Journal of the Association of Public Analysts (Online) 2019 **47 001-035**
Gray *et al*

Validation of a Method for the Determination of Theobromine in Feed Materials and Compound Feeds by Liquid Chromatography with UV Detection or with Tandem Mass Spectrometry

Kirstin M Gray [a] and D Thorburn Burns[b]

[a] LGC, Teddington, Middlesex, TW11 0LY, UK
[b] Institute for Global Food Security, The Queen's University of Belfast, Belfast BT9 5HN, Northern Ireland, UK

Summary

An inter-laboratory validated HPLC separation coupled with UV detection method for the determination of theobromine in animal feed materials and in compounded feeds is described using 7-(β-hydroxyethyl) theophylline as the internal standard. If more forensically robust identification of the presence of theobromine is required, the same HPLC separation can be coupled to a tandem mass spectrometer and theobromine-d3 used as the internal standard. At the time of publishing, the method described in this paper is in the process of a formal vote before being published as a CEN standard.

Introduction

Theobromine (3,7-dihydro-3,7-dimethyl-*H*-purine-2,6-dione) is a colourless and odourless alkaloid with a slightly bitter taste that is present naturally in the cacao tree (*Theobroma cacao* L) and its seeds. It has a long history[1], discovered in 1841 by Woskresensky[2], its toxicity to kittens and other small animals was noted soon after by Mitscherlich[3], and it was synthesised by Fisher in 1897[4]. The composition of cocoa and the determination of theobromine in various products has been of interest to Public Analysts since the 1870's to date[5-9]. Theobromine is a metabolite of caffeine in mammals and its presence in post-race horse urine samples above 2 μg/ml raises questions as to its source, possibly as a residual from feed or from administration of caffeine to alter racehorse performance[10].

As cocoa pod husk is used in animal feed material in developing countries where cacao is grown, and in Europe using cocoa bean shells, cocoa bean meal, cocoa germs and discarded confectionary, the toxicology of theobromine was reviewed in detail by the European Food Safety Authority (EFSA) in 2008. It reported theobromine to exhibit moderate acute oral toxicity in experimental animals with LD_{50} values ranging from around 300 to 1350 mg/kg body weight. The toxic effects in a range of farmed and domesticated animals were also reported along with a review of the then extant analytical methods[11]. Since this report, Adamafio has noted the large quantities of cocoa by-products generated in cocoa producing and processing countries and that theobromine toxicity imposes limitations on their use in animal feed necessitating studies on detheobromination methods[12]. Current EU regulations on

maximum levels (ML) of theobromine in feed material (300 mg/kg for complete feeding stuffs with the exception of 200 mg/kg for complete feed for pigs and 50 mg/kg for complete feed for dogs, rabbits, horses and fur animals)[13] may not be fully protective for some target animal species, eg as effects on milk production in dairy cows and adverse effects in pigs may occur. Owing to the recognized susceptibility to theobromine toxicity, information from industry indicates that UK animal feed manufacturers do not include by-products of cocoa manufacture or confectionary by-products in feeds for dogs or for horses.

Numerous methods for the analysis of theobromine[11] have been developed including liquid chromatography with a variety of detectors[14,15] and directly using near infrared spectroscopy[16].

The aim of this study, in response to a call to tender from CEN/TC 327[17], was to develop and validate a method for the determination of theobromine in animal feed materials and compound feed, in particular for those with cocoa derived content. CEN/TC 327 is the CEN technical committee for animal feeding stuffs – methods of sampling and analysis with a scope of standardisation of methods of sampling and analysis for animal feeding stuffs, including chemical, biochemical, microbiological, physical and microscopical methods. Working group 5 of CEN/TC 327 deals with natural toxins.

The initial method involved solvent extraction followed by solid phase extraction (SPE) clean-up. In the final developed method clean-up using Carrez solutions was adopted.

In addition to determination by LC-MS/MS, at the request of the CEN working group, an LC-UV detection method using the same extraction, clean-up and separation was also examined to provide method versatility.

The initial study followed a staged approach as summarised in Table 1.

Table 1 – Staged Approach to the Development of the Validated Method

Stage	Study Objective	Task	
1	In-house Validation of Method	1.1	Demonstration of SPE clean-up with LC-MS/MS detection
		1.2	In-house validation for theobromine in feeds using LC-MS/MS
		1.3	Preparation of draft SOP for collaborative trial
2	Collaborative Trial	2.1	Design of collaborative trial and recruitment of participating laboratories
		2.2	Homogeneity check for collaborative trial materials
		2.3	Pre-trial (method familiarisation)
		2.4	Statistical analysis of pre-trial data
		2.5	Main collaborative trial
		2.6	Statistical analysis of collaborative trial data

Stage	Study Objective	Task	
3	Preparation of a Draft European Standard	3.1	Preparation and circulation of draft standard
		3.2	Consideration of responses
		3.3	Preparation of final draft of the European standard

Materials and Method

Reagents

Supplier	Reagents
Fisher Scientific, Loughborough, UK	• Solvents (methanol, chloroform, hexane, acetonitrile) • SPE Cartridges C18 ec, 1ml, 100mg • Zinc acetate dihydrate • Potassium ferrocyanide trihydrate • Glacial acetic acid • Ammonium formate • Potassium dihydrogen phosphate
Sigma-Aldrich, Dorset, UK	• 18MΩ water • Theobromine • 7-(β-Hydroxyethyl) theophylline • Paraxanthine • 2.5M ammonium acetate buffer, pH 5.5 (ammonium acetate and acetic acid)
CDN Isotopes, Quebec, Canada)	• Theobromine-d3

Carrez reagent I was prepared by weighing 219 g±1g zinc acetate dihydrate into a 1l beaker, add 30ml glacial acetic acid and approximately 800ml water. Mix thoroughly until dissolved, transfer to a 1l volumetric flask and dilute to volume with water. Mix well before use. This solution is stable for up to 3 months when stored at room temperature.

Carrez reagent II was prepared by weighing 106 g±1g potassium ferrocyanide trihydrate into a 1l beaker and add approximately 800ml water. Mix thoroughly until dissolved, transfer to a 1l volumetric flask and dilute to volume with water. Mix well before use. This solution is stable for up to 3 months when stored at room temperature.

The following feed samples were obtained:

Supplier	Sample
Amazon, London, UK	• Saracen Show Improver Pencils (Ingredients: Wheat feed, soya hulls, wheat, molasses, dried alfalfa, Equi-jewel, soya bean meal, vegetable oil, limestone, vitamins and minerals, dicalcium phosphate, salt)

	• Saracen Show Improver Mix (Ingredients: lucerne pellets, barley flakes, soya hulls, extruded barley, maize flakes, molasses, pea flakes, soya flakes, rice bran with calcium carbonate, Equi-jewel, soya oil, vitamins and mineral, yeast and mixture of flavouring compounds) • Purina One Adult (Ingredients: dehydrated poultry protein, wheat, maize, beef (14%), animal fat, rice (4%), soya meal, maize gluten meal, minerals, glycerol, propylene glycol, maize grits, malt flour, fish oil)
Tesco, Welwyn Garden City, UK	• Pedigree Vital Protection adult dog food with chicken (Ingredients: Cereals, meat and animal derivatives (including 4 % chicken), oils and fats (including 0.2% fish oil, 0.2% sunflower oil), derivatives of vegetable origin (including 2% dried beet pulp), minerals (including 0.7 5 sodium tripolyphosphate) • Winalot Shapes (Ingredients: Cereals, oils and fats, derivatives of vegetable origin, meat and animal derivatives, vegetables, minerals)
Wilko, Worksop, UK	• Pets Inc Chocolate Treat Bar for Dogs (Ingredients: Wheat flour, hydrogenated vegetable oil, fat reduced cocoa powder, emulsifier (lecithin E322), vitamins) • Choc Drops for Small Animals (Ingredients: oils and fats, cereals, various sugars, milk and milk derivatives, derivatives of vegetable origin, minerals)

Apparatus

In addition to normal laboratory apparatus and disposables the following two LC systems were used:

UV Detection

HPLC System	• Jasco AS-1555-10, PU-1580, CO-965, UV-1575 (Jasco, Essex, UK)
HPLC Column	• Luna C18 (2), 250mm × 4.60mm, 5 micron column (Phenomenex, Cheshire, UK)

