

Determination of Theobromine in Animal Feeding Stuffs

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A method is described for the determination of theobromine in animal feeding stuffs. The sample is extracted with hot water and, after clarification, examined by High Performance Liquid Chromatography using benzoic acid as an internal standard.

Recovery of added theobromine is of the order of 90 per cent., down to a level of about 20 mg/kg in the feeding stuff.

Under the provisions of The Fertilisers and Feeding Stuffs (Amendment) Regulations 1976 there are prescribed maximum limits of theobromine of 300 mg/kg in whole feeding stuffs and 700 mg/kg in feeding stuffs for adult cattle, but these Regulations do not include a prescribed method of analysis for theobromine.

It was found that theobromine was readily extracted from animal feeding stuffs by digestion at boiling point with water and that such an extract, after clarification with Carrez Reagents (zinc and ferrocyanide), was suitable for direct injection on to an HPLC column of Partisil P x S 10/25 ODS with u.v. detection at 272 nm.

The main problem encountered was the swelling of the sample portion due to the gelatinisation of the starch which limited the amount of sample that could be taken for the determination. At ratios of water:feed of 20:1 or greater, however, little difficulty was experienced. There was some indication that the recovery was better at the higher ratios.

Experimental

APPARATUS

Pye-Unicam LC3 Liquid Chromatograph, u.v. Detector.

Partisil P x S 10/25 ODS Column.

High Pressure Syringe, 10 μ l.

Operating conditions: Operating temperature, ambient.

Flow rate, 0.8-1.0 ml/min.

Detector, 272 nm.

REAGENTS

1. *Carrez Solution 1.* Dissolve 21.9 g of zinc acetate dihydrate in water, add 3 ml of glacial acetic acid and dilute to 100 ml with water.
2. *Carrez Solution 2.* Dissolve 10.6 g of potassium ferrocyanide in water and dilute to 100 ml with water.

3. 4 per cent. v/v acetic acid.
4. Methanol.
5. *Eluting solvent.* 4 per cent. v/v acetic acid: methanol in the proportions of 4:1 by volume.
6. *Standard theobromine.* Dissolve 0.050 g of theobromine in 200 ml of hot water, cool and dilute to 500 ml with eluting solvent (100 mg/litre).
7. *Internal standard.* Dissolve 0.2500 g of benzoic acid in 100 ml of methanol and dilute to 500 ml with 4 per cent. v/v acetic acid (500 mg/litre). Prepare further dilutions with 4 per cent. v/v acetic acid as required.

Procedure

(a) CALIBRATION

Prepare standard solutions containing from 1.0 to 25.0 mg/litre of theobromine.

Transfer 5.00 ml of each into dry stoppered tubes and add 1.00 ml of the appropriate internal standard solution.

Inject each solution, in turn, and calculate the peak height ratios in each case.

$$\frac{\text{Peak height theobromine}}{\text{Peak height benzoic acid}} = H_s.$$

TABLE I
THEOBROMINE CALIBRATION

Standard theobromine solution, mg/litre	Standard benzoic acid solution, mg/litre	Peak height ratio*
25.0	500	2.40
10.0	500	0.95
10.0	375	1.33
5.0	375	0.65
2.5	150	0.81
1.0	75	0.61

* These are typical results. The actual values vary with changes in the working conditions and should, therefore, be determined for each analysis.

(b) DETERMINATION

Weigh 5 g $\pm$ 0.01 g of a feed containing 100 mg/kg or more of theobromine, or up to 10 g of a feed containing less than 100 mg/kg of theobromine, into a 250 ml flat-bottomed flask and add approximately 150 ml of distilled water.

Bring the liquid to the boil, with constant mixing of the contents of the flask, and then continue to boil gently under a reflux condenser for 30 minutes, with frequent mixing.

Cool to 20°C, transfer to a 200 ml calibrated flask, add 5 ml each of Carrez Solution 1 and Carrez Solution 2, in the accustomed manner, dilute to the mark with distilled water and mix well.

Centrifuge, or filter through a dry Whatman No. 542 paper.

To 5.00 ml of the clear liquid add 1.00 ml of the appropriate internal standard solution, according to the amount of theobromine expected. Inject, and measure the peak height ratio as in the calibration procedure.

$$\frac{\text{Peak height theobromine}}{\text{Peak height benzoic acid}} = H_t.$$

$$\text{Theobromine} = \frac{H_t \times S \times 200}{H_s \times W} \text{ mg/kg}$$

where H_t = peak height ratio for the sample,

H_s = peak height ratio for the standard—at the same concentration of the internal standard as for H_t ,

S = concentration of standard theobromine, mg/litre, and

W = weight of sample in grams.

TABLE II
RECOVERY OF THEOBROMINE ADDED TO FEEDING STUFFS

Feeding stuff	Weight taken, g	Theobromine		
		Added, mg/kg	Recovered, mg/kg	Recovery,* per cent.
Bullock mixture (FS78/9)	5	Nil	Nil	—
	10	26	25	96.2
	10	50	43	86.0
	5	94	76	80.9
	5	95	78	82.1
	5	300	271	90.3
	5	302	268	88.7
Meal (FS78/12)	5	Nil	Nil	—
	5	118	99	83.9
	5	148	125	84.5
	5	333	295	88.6
	5	334	285	85.3
Dairy nuts (FS78/14)	5	Nil	Nil	—
	10	25	23	92.0
	10	50	42	84.0
	5	96	82	85.4
	5	98	83	84.7
	5	300	288	96.0
Dairy pellets (FS78/18)	5	Nil	Nil	—
	5	299	270	90.3
	5	300	259	86.3
Dairy meal (FS79/25)	5	Nil	10	—
	10	23	33	100.0*
	10	50	55	90.0*
Dairy meal (FS79/32)	5	Nil	12	—
	10	25	32	80.0*
	10	47	51	83.0*

* Recovery corrected, where necessary, for theobromine in original feeding stuff.

Discussion

Under the operating conditions described, theobromine was eluted after approximately 11 minutes and benzoic acid after approximately 15 minutes.

A small peak at 8 minutes was not identified.

The peak heights were measured from a smooth curve drawn through the bases of the peaks, as is the convention when the peaks occur on the "tail" of the injection peak.

Results obtained by this method are given in Tables II and III.

TABLE III
THEOBROMINE FOUND IN SAMPLES OF FEEDING STUFFS
EXAMINED UNDER THE AGRICULTURE ACT 1970

Feeding stuffs		Theobromine, mg/kg
FS78/1	Pig meal	13
FS78/6	Cattle nuts	45
FS78/9	Bullock mixture	Nil
FS78/10	High maize meal	323
FS78/12	Meal	Nil
FS78/14	Dairy nuts	Nil
FS78/16P	Grass cubes	7
FS78/18	Dairy pellets	Nil
FS78/29	Dairy meal	235
FS78/41	Pig meal	13
FS78/43	Horse feed	20
FS78/45	Dairy nuts	35
FS78/50	Pig meal	36
FS78/58	Layers pellets	12
FS78/59	Rabbit pellets	Nil
FS79/14	Dairy balancer	323
FS79/16	Calf starter	22
FS79/25	Dairy mixture	10
FS79/27	Dairy mixture	18
FS79/32	Dairy meal	12
FS79/33	Dairy concentrate	99

Effect of Other Medicinal Substances

Various medicinal substances commonly used in animal feeding stuffs were extracted as in the method used and examined by HPLC.

Under these conditions no interference was observed, there being either no peaks or peaks that were distinct from those produced by theobromine or by benzoic acid.

The following were examined: Clopidol, Ethopabate, Fenbendazole, Halquinol, Nicarbazin, Nitrovin, Pyrimethamine, Robendine, Sulphoquinoxaline, Amprolium, Dimetridazole, Halofuginone, Iprnidazole, Nifursol and Ronidazole.

Although not a "medicinal substance" for the purpose of the Agriculture Act 1970, the effect of caffeine was investigated as a possible interfering substance. It was found, however, that caffeine was eluted from the column *after* the benzoic acid and could not, therefore, be confused with theobromine.