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### The HPLC Analysis of Caffeine and Theobromine in Animal Diets

W. Jeffrey Hurst<sup>a</sup>; Robert A. Martin Jr.<sup>a</sup>

<sup>a</sup> Hershey Foods Corporation, Technical Center, Pennsylvania

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The HPLC Analysis of Caffeine and  
Theobromine in