Mobile Phase	• 90% 0.0125mol/l potassium dihydrogen phosphate, pH 3.5, 10% acetonitrile
Column Temperature	• 30°C
Injection Volume	• 20µl
Flow Rate	• 1.0ml/min
UV Detector	• Jasco UV-1575, wavelength 278 nm (Jasco, Essex, UK)

LC-MS/MS Detection

HPLC System	• Agilent 1260 Infinity (Agilent, Cheshire, UK)
HPLC Column	• Atlantis T3 C18, 3µm, 2.1mm x100mm column (Waters, Hertfordshire, UK)
Mobile Phase	• Gradient elution of 12mM ammonium formate in methanol/12mM ammonium formate in water • time (min)=0 – 1% 12 mM ammonium formate in methanol • time (min)=2 – 1% 12 mM ammonium formate in methanol • time (min)=4 – 20% 12 mM ammonium formate in methanol • time (min)=14 – 80% 12mM ammonium formate in methanol • time (min)=15 – 98% 12mM ammonium formate in methanol • time (min)=16 – 1% 12mM ammonium formate in methanol • time (min)=24 – 1% 12 mM ammonium formate in methanol
Column Temperature	• 25°C
Injection Volume	• 20µl
Flow Rate	• 0.2ml/min
MS Detector	• AB Applied Biosystems MDS SCIEX, Q Trap LC-MS/MS system (AB Sciex UK Ltd, Cheshire, UK)
Curtain Gas	• 20.00 (arbitrary units)
Ion Spray	• 4,500V
Temperature	• 450.0°C
Gas 1	• 30.0 (arbitrary units)
Gas 2	• 30.0 (arbitrary units)
CAD Gas	• Medium (arbitrary units)

The transitions monitored are given in Table 2

Table 2 – Ion Transitions

Analyte	Approximate Retention Time (min)	Transitions (m/z)	Time (msec)	Declustering Potential (V)	Collision Energy (eV)
Theobromine	12.7	181.2 > 138.2	50	40	30
		181.2 > 163.1			
Theobromine-d3	12.7	184.2 > 141.1	50	80	35
		184.2 > 166.2			
7-(β-hydroxyethyl) theophylline	14.4	225.2 > 124.1	50	30	40
		225.2 > 181.3			15
Caffeine	15.3	195.1 > 138.0	50	30	30
Paraxanthine	13.8	181.1 > 124.0	50	30	35
Theophylline	14.1	181.1 > 124.0	50	30	35

The conditions for caffeine are provided for information only.

It is recommended that paraxanthine and theophylline are run to confirm chromatographic separation from theobromine.

The initial approach to sample preparation, summarised in Table 3, was investigated and amended in the light of this experience to that summarised in Table 4.

Table 3 – Summary of the Initial Method of Sample Extraction for Analysis

Step	Action
1	Homogenise the sample using an appropriate blender or grinder such that the ground-up sample can be passed through a 500 micron sieve
2	Weigh out accurately 2.5g of each sample into separate 50ml polypropylene tubes. (A sample weight smaller than 2.5g is inadvisable for animal feed as it is unlikely to be representative as this matrix type is inherently difficult to homogenise)
3	Spike each sample with theobromine-d3 as the internal standard or 7-(β-hydroxyethyl) theophylline as the surrogate standard
4	Add 25ml of 2.5M ammonium acetate buffer, pH 5.5, to the sample and briefly vortex mix. Place the tubes in an ultrasonic water bath at room temperature and sonicate for a minimum of 20 minutes

Step	Action
5	Remove the tubes from the water bath, briefly vortex mix and centrifuge at 4000rpm for 20 minutes using a centrifuge, cooled if possible
6	Condition SPE Cartridges (C18 ec, 1ml, 100mg) with 2x1ml methanol followed by 2x1ml water ensuring that the cartridges do not dry out after the conditioning stage
7	Add 1ml of the sample extract to a conditioned SPE cartridge and pull this through slowly under vacuum until the meniscus is just above the silica bed. Wash the column with 1ml of water and dry the cartridge under vacuum for a minimum of 10 minutes
8	Elute the cartridge with 2x500µl of chloroform collecting the eluent in a 9ml glass tube. Evaporate the tube contents to dryness using a Turbovap at 45°C and add 1ml of water. Dissolve any solid material that is present in the tube using the vortex mixer and filter the solution through a 0.45µm filter into a 2ml crimp cap auto-sampler vial ready for analysis

Table 4 – Final Method of Analysis

Step	Action
1	Homogenise the sample using an appropriate blender or grinder such that the ground-up sample can be passed through a 500micron sieve
2	Weigh out accurately 2.5g of each sample into separate 50ml polypropylene tubes. (A sample weight smaller than 2.5g is inadvisable for animal feed as it is unlikely to be representative as this matrix type is inherently difficult to homogenise)
3	Add 3ml hexane and warm in a water bath set at 40°C
4	Mix thoroughly by vortex then centrifuge at 5000 rpm for 10 minutes
5	Discard the top hexane layer
6	Repeat hexane extraction 3 more times
7	Dry sample with a stream of nitrogen to remove last traces of hexane
8	Add 0.2ml of 1mg/ml 7-(β-hydroxyethyl) theophylline internal standard solution
9	Add 25ml of 2.5M ammonium acetate. Warm to 40°C in a water bath, vortex, then place in an ultrasonic bath and sonicate for 20 minutes
10	Transfer to 100ml volumetric flask
11	Add 5ml of Carrez Reagent I and mix well by shaking then add 5ml of Carrez Reagent II and mix well by shaking
12	Dilute to volume with water and mix well
13	Filter through a Whatman 541 filter paper followed by a 0.45µm syringe filter
14	Pipette 1ml of the filtrate into an autosampler vial and add 25µl of 200µg/ml theobromine-d3, if required
15	Analyse by LC-MS/MS or HPLC-UV

Note – pre-collaborative trial work led to some further amendments to this procedure

Selection of External Reference Materials and Preparation of Test Feed Samples

Preliminary Considerations

A critical issue to be taken into account when carrying out the determination of theobromine is the solubility of the theobromine standard material compared to the maximum permitted concentration in feed. The aqueous solubility of theobromine at room temperature is low, at about 0.4 mg/ml[16] and the maximum permitted concentration of theobromine in complete feed with the exception of feed for pigs (200 mg/kg), dogs, rabbits, horses and fur animals (50 mg/kg) is 300 mg/kg (Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on Undesirable Substances in Animal Feed). Therefore, the recommended 2.5g of sample would need to be spiked with 1.875ml of 0.4mg/ml aqueous theobromine solution to provide a theobromine concentration at the maximum permitted level. Spiking with such a comparatively large volume of an aqueous solution is not recommended. It has been noted that other laboratories have used boiling water to prepare stock theobromine standards, but the small amount of gain in solubility is outweighed by the potential for solute precipitation upon cooling, especially when making the stock standard up to volume. Also, the slight increase in solubility achieved by heating is not sufficient to allow an appropriately low spiking volume.

Theobromine is not soluble in any of the common organic solvents such as ethanol, acetone or dichloromethane[18] ruling out, therefore, a conventional spiking procedure where spiking compounds would be added as a concentrated solution in a small volume of a semi-volatile organic solvent that would evaporate prior to extraction.

The solubility of theobromine was explored for other solvents such as iso-propanol and in the ammonium acetate extraction buffer, but these experiments were unsuccessful and failed to increase the solubility above that previously obtained with room temperature water.

Further investigations showed that 0.5g theobromine is readily soluble in 50ml 0.5N sodium hydroxide. However standard solutions prepared thus proved less than ideal due to the high pH which had an adverse effect on the chromatography.

Use of the Standard Addition Method was considered and rejected as it is only practical if:

- there is an estimate of the expected amount of theobromine to be found in the sample
- the expected level of theobromine is very low thus allowing reasonable volumes of spiking solution to be added.

For routine analysis, standard additions to all samples would be very labour-intensive and would be expensive with regards to reagent and other consumable costs thus an internal reference method was adopted.

For method validation and collaborative trials, bulk quantities of each ground test feed material were spiked with suitable volumes of 125µg/ml aqueous theobromine solution and freeze dried. Once the materials had been dried, they were homogenised and mixed thoroughly before use.

