3-methylindole to 10 g colonic contents (see Table 2) obtained by pooling material previously

shown to contain low but detectable amounts of indole and 3-methylindole. The reproducibility of the method was determined by repeated measurement of the same sample either as injection variation (5 injections of the same extract), within-sample variation (5 extractions of the same homogenate) or between-sample variation (5 homogenations and extractions of different batches of the same material).

3. Results

As the chemical composition of intestinal contents and faeces is extremely complex it is desirable to use a specific detection method to get less complicated chromatograms. This is possible with the NPD [16] which selectively detects nitrogen or phosphorus-containing compounds depending on the settings of the detector. The NPD has the additional advantage of being more sensitive than the flame ionization detector (FID). This is evident in Fig. 1 where a sample from pig stomach was analyzed with both FID and NPD. The chromatogram with NPD shows only one peak originating from the internal standard and does not show any injection peak. The chromatogram with FID shows a large injection peak and in addition several other peaks, including some in the area of indole and 3-methylindole. The NPD chromatogram shows that neither indole nor 3-methylindole is present in the sample; chromatograms with FID have therefore to be carefully interpreted. The situation is less complicated if there are relatively high amounts of indole and 3-methylindole, as in the example shown in Fig. 2 where a sample from the lower part of pig large intestine has been analyzed with both types of detectors. In the FID chromatogram the components of interest are easily identified but the chromatogram is still more complex than that obtained with NPD. The advantage of the NPD is particularly evident in the analysis of samples from the upper parts of the large intestine and caecum, where the concentrations of indole and 3-methylindole are much lower than those of interfering substances normally detected by the FID.

The effects of the ratio of sample to homogenization medium on the efficiency of the extraction procedure are shown in Table 1. Recovery of indole and 3-methylindole from digesta of pigs fed a standard diet was close to 100%, even when the amount of sample in the homogenization medium was 25%. In a special case where the pigs received a diet with a high fat content the recovery decreased with increasing amounts of sample. This was particularly the case for 3-methylindole which is more hydrophobic than indole. Results documenting the accuracy of the method are given in Table 2. Recovery of indole varied from 101% to 117% (mean 110%), the corresponding values for 3-methylindole were 99% to 108% (mean 102%). The mean between sample coefficient of variation was 3.5% for indole and 3.0% for 3-methylindole. Fig. 3 shows a typical experiment where indole and 3-methylindole were determined in samples from different intestinal seg-

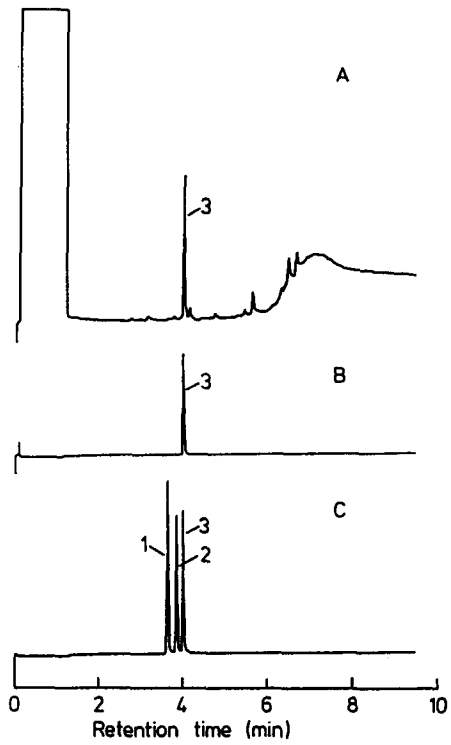

Fig. 1. GC analysis of samples from pig stomach with 5-methylindole added as internal standard. Detection was by FID (A) and NPD (B). (C) Mixture of standards (5 mg/l of each compound); detection by NPD. Peak identification: 1 = indole, 2 = 3-methylindole, 3 = 5-methylindole.

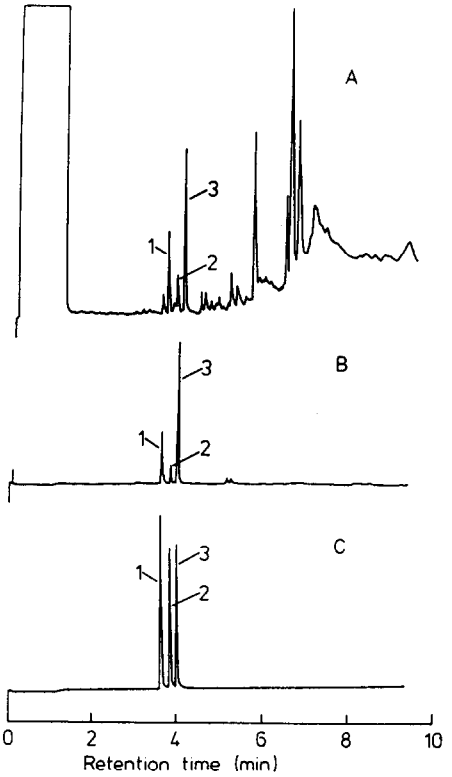

Fig. 2. GC analysis of samples from the lower part of pig large intestine. Detection was by FID (A) and NPD (B). (C) Mixture of standards (detection by NPD) as described in the legend to Fig. 1.

ments from a pig fed a normal diet. Recoveries of added indole and 3-methylindole were higher than 90%, except for the sample from the rectum.

4. Discussion

The active element of a NPD is a small electrically heated bead coated with a rubidium salt, positioned directly above the jet in the detector. Nitrogen and phosphorus containing substances are ionized at the surface of the active element and detected by the collector. The sensitivity of the analysis depends on the power applied to the active element and the time for which it has been in use. Greater sensitivity can be achieved by applying a higher power, however at the expense of the lifetime of the active

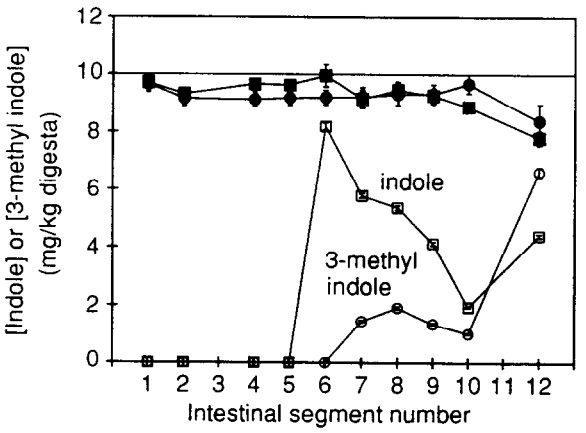

Fig. 3. Typical results from an analysis of indole and 3-methylindole in intestinal segments of a pig fed a normal diet. The open symbols show the mean of three separate determinations using 10-g aliquots from the homogenized intestinal contents. The solid symbols show the mean calculated recoveries from three samples which were spiked with 10 mg/kg digesta of either indole or 3-methylindole; the error bars show the sum of the standard deviations of the unspiked and the spiked samples. The intestinal segments are: 1, 2: stomach; 3–5: small intestine; 6: caecum; 7–12: large intestine.

element. The detection limit under the conditions employed is 0.02 ppm, which is well below the values found in intestinal contents and faeces samples (<0.1 up to 100 mg/kg). Under these conditions the detector retains its activity for *ca.* 500 assays, but can be reactivated by recoating. The short column used in this analysis decreases the time and the maximum oven temperature required to elute the substances of interest. This also halves the costs of columns. The shortening of the column does not seem to decrease its lifetime.

The use of relatively large amounts of sample (10 g) was found to be desirable because this type of material is not homogenous but often contains undigested feed components such as straw or particles from crude ground feed components. In the methods described in refs. 10 and 11 sample amounts of only 0.5 and 1.0 g are used, which in our experience is too small. Our method involves only a simple homogenization and a single extraction step. Other published methods are more complicated and time-con-

Table 1
Effect of the ratio of sample to homogenization medium on the efficiency of extraction

Amount of faeces to homogenization media (%) in stomacher bags	Concentration added (mg/kg faeces)	Recovery from faeces mixed from four pigs receiving a standard diet			Recovery from faeces from a pig receiving a diet with high fat content		
		Mean ± S.D. (mg/kg faeces)	Coefficient of variation (%)	Recovery (%)	Mean ± S.D. (mg/kg faeces)	Coefficient of variation (%)	Recovery (%)
Indole							
1	0	10.8 ± 0.2	1.9		19.8 ± 0.5	2.7	
5	0	10.3 ± 0.2	2.1		23.4 ± 0.3	1.1	
10	0	10.0 ± 0.3	2.7		21.6 ± 0.1	0.3	
25	0	8.8 ± 0.2	1.9		20.8 ± 0.2	0.7	
1	250	296.4 ± 2.1	0.7	114	296.8 ± 0.1	0.0	111
5	50	62.6 ± 0.9	1.4	105	74.7 ± 0.1	0.2	102
10	25	39.2 ± 1.2	3.0	117	46.6 ± 0.1	0.2	100
25	10	19.1 ± 0.4	1.9	103	27.6 ± 0.6	2.2	69
3-Methylindole							
1	0	20.0 ± 0.2	1.0		52.5 ± 0.9	1.7	
5	0	20.8 ± 0.6	2.8		64.7 ± 0.4	0.6	
10	0	21.7 ± 0.9	4.1		62.0 ± 0.2	0.3	
25	0	19.3 ± 0.4	2.2		58.1 ± 0.5	0.8	
1	250	278.9 ± 0.8	0.3	104	325.8 ± 1.5	0.5	109
5	50	71.9 ± 0.1	0.1	102	113.9 ± 0.2	0.1	98
10	25	44.4 ± 0.4	0.9	91	84.8 ± 0.4	0.4	91
25	10	29.4 ± 0.4	1.3	100	62.0 ± 0.3	0.5	39

Faeces from four pigs fed a standard diet and faeces from a pig fed a diet with high fat content. The amount of faeces to homogenization media was varied from 1 to 25% with addition of indole and 3-methylindole to a concentration in homogenate of 10 mg/l. Each determination was performed in triplicate. S.D. = Standard deviation.

Table 2
Accuracy and injection, within- and between-sample coefficients of variation of 3-methylindole and indole determinations

Concentration (mg/kg)		Coefficient of variation (%)			Recovery (%)
Added	Measured (mean ± S.D., $n = 5$)	Injections ($n = 5$)	Within-sample ($n = 5$)	Between-sample ($n = 5$)	
Indole					
0	1.08 ± 0.04	2.8	3.9	3.7	
1.0	2.09 ± 0.02	0.9	0.5	1.0	101
5.0	6.47 ± 0.25	0.2	1.2	3.9	109
20.0	24.5 ± 0.8	0.9	0.5	3.4	117
100.0	111.6 ± 7.2	0.2	1.5	6.5	111
3-Methylindole					
0	0.79 ± 0.03	3.7	3.9	3.8	
1.0	1.78 ± 0.02	1.7	1.1	1.1	99
5.0	5.81 ± 0.19	0.2	1.9	3.3	100
20.0	22.41 ± 0.67	0.2	0.4	3.0	108
100.0	102.2 ± 3.8	0.1	0.6	3.7	101

suming, involving steam distillation [12], three fold extraction with dichloromethane and sample concentration by evaporation [9] or a solid-phase extraction [11]. A simple homogenization and single extraction step were employed in one method [10] but the elution time in this HPLC analysis is 22 min and a filtration step with a Minisart filter is involved. Filtration steps have to be used with caution as some types of filters can effectively absorb all the 3-methylindole and indole present in a sample. Although the extraction procedure which we employ is simple, this does not effect the recovery as long as samples with a low fat content are analyzed. If samples with a high fat content are to be analyzed, recovery can still be acceptable if a low ratio of sample to homogenization medium is used (Table 1). 3-Methylindole seems to be particularly affected by a high fat content, probably as a consequence of its more lipophilic nature compared to indole. Recovery values for indole are generally higher than for 3-methylindole (Tables 1 and 2); this could be explained by the fact that 5-methylindole (the internal standard) and 3-methylindole are both more hydrophobic than indole and are thus less efficiently extracted into the organic phase from non-polar sites on the digesta particles.

The method described is simple and rapid and the accuracy, precision and sensitivity are comparable with other methods, which require a much more time-consuming sample preparation. Each analysis takes 10 min, so more than 100 samples can be analyzed in one day if an autosampler is used. The method has demonstrated its practical value in the analysis of more than 50 000 samples in our laboratory.

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6. References

[1] K. Lundström, B. Malmfors, G. Malmfors, S. Stern, H. Peterson, A.B. Mortensen and S.E. Sørensen, *Livestock Prod. Sci.*, 18 (1988) 55.
[2] M.T. Yokoyama and J.R. Carlson, *Am. J. Clin. Nutr.*, 32 (1979) 173.
[3] B.B. Jensen and M.T. Jensen, in M. Bonneau (Editor), *Measurment and Prevention of Boar Taint in Entire Male Pigs*, Les Colloques no. 60, INRA, Paris, 1993, p. 99.
[4] J.C. Peleran and G.F. Bories, *J. Chromatogr.*, 324 (1985) 469.
[5] M.G. Porter, S.M. Hawe and N. Walker, *J. Sci. Food Agric.*, 49 (1989) 203.
[6] J. Hansen-Møller, *J. Chromatogr.*, 624 (1992) 479.
[7] J. Garcia-Regueiro and I. Diaz, *Meat Sci.*, 25 (1989) 307.
[8] A.B. Mortensen and S.F. Sørensen, in *Proceedings of the 30th European Meeting of Meat Research Workers, Bristol, 1984*, paper 8:11, p. 394.
[9] D.A. Karlin, A.J. Mastromarino, R.D. Jones, J.R. Stroehlein and O. Lorentz, *J. Cancer Res. Clin. Oncol.*, 109 (1985) 135.
[10] C.K. Wilkins, *Int. J. Food Sci. Technol.*, 25 (1990) 313.
[11] M. Dehnhard, H. Bernal-Barragan and R. Claus, *J. Chromatogr.*, 566 (1991) 101.
[12] I. Yoshihara and K. Maruta, *Agric. Biol. Chem.*, 41 (1977) 2083.
[13] G.M. Anderson, *J. Chromatogr.*, 105 (1975) 323.
[14] M.T. Yokoyama and J.R. Carlson, *Appl. Microbiol.*, 27 (1974) 540.
[15] S.R. Elsden, M.G. Hilton and J.M. Valler, *Arch. Microbiol.*, 107 (1976) 283.
[16] G.R. Verga and F. Poy, *J. Chromatogr.*, 116 (1976) 17.