Commercially Available Reference Materials

Several reference materials were available (see Table 5) however none were an ideal matrix match to animal feed. Baking chocolate (NIST SRM 2384) was chosen as the most appropriate quality control material as the theobromine was from a cocoa-derived material despite the reference concentration of theobromine being greater than that expected in animal feed.

Table 5 – Reference Materials

NIST SRM 2384 Baking chocolate, certified theobromine content 11600±1100 mg/kg
NIST SRM 3254 Green tea (*Camellia Sinensis*) leaves, certified theobromine content 463±52 mg/kg
NIST SRM 3256 Green tea containing solid oral dosage form, certified theobromine content 1040±150 mg/kg
NIST SRM 3255 Green tea (*Camellia Sinensis*) extract, certified theobromine content, 867±76 mg/kg
MUVA-S-0813 Nut-nougat-cream, certified theobromine content, 1724±35 mg/kg

Test Samples

It was originally intended to validate the method using three materials:

- a horse feed
- a dog feed
- a feed containing cocoa-derived material

Two different types of horse feed (improver mix and improver pencils) and three different dog feeds (biscuits, complete feed with chicken and adult feed with chicken and beef) were obtained and analysed and it was found that none contained theobromine. After discussions with several manufacturers it was understood that cocoa-derived ingredients such as waste from confectionery plants can be added to feed for animals such as pigs, however it was not possible to procure such a product. Following a survey of available animal feeds, a “chocolate” treat bar for dogs was obtained that contained theobromine.

In the absence of appropriate samples containing known quantities of theobromine nine samples, five of which were spiked, were prepared and used for the single laboratory validation. The blank complete dog feed with chicken and the blank horse improver pencils were spiked at two different concentrations with the dog treat bar containing theobromine. The dog

chocolate was also mixed with the blank carob chocolate drops for small animals to provide test materials with different concentrations of theobromine. Each of the nine samples were analysed in triplicate using SPE clean up on each of three days by two different analysts, giving 18 results for each sample.

There are however issues associated with this approach, for example the method is being validated against the extraction of theobromine from chocolate in a background of either dog or horse feed rather than from the feeds alone.

Before analysis the dog treat bar containing theobromine was homogenised thoroughly, however the 18 results for this material gave a %RSD (Relative Standard Deviation = 100*standard deviation/mean result) in the region of 70%. There was some within-day variation in the results as well as day-to-day variation which were not contributable to the analysts, Table 6. Due to the variation in results it was considered that the method, as it then stood, was not suitable for a collaborative trial.

Table 6 – Initial Validation Results

	Mean Theobromine mg/kg	**RSD %**
Blank horse feed	1.3	176
Horse feed spiked with dog treat bar	632	69
Horse feed spiked with dog treat bar	2667	49
Blank dog food	2.9	132
Dog food spiked with dog treat bar	1132	154
Dog food spiked with dog treat bar	1538	45
Blank chocolate	0.7	172
Dog treat bar	18846	71
Blank chocolate spiked with dog treat bar	4398	63

Internal Standards

In an ideal situation an isotopically-labelled standard of the compound of interest would be added prior to extraction for analyses where the end point determination is by mass spectrometry. The use of theobromine-d3 was investigated but the price (approximately €450 for 10mg) is prohibitively expensive for most laboratories to use routinely. However, theobromine-d3 could, if need arises, be added to the sample extract just prior to injection both to confirm the identity of any theobromine detected in the samples and show where theobromine would have eluted had there been any present in the extracted sample.

Instead the method was optimised with the use of 7-(β-hydroxyethyl) theophylline as the internal standard and the results calculated using a ratio of the response for theobromine to that for 7-(β-hydroxyethyl) theophylline.

Further Optimisation of Extraction

Tests were carried out first to establish if the SPE or an alternative clean-up was necessary. The resulting chromatograms indicated that a clean-up procedure was needed to remove interferences. Further trials were then carried out with the aim of improving the extraction efficiency and repeatability. The following were considered to be the most critical steps of the extraction procedure:

- prior defatting
- extraction solvent (water or acetate buffer)
- clean-up (SPE or Carrez reagents).

The Carrez reagents[19] were prepared in the laboratory: (Carrez I) zinc acetate dihydrate and (Carrez II) acetic acid and potassium ferrocyanide. The SPE cartridges used were C18 ec, 1ml/100mg.

The NIST baking chocolate, SRM 2384, was extracted using combinations of these techniques to establish the optimum extraction procedure. Table 7 summarises the results obtained.

The certified value for theobromine in NIST SRM 2384 baking chocolate is 11600±1100mg/kg and the results in Table 7 within this range are highlighted. None of the results obtained were below the certified range implying that interferences or poor clean-up were more likely an issue than was the extraction efficiency. The samples that were defatted prior to extraction generally gave more accurate results therefore it was considered important to include this step in the final protocol. The repeatability was generally better when acetate buffer had been used as opposed to water. There was little difference between the results obtained using acetate buffer when SPE clean-up was used rather than when using Carrez solutions, therefore it was decided that clean-up with Carrez solutions would be used as it is quicker and cheaper than SPE.

Table 7 – Optimisation of Extraction Procedure for NIST SRM 2384

Extraction Procedure				
Defat Sample with Hexane?	**Extraction Solvent**	**Clean-up Procedure**	**Average Theobromine Concentration (mg/kg)**	**RSD%**
No	Acetate buffer	None	13289	2.7
No	Acetate buffer	SPE	12856	11.8
No	Acetate buffer	Carrez reagents	12967	2.4
No	Water	None	12699	4.7
No	Water	SPE	13008	8.0
No	Water	Carrez reagents	13282	6.5
Yes	Acetate buffer	None	12412	0.2
Yes	Acetate buffer	SPE	11979	4.5
Yes	Acetate buffer	Carrez reagents	12022	4.0
Yes	Water	None	12175	4.6
Yes	Water	SPE	11587	17.1
Yes	Water	Carrez reagents	11770	3.7

Note – Shaded data are within the certified range of the SRM

Method Evaluation

Six 1.00g±0.1g replicates of the dog treat bar were analysed to establish the repeatability of the updated method; the results are presented in Table 8.

Table 8 – Replicate Analysis of Dog Treat Bar

Replicate	Theobromine Concentration (mg/kg) (Average of Duplicate Injections)
1	859
2	849
3	859
4	838
5	879
6	845
Mean	855
Standard deviation	14.5
% RSD	1.70

As the data in Table 8 indicated that the results for the dog treat bar were reproducible when a sample weight of 1g was used the repeatability and reproducibility of the method was further evaluated by triplicate extractions of three different weights (0.5, 1 and 2g) on three days. The results are summarised in Table 9.

Overall the results looked promising however there was an unexplainable low bias to the results obtained on day 3. As the product used to evaluate the extraction procedure was a retail product containing fat-reduced cocoa powder and the theobromine content was not stated on the label, the repeatability and precision but not the accuracy of the method could be estimated.

Table 9 – Multiple Replicates of Dog Treat Bar Analysed on Multiple Days

Replicate	Day	Sample Weight (g)	Concentration in Sample (mg/kg)	Mean	SD	% RSD
1	1	2	861	801	74	9
2	1	2	770			
3	1	2	893			
1	2	2	827			

Replicate	Day	Sample Weight (g)	Concentration in Sample (mg/kg)	Mean	SD	% RSD
2	2	2	835			
3	2	2	869			
1	3	2	765			
2	3	2	684			
3	3	2	704			
1	1	1	900	830	54	7
2	1	1	804			
3	1	1	790			
1	2	1	889			
2	2	1	846			
3	2	1	902			
1	3	1	779			
2	3	1	781			
3	3	1	778			
1	1	0.5	938	892	96	11
2	1	0.5	962			
3	1	0.5	940			
1	2	0.5	1019			
2	2	0.5	843			
3	2	0.5	980			
1	3	0.5	817			
2	3	0.5	780			
3	3	0.5	750			

The results indicated that the extraction was suitable for a matrix such as a chocolate product when 0.5, 1 or 2g of sample was taken. However, due to possible homogeneity issues with complete and compound feeds, it is recommended that for an animal feed a sample weight of no less than 2.5g is taken for each extraction.

The method was shown to work in principle for chocolate products and be suitable as a screening method. Analysis of the NIST SRM 2384 baking chocolate provided an indication of the accuracy of the method in chocolate.

Single Laboratory Validation of Spiked samples by LC-MS/MS

As it was not possible to obtain commercial samples of animal feed containing known amounts of theobromine, validation was carried out on spiked materials and a NIST reference material (NIST SRM 2384, baking chocolate). Bulk quantities of ground complementary feed for adult dogs and complementary compound feedstuff for horses were spiked with appropriate volumes of an aqueous solution of theobromine (125µg/ml) and then freeze dried. The resulting dried samples were ground to pass a 500micron sieve and mixed thoroughly before being used for the validation.

The method was validated using the following samples:

- Blank dog food
- Dog food spiked at approximately 50mg/kg (53mg/kg)
- Dog food spiked at approximately 150mg/kg (156mg/kg)
- Dog food spiked at approximately 300mg/kg (311mg/kg)
- Blank horse feed
- Horse feed spiked at approximately 50mg/kg (53mg/kg)
- Horse feed spiked at approximately 300mg/kg (315mg/kg)
- Horse feed spiked at approximately 700mg/kg (734mg/kg)
- NIST SRM 2384 (baking chocolate)

These concentrations were chosen as they reflected the current maximum permitted concentrations stated for various feeds in Commission Directive 2002/32/EC as amended, and those in the original Directive.

The nine materials were analysed in triplicate by LC-MS/MS on each of three days by two different analysts to give 18 data points for each material. Table 10 presents the estimated mean recoveries for the dog food and horse feed. Standard errors are given in parentheses. All six estimates are not significantly different from 100% and have 17 degrees of freedom ($k = 2.11$). Table 11 presents the within-batch standard deviation (repeatability) and between-batch standard deviation; standard errors are given in parentheses.

Table 10: Mean Recoveries

Matrix	Concentration (mg/kg)	Mean Recovery (%)*
Dog food	50	89.2 (5.5)
	150	95.8 (2.9)
	300	99.1 (2.9)
Horse feed	50	98.1 (4.7)
	300	105.1 (3.9)
	700	107.4 (4.3)

* Standard errors are given in parentheses

Table 11 – Within and Between-batch Standard Deviations

Matrix	Nominal Concentration (mg/kg)	Repeatability SD* (mg/kg) S_r	Between-batch SD* (mg/kg) S_b
Dog food	50	12.0 (25.4%)	2.7 (5.6%)
	150	20.4 (13.7%)	0.0
	300	41.4 (13.4%)	0.0
Horse feed	50	6.5 (12.4%)	8.8 (16.8%)
	300	53.8 (16.3%)	0.0
	700	151.9 (19.3%)	0.0
NIST SRM 2384, baking chocolate	11600	901 (8.5%)	840 (7.9%)

* Standard errors are given in parentheses

Determination of Theobromine Content by HPLC-UV

An additional trial was carried out to establish the feasibility of using the extracts from the validated LC-MS/MS method for analysis by LC-UV.

The following samples were extracted in duplicate using the revised protocol (see Table 4) and the extracts of samples listed below analysed both by LC-MS/MS and LC-UV.

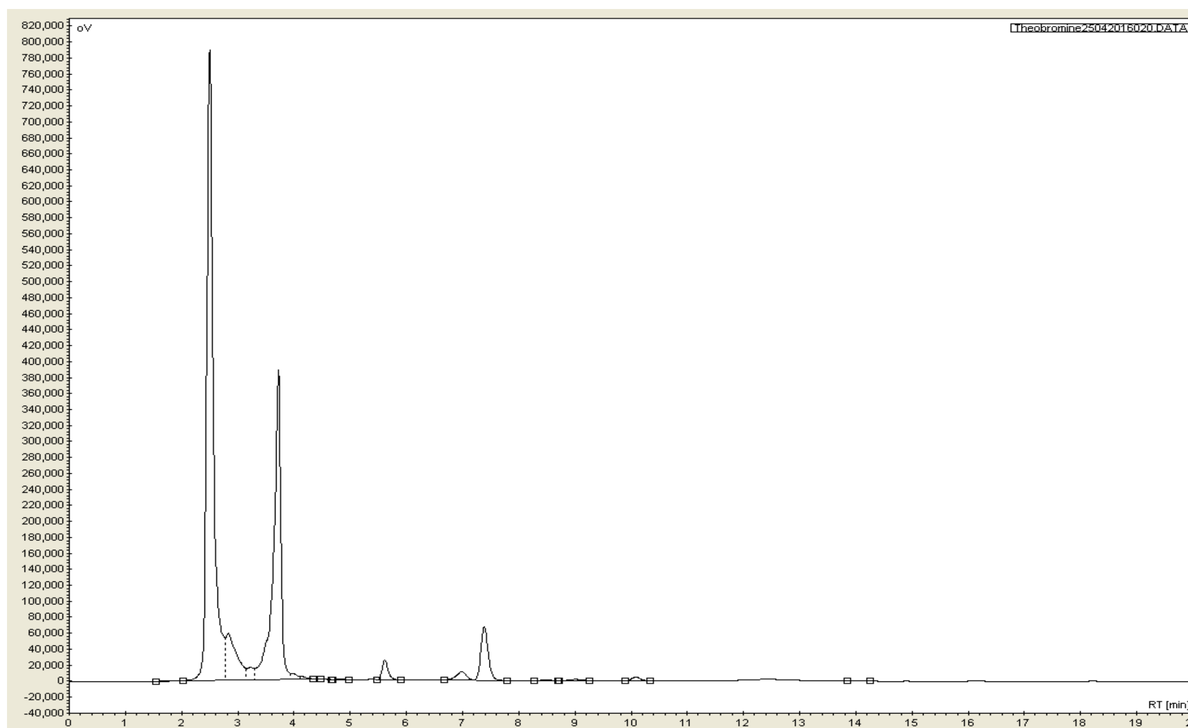
- Blank dog food
- Dog food spiked at approximately 50 mg/kg
- Dog food spiked at approximately 150 mg/kg

- Blank horse feed
- Horse feed spiked at approximately 300 mg/kg
- NIST SRM 2384 (Baking chocolate)

The theobromine content for the extracts analysed using both techniques was calculated using the ratio of the responses of theobromine to those of 7-(β-hydroxyethyl) theophylline, the internal standard.

A set of calibration standards was prepared and run on both systems (LC-MS/MS and LC-UV) and acceptable responses and linearities were achieved for both. The lowest calibration standard analysed was 0.5µg/ml theobromine which is equivalent to 20mg/kg theobromine in a sample when 2.5g of sample is extracted into 100ml. Figure 1 presents an example chromatogram of a UV chromatogram for a spiked sample of horse feed.

Figure 1 - Example Spiked Horse Feed LC-UV Chromatogram



Theobromine – 5.6 minutes
7-(β-Hydroxyethyl) theophylline – 10.1 minutes

With UV detection, theobromine was not detected in either the blank dog food or blank horse feed. Acceptable recoveries were obtained for the spiked samples (> 90%) by UV and the results were comparable to those obtained by LC-MS/MS. The results for the NIST SRM 2384 baking chocolate were within the certified limits.

The HPLC conditions were based on an earlier in-house LGC method for the determination of theobromine in foods. With these conditions it was found that paraxanthine and theophylline elute closely however both of these compounds, as well as caffeine, were clearly resolved from theobromine.

In summary, the additional trial requested by CEN/TC 327 Working Group 5 indicated that extracts obtained using the validated extraction procedure intended for end determination by LC-MS/MS were also suitable for analysis by LC-UV.

Following the completion of the trial of the LC-UV method it was agreed to adjust the method to give one extraction procedure but with the option to use either LC-MS/MS or LC-UV. This approach is more versatile, applicable to a wider range of laboratories and provides the option to use the UV method as a routine screening method with MS confirmation of the identity of any theobromine detected.

It was also agreed by the CEN Working Group that, as the extraction procedure had been validated by LC-MS/MS, a LC-UV validation of the procedure need not be repeated. The Group considered that any data for single laboratory validation obtained using LC-MS/MS would also be applicable to determination by HPLC-UV and requested that a collaborative trial proceed on this basis.

Collaborative Trial

Spiked Sample Preparation

Sufficient quantities of the horse and dog feed as used for the single laboratory validation were ground to pass through a 500µm sieve. Bulk quantities were then spiked with aqueous solutions of theobromine and freeze dried. The dried samples were re-ground and sieved. The prepared materials were divided into sample containers and stored at room temperature until required for analysis. The NIST SRM 2384 baking chocolate material was grated by hand and sub-sampled into sample containers. As an SRM, further homogeneity testing was not required.

A sample labelled as “cocoa feed” was kindly provided by a laboratory in France. The material was described as being used in the manufacture of cattle/pig/poultry feeding stuffs and contained recycled cakes/biscuits (sweet or salted). The exact ingredients were not declared, but visually, the feed appeared to be mainly cereal based with the addition of some cocoa/chocolate material. Even though the exact concentration of theobromine in the product was not known the material was included in the main collaborative trial as an example of a real-world product containing cocoa-derived material.

Homogeneity

Ten samples of each matrix were taken at random and analysed in duplicate, results of the test are given in Table 12 and the statistical analysis is presented in Table 13. It was concluded that the samples were suitable for use as test materials in a collaborative trial.

Table 12 – Homogeneity Testing of Spiked Test Samples

Sample Type	Dog Feed	Dog Feed	Horse Feed	Horse Feed	Cocoa Feed
Spiking Concentration	**27 mg/kg**	**158 mg/kg**	**49 mg/kg**	**307 mg/kg**	
	Theobromine (mg/kg)				
1A	27.9	151.6	67.3	319.4	176.1
1B	28.8	152.4	62.8	336.5	194.2
2A	28.5	145.2	67.6	360.5	187.3
2B	33.2	148.9	68.0	322.3	188.2
3A	27.8	151.2	67.5	325.2	187.6
3B	28.4	146.5	68.3	331.6	184.8
4A	28.5	146.1	68.3	332.6	192.7
4B	27.8	147.8	67.4	304.0	189.3
5A	28.6	153.9	69.3	309.7	187.3
5B	28.6	146.4	67.8	341.8	187.8
6A	28.4	147.4	67.6	292.0	188.4
6B	28.2	148.8	65.2	310.8	189.1
7A	28.1	150.7	69.1	315.8	190.3
7B	28.0	147.3	66.6	337.0	190.7
8A	28.3	154.0	70.4	331.6	192.4
8B	28.1	144.3	66.2	323.0	188.3
9A	28.3	148.1	68.7	351.5	189.9
9B	27.8	141.7	68.4	312.2	191.9
10A	23.7	147.4	66.2	328.8	190.4
10B	23.4	143.6	67.3	292.4	186.8

Table 13 – Results of Homogeneity Testing of the Main Trial Samples

Sample	Mean (µg/g)	MS_b	MS_w	s_w (µg/g)	s_b (µg/g)	s_b (%)
Cocoa Feed (unit 1 removed)	189.1	5.87	3.04	1.74	1.19	0.63
Dog Feed (repeat data set)	23.43	0.880	0.828	0.91	0.161	0.69
Horse Feed*	67.50	-	-	1.61	0.00016	2.4×10^{-4}
Horse Feed (second sample)*	266.3	-	-	2.25	0.754	0.28

* Estimates obtained using a mixed effects model
MS_b is the between-unit mean square
MS_w is the within-unit mean square.

Pre-Trial Familiarisation

A pre-trial study was carried out to enable the participants to become familiar with the method. 15 laboratories (12 LC-UV, 6 LC-MS/MS, 3 both) volunteered to participate in the collaborative trial. The pre-trial consisted of triplicate aliquots of a sample of dog feed spiked with approximately 150mg/kg theobromine and triplicate aliquots of a sample of horse feed spiked with approximately 300mg/kg theobromine.

By the reporting date, 10 sets of results had been received for LC-UV and 2 sets for LC-MS/MS. As several laboratories pulled out of the trial due to a variety of reasons including instrument breakdowns and pressure of other work, additional participants were recruited and the pre-trial re-run for the new laboratories. An additional 6 sets of results for LC-UV and 7 sets of results for LC-MS/MS were received. Therefore, in total, pre-trial results were returned by 16 participants for LC-UV and 9 for LC-MS/MS (6 laboratories reported results for both techniques). The results are presented in Tables 14 (LC-MS/MS) and 15 (LC-UV). Statistical evaluation (Tables 16 and 17) showed that the distribution of the means determined by LC-MS/MS showed no departure from a normal distribution and no inhomogeneity of variance. Evaluation of the LC-UV data showed some outliers.

Table 14 – LC-MS/MS Pre-Trial Results

		Laboratory								
		4	**13**	**17**	**18**	**20**	**21**	**22**	**23**	**24**
Sample Number	**Matrix**	**Theobromine Concentration (mg/kg)**								
1	Dog feed	175.3	223.2	123.3	176.4	148.7	202.4	147.4	154.5	142.2
2	Dog feed	151.8	219.6	134.7	167.0	152.9	198.2	140.8	151.0	140.6
3	Dog feed	140.1	218.8	137.7	206.8	151.6	206.4	169.4	149.0	140.4
4	Horse feed	272.6	468.0	279.6	365.9	286.9	373.1	288.8	258.5	314.7
5	Horse feed	280.3	464.0	277.8	358.0	285.0	368.2	268.6	274.0	338.3
6	Horse feed	289.2	357.2	296.8	375.4	286.1	368.5	271.9	278.5	329.0

Table 15: LC-UV Pre-Trial Results

Laboratory																	
		1	**2**	**4**	**5**	**6**	**7**	**9**	**10**	**12**	**14**	**18**	**20**	**22**	**23**	**24**	**25**
Sample Number	**Matrix**	**Theobromine concentration (mg/kg)**															
1	Dog feed	153.2	148.5	136.7	144.2	150.6	147.6	143.7	136.1	151.0	140.3	145.4	126.3	219.3	150.2	148.3	154.2
2	Dog feed	164.2	142.4	134.8	146.4	155.8	147.1	143.0	146.6	153.4	140.9	165.0	124.8	196.0	160.2	136.7	157.8
3	Dog feed	163.2	146.7	134.7	148.8	146.8	137.3	144.5	141.8	150.6	140.5	162.2	125.5	193.8	152.2	151.0	154.1
4	Horse feed	283.8	295.3	292.1	287.6	499.9	403.6	316.9	304.7	288.9	299.0	329.6	283.6	399.2	475.7	290.9	349.8
5	Horse feed	291.3	300.0	288.4	289.6	506.3	412.9	322.3	319.8	286.9	297.1	325.6	281.7	375.0	491.1	306.5	356.0
6	Horse feed	290.3	302.6	294.9	291.3	485.8	419.4	322.9	291.3	294.3	298.7	325.2	283.7	373.9	492.3	288.1	359.1

Table 16 – Statistical Evaluation of LC-MS/MS Pre-Trial Results

	Dog Feed 158 mg/kg	Horse Feed 319 mg/kg
Mean value (mg/kg)	166	308
Number of participants	9	9
Outliers	0	1
% Recovery	105	96
RSD_R	18.9	13.6
Horwitz Ratio	2.5	2.0

Table 17 – Statistical Evaluation of LC-UV Pre-Trial Results

	Dog Feed 158 mg/kg	Horse Feed 319 mg/kg
Mean value (mg/kg)	146	317
Number of participants	16	16
Outliers	2	2
% Recovery	92	99
RSD_R	6.4	12.6
Horwitz ratio	0.9	1.9

Following discussions on the outlying results for LC-UV determinations and comments from participants further investigation into the determination of theobromine by LC-UV indicated a potential interference, seen particularly in the horse feed, at 220nm. Following further work, the suggested detection wavelength for the main trial was 278 nm which gave more repeatable results (data not shown).

Comments from the pre-trial participants led to the following amendments to the procedure given in Table 4:

- To avoid pipetting small volumes, 10ml of each calibration standard was to be prepared rather than 1ml
- 2ml of 1mg/ml 7-(β-Hydroxyethyl) theophylline internal standard was to be added to SRM 2384, baking chocolate, rather than 0.2ml
- Reference to PTFE was removed from the description of the syringe filters
- It was recommended that the sample extracts were diluted prior to analysis by LC-MS/MS
- The suggested wavelength was amended from 220nm to 278nm

Main Trial

Blind duplicates of the following samples were sent to the participants:

- Blank horse feed
- Horse feed spiked with theobromine at approximately 300 mg/kg
- Horse feed spiked with theobromine at approximately 50 mg/kg
- Blank dog feed
- Dog feed spiked with theobromine at approximately 150 mg/kg
- Dog feed spiked with theobromine at approximately 25 mg/kg
- Feed containing cocoa-derived material
- SRM 2384 Baking chocolate

Tables 18 and 19 present the results for the main trial as determined by LC-MS/MS (Table 18) and LC-UV (Table 19). The precision data for the two detection techniques are presented in Tables 20 and 21.

Table 18 – LC-MS/MS Main Trial Results (formats as presented by the participants)

	Laboratory Number	**4**	**7**	**13**	**17**	**18**	**20**	**21**	**23**	**22**	**24**
Sample Description	**Sample Number**	**Theobromine Concentration (mg/kg)**									
Blank dog feed	7	/	0	ND	< 0.1	0	nd	0	0	< 5,00	< 1
Blank dog feed	12	/	0	ND	< 0.1	0	nd	0	0	< 5,00	< 1
Dog feed 25 mg/kg	9	24	7	21	15	0	18	26	14	26	26
Dog feed 25 mg/kg	11	18	9	22	15	0	19	28	15	27	26
Dog feed 150 mg/kg	8	175	92	145	143	159	161	157	163	185	147
Dog feed 150 mg/kg	10	266	105	142	166	164	158	155	158	146	151
Blank horse feed	2	/	0	ND	< 0.1	0	nd	0	0	< 5,00	< 1
Blank horse feed	5	/	0	ND	9	0	nd	0	0	< 5,00	< 1
Horse feed 50 mg/kg	4	46	24	48	27	34	51	54	42	40	51
Horse feed 50 mg/kg	6	45	20	42	26	31	49	53	45	50	51
Horse feed 300 mg/kg	1	217	217	282	348	321	348	293	248	308	327
Horse feed 300 mg/kg	3	234	205	272	319	313	358	283	305	349	322
Cocoa feed	13	371	140	180	209	221	212	194	226	210	197
Cocoa feed	15	260	139	191	215	205	217	190	233	223	200
SRM baking chocolate	14	13934	10025	10166	11625	9711	11294	9200	11593	7889	10577
SRM baking chocolate	16	11395	9192	10200	11575	9564	11148	9337	11186	7754	10770

Table 19: LC-UV Main Trial Results (formats as presented by the participants)

	Laboratory Number	**1**	**2**	**4**	**5**	**6**	**7**	**9**	**10**	**12**	**14**	**18**	**20**	**22**	**23**	**24**	**25**
Sample Description	**Sample Number**	**Theobromine Concentration (mg/kg)**															
Blank dog feed	7	ND	0	/	6	0	14	ND	0	0	ND	0	ND	60	<10	82	3
Blank dog feed	12	ND	0	/	7	0	17	ND	0	0	ND	0	ND	63	<10	73	3
Dog feed 25 mg/kg	9	10	29	23	30	29	24	24	26	24	28	22	26	57	30	82	29
Dog feed 25 mg/kg	11	11	27	24	30	28	36	24	27	25	28	24	29	46	28	69	27
Dog feed 150 mg/kg	8	139	149	138	147	149	137	137	147	149	148	139	152	195	155	193	153
Dog feed 150 mg/kg	10	139	148	132	149	145	139	138	152	148	151	141	172	165	149	183	153
Blank horse feed	2	ND	0	/	2	0	9	ND	0	1	ND	0	ND	98	<10	156	5
Blank horse feed	5	ND	0	/	1	0	7	ND	0	1	ND	0	ND	74	<10	173	5
Horse feed 50 mg/kg	4	39	53	42	49	50	51	45	44	42	51	54	41	117	52	64	55
Horse feed 50 mg/kg	6	40	54	43	49	49	49	41	45	44	52	56	41	123	52	62	49
Horse feed 300 mg/kg	1	296	294	257	280	277	314	262	278	264	315	294	311	392	310	351	313
Horse feed 300 mg/kg	3	276	293	260	282	275	309	258	279	271	320	296	330	372	298	329	306

	Laboratory Number	**1**	**2**	**4**	**5**	**6**	**7**	**9**	**10**	**12**	**14**	**18**	**20**	**22**	**23**	**24**	**25**
Sample Description	**Sample Number**	**Theobromine Concentration (mg/kg)**															
Cocoa feed	13	194	189	179	183	180	177	179	199	193	195	183	250	215	192	272	231
Cocoa feed	15	194	193	178	187	178	160	177	200	199	188	179	239	239	193	250	210
SRM baking chocolate	14	9973	10117	11059	10798	10969	8805	9488	10618	10897	11184	10758	11727	7886	10887	11185	9422
SRM baking chocolate	16	9878	10111	10662	10729	10992	8860	9361	10695	10795	11023	10352	11296	7530	10966	10882	10089

Table 20 – Statistical Evaluation of LC-MS/MS Main Trial Results

Sample	Dog Feed 27 mg/kg	Dog Feed 158 mg/kg	Horse Feed 49 mg/kg	Horse Feed 307 mg/kg	Cocoa Feed	SRM Baking Chocolate
Mean value (mg/kg)	17.9		41.6	293	200	10,200
Number of laboratories	10	10	10	10	10	10
Number of outliers (laboratories)	0	≥ 3	0	0	1	1
Number of accepted results	10	Note 1	10	10	9	9
Repeatability limit r	4.62		8.53	51.1	17.1	654
Repeatability standard deviation s_r (mg/kg)	1.65		3.05	18.3	6.12	233
Repeatability relative standard deviation RSD_r (%)	9.23		7.33	6.22	3.06	2.30
Reproducibility limit R	24.8		30.6	137	75.0	3,430
Reproducibility standard deviation s_R (mg/kg)	8.86		10.9	48.9	26.8	1,230
Reproducibility relative standard deviation RSD_R (%)	49.6		26.3	16.7	13.4	12.1
Horwitz ratio	4.78		2.88	2.45	1.86	3.03

Note 1 – No precision data was produced for the dog feed spiked at 158 mg/kg theobromine due to the proportion of outliers

Table 21 – Statistical Evaluation of LC-UV Main Trial Results

Sample	Dog Feed 27 mg/kg	Dog Feed 158 mg/kg	Horse Feed 49 mg/kg	Horse Feed 307 mg/kg	Cocoa Feed	SRM Baking Chocolate
Mean value (mg/kg)		145	47.5	299	199	10,300
Number of laboratories	16	16	16	16	16	16
Number of outliers (laboratories)	= 5	3	2	0	0	0
Number of accepted results	Note 2	13	14	16	16	16
Repeatability limit r		6.13	4.17	22.2	22.6	554
Repeatability standard deviation s_r (mg/kg)		2.19	1.49	7.93	8.06	198
Repeatability relative standard deviation RSD_r (%)		1.51	3.13	2.66	4.04	1.92
Reproducibility limit R		17.7	14.6	90.9	74.4	2,790
Reproducibility standard deviation s_R (mg/kg)		6.31	5.20	32.5	26.6	998
Reproducibility relative standard deviation RSD_R (%)		4.35	10.9	10.9	13.3	9.67
Horwitz ratio		0.58	1.22	1.60	1.85	2.43

Note 2 – No precision data was produced for the dog feed spiked at 27 mg/kg theobromine due to the proportion of outliers (possibly due to issues with selectivity)

To summarise, the results of the collaborative trial showed:

- The precision data for the lowest concentration (dog feed spiked with theobromine at 27mg/kg) is generally poorer than for the other samples and this is thought to be possibly due to selectivity issues in some of the participating labs

- The collaborative trial data appears to be poorer for the LC-MS/MS than for HPLC-UV and this may be due to the outlying results having a greater effect due to the lower number of participants (16 for determination by HPLC-UV and 10 for LC-MS/MS)

It was noted that two participants (labs 22 and 24) reported significant results (average of 97mg/kg) for both the blank horse feed and blank dog feed by UV, however theobromine was not detected by either lab when the same extracts were analysed by LC-MS/MS. Both labs were approached and asked to supply copies of the LC-UV chromatograms for the blank samples. Chromatograms were received from lab 22 and are presented in Figures 2 and 3. A small poorly shaped peak can be seen at a similar retention time as theobromine.

Figure 2 - Example UV Chromatogram for Blank Horse Feed

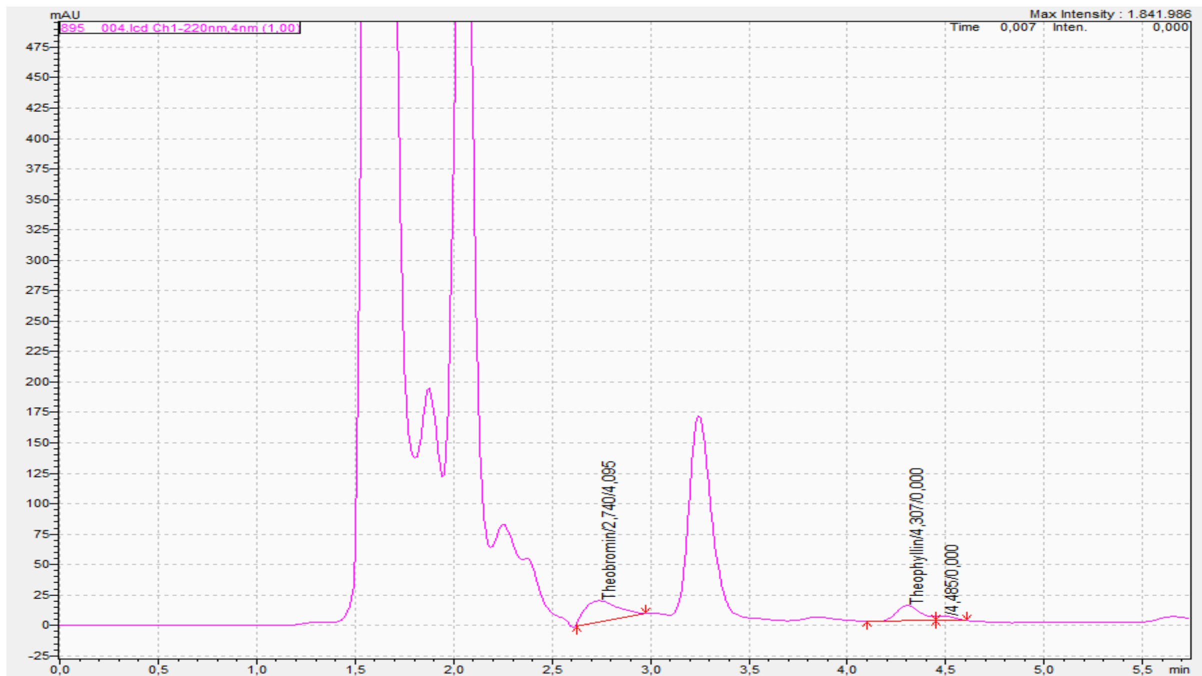
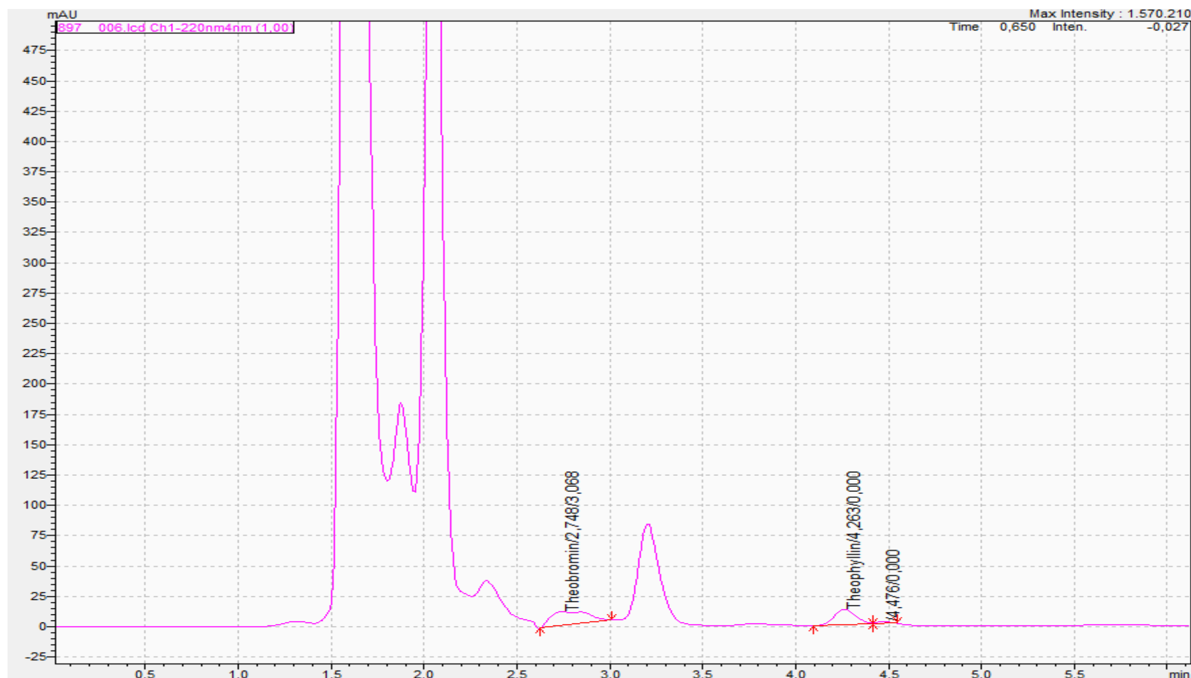


Figure 3 – Example LC-UV Chromatogram for Blank Dog Feed



In the protocol it was not stated that for detection by LC-MS/MS quantification should be carried out using theobromine-d3 as an internal standard as the cost of the standard was considered to be prohibitively expensive for most laboratories for this type of analysis. At the request of the Working Group the participants who had determined the theobromine concentration of the trial samples using LC-MS/MS were asked to provide the raw data so that the theobromine concentration could be calculated without the use of an internal standard so that the effect of the 7-(β-hydroxyethyl) theophylline internal standard could be assessed.

The 10 participants who analysed the collaborative trial samples by LC-MS/MS were contacted and data was received from 7 of these participants. The theobromine concentrations were then calculated with and without the internal standard. As an example, the results from lab 18 are presented in Table 22. It can be seen that the recoveries for this laboratory, based on the actual spiking concentrations, are more accurate and show less variation between duplicates when the theobromine content is calculated using the ratio of the responses for theobromine and 7-(β-hydroxyethyl) theophylline rather than the response for theobromine alone. The variation in results, calculated with and without internal standard, for the 6 participants where results could be calculated from the data, which was supplied, is presented in Table 23.

Table 22 – LC-MS/MS Results from Lab 18 Calculated with and without the Internal Standard

Sample	% Recovery	
	Without Internal Standard	With Internal Standard
Horse feed 50mg/kg	13.4	70.2
Horse feed 50mg/kg	0.1	63.7
Horse feed 300mg/kg	87.0	104.6
Horse feed 300mg/kg	76.3	101.8
Dog feed 25mg/kg	0.0	0.0
Dog feed 25mg/kg	0.0	0.0
Dog feed 150mg/kg	54.0	100.7
Dog feed 150mg/kg	41.1	103.8

Table 23: Variation in LC-MS/MS Results when Calculated with and without the Internal Standard

Lab No	4	13	17	18	22	23
Horse Feed 50mg/kg						
%CV - With IS	1.48	8.17	3.32	7.27	16.29	6.35
%CV - Without IS	1.33	2.36		139.31	13.48	
Horse Feed 300mg/kg						
%CV - With IS	5.38	2.45	6.28	1.88	8.76	14.74
%CV - Without IS	33.92	2.45	141.42	9.26	15.86	12.04
Dog Feed 25mg/kg						
%CV - With IS	20.19	4.29	1.58		2.64	7.86
%CV - Without IS	2.20	5.16			13.61	
Dog Feed 150mg/kg						
%CV - With IS	29.22	1.78	10.79	2.22	16.82	2.33
%CV - Without IS	8.35	0.55	18.29	19.22	29.65	2.38
Cocoa Feed						
%CV - With IS	25.05	4.28	2.21	5.18	4.20	2.16
%CV - Without IS	0.06	6.43	4.36	4.11	1.85	0.03
QC Baking Chocolate						
%CV - With IS	14.18	0.24	0.30	1.08	1.22	2.53
%CV - Without IS	9.79	28.06	9.17	7.31	135.52	2.67

Due to the existence of outliers in the data, at the suggestion of the Working Group, the results from the main trial were evaluated using robust methods rather than the classical statistics

reported above. The results are presented in Tables 24 and 25. The validation data from the collaborative trial was satisfactory (Horwitz ratio of <2)[20] for the determination of theobromine by HPLC-UV in the range 27 to 307mg/kg. The method was also shown to be suitable for the determination of theobromine in SRM 2384, baking chocolate, at 10700mg/kg.

For the determination of theobromine by LC-MS/MS, the validation was satisfactory in the range 49 to 307mg/kg. The method was also shown to be fit for purpose for the determination of theobromine in SRM 2384, baking chocolate, as an acceptable RSD_R was obtained (<25%).

Table 24 – Statistical Evaluation of LC-MS/MS Main Trial Results using Robust Statistics

	Dog Feed 27 mg/kg	**Dog Feed 158 mg/kg**	**Horse Feed 49 mg/kg**	**Horse Feed 307 mg/kg**	**Cocoa Feed**	**SRM Baking Chocolate**
Median value (mg/kg)	18.8	157	45.5	307	209	10,400
Number of laboratories	10	10	10	10	10	10
Repeatability limit r	3.56	14.8	7.45	31.6	19.5	420
Repeatability standard deviation s_r (mg/kg)	1.27	5.29	2.66	11.3	6.96	150
Repeatability relative standard deviation RSD_r (%)	6.77	3.37	5.86	3.68	3.33	1.44
Reproducibility limit R	23.5	26.3	23.4	111	65.0	3710
Reproducibility standard deviation s_R (mg/kg)	8.40	9.39	8.34	39.7	23.2	1320
Reproducibility relative standard deviation RSD_R (%)	44.7	5.97	18.4	12.9	11.1	12.8
Horwitz ratio	4.34	0.80	2.04	1.92	1.55	3.21

Table 25 – Statistical Evaluation of LC-UV Main Trial Results using Robust Statistics

	Dog Feed 27 mg/kg	Dog Feed 158 mg/kg	Horse Feed 49 mg/kg	Horse Feed 307 mg/kg	Cocoa Feed	SRM Baking Chocolate
Median value (mg/kg)	27.7	149	49.2	295	193	10,700
Number of laboratories	16	16	16	16	16	16
Repeatability limit r	2.63	6.96	3.12	14.4	12.1	332
Repeatability standard deviation sr (mg/kg)	0.94	2.49	1.11	5.15	4.31	119
Repeatability relative standard deviation RSDr (%)	3.39	1.67	2.27	1.74	2.24	1.11
Reproducibility limit R	9.78	27.2	21.9	73.9	50.4	1506
Reproducibility standard deviation sR (mg/kg)	3.49	9.71	7.83	26.4	18.0	538
Reproducibility relative standard deviation RSDR (%)	12.6	6.54	15.9	8.94	9.34	5.02
Horwitz ratio	1.30	0.87	1.79	1.32	1.29	1.27

Conclusions

1 Theobromine in animal feed materials and in compounded animal feeds can be determined by an inter-laboratory validated HPLC-UV method using 7-(β-hydroxyethyl) theophylline as internal standard. When more forensically robust confirmatory data are required the same HPLC separation can be coupled with tandem mass spectrometry using theobromine-d3 as the internal reference standard

2 In the current absence of internationally agreed standardised animal feed components and compounded feeds for theobromine, methods are described for the production of spiked materials to use in their absence

3 At the time of publishing, the method described in this paper is in the process of a formal vote before being published as a CEN standard (prEN 17270:2018 Animal Feeding Stuffs: Methods of Sampling and Analysis — Determination of Theobromine in Feed Materials and Compound Feed, including Cocoa-derived Ingredients by Liquid Chromatography).

Acknowledgements

The authors wish to thank Malvinder Singh, Magda Mazur, Malcolm Burn, Andrew Campbell and Richard Brown of LGC's Food and Consumer Products Testing team, Simon Cowen of LGC's statistics team for assistance with the validation of the method, CEN/TC 327/WG 5 for financial support and the following laboratories for participating in the collaborative trials:

- Chemisches und Veterinäruntersuchungsamt Rhein-Ruhr-Wupper , Germany
- Food Control Laboratory, Saudi Arabia
- Glasgow Scientific Services, UK
- Institute of Food Safety, Feed and Environment, Slovenia
- Institutul de Igienã si Sãnãtate Publicã Veterinarã, Romania
- Lancashire County Scientific Services, UK
- Landesbetrieb Hessisches Landeslabor, Germany
- Landwirtschaftliche Kommunikations-und Servicegesellschaft mbH, Germany
- National Research Institute of Animal Production, Poland
- National Veterinary Institute, Sweden
- Public Health Laboratory, Malta
- SGS Institut Fresenius GmbH, Germany
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, Germany
- Tayside Scientific Services, UK
- The State Laboratory, Ireland
- Thüringer Landesanstalt Für Landwirtschaft, Germany
- ÚKZÚZ, Opava, The Czech Republic
- ÚKZÚZ, Praha, The Czech Republic
- Worcestershire County Council Scientific Services, UK

References

1 K Pejml, Sto let theobromine [A hundred years of Theobromine], Cas Cesck Lekarnic, 21 210-122, 1941

2 A Woskresensky, Ueber das Theobromin. Ann Chem Pharn, 41 125-127, 1841

3 A Mitscherlich, Der cacao und die Chocolade, A Hirschwald, Berlin, 1859

4 E Fischer, Synthesis of Theobromine, Ber Deut Chem Ges, 30 1839-1849, 1897

5 C Heisch, On the Composition of Different Kinds of Cocoa, Analyst, 1 142, 1876

6 A Wynter Blyth, Foods: their Composition and Analysis, Third Edition, C Griffen and Co, London (1888), 390-401

7 RV Wadsworth, The Estimation of Theobromine in Cocoa and its Products, Analyst, 46, 32-37, 1921

8 RS Hatfull, I Milner and V Stanway, Determination of Theobromine in Animal Feeds, J Assoc Publ Analysts, 18 19-22, 1980

9 A Richards and B Wailes, Estimation of Fat-free Cocao Solids in Chocolate and Cocao Products – Global Survey of Typical Concentrations of Theobromine and Caffeine Determined by HPLC, J Assoc Public Analysts, 40 1-12, 2012

10 TM Dyke and RA Sams, Detection and Determination of Theobromine and Caffeine after Administration of Chocolate-coated Peanuts to Horses J Anal Toxicol, 22 112-116, 1998

11 Scientific Opinion of the Panel on Contaminants in the Food Chain on a Request from the European Commission on Theobromine as Undesirable Substances in Animal Feed, The EFSA Journal, 725 1-66, 2008

12 NA Adamafio, Theobromine Toxicity and Remediation of Cocoa By-products: An overview, J Bio Sci, 13 570-576, 2013

13 http://data.europa.eu/eli/dir/2002/32/2017-12-25 Accessed 20 May 2019.

14 AR de Sena, SA de Assis and A Branco, The Analysis of Theobromine and Related Compounds by Reversed Phase High-performance Liquid Chromatography with Ultraviolet Detection: an update (1992-2011), Food Technol Biotechnol, 49 413-423, 2011

15 I Baranowska and J Plonka, Simultaneous Determination of Biogenic Amines and Methylxanthines in Foodstuff – Sample Preparation with HPLC-DAD-FL Analysis, Food Anal Methods, 8 963-973, 2015

16 C Álvarez, E Pérez, E Cros, M Lares, S Assemat, R Boulanger and F Davrieux, The Use of Near Infrared Spectroscopy to Determine the Fat, Caffeine, Theobromine and [-]-Epicatechin Contents in Unfermented and SCriollo Cocoa, J Near IR Spectros, 26 307-316, 2012

17 CEN/TC 327 document N1269, Second Call for Tender for Technical Project Leaders for the Development of Standardised Methods of Analysis of Animal Feed

18 J Zhong, N Tang, B Asadzadeh and W Yan, Measurement and Correlation of Solubility of Theobromine, Theophylline and Caffeine in Water and Organic Solvents at Various Temperatures, J Chem Eng Data, 62 2570-2577, 2017

19 C Carrez, Determination of Lactose in Milk, Rep Pharm 19 533-538, 1908 – for a detailed abstract see Analyst, 21, 400, 1909

20 W Horwitz, Evaluation of Analytical Methods used for Regulation of Foods and Drugs, Anal Chem 54, 67A- 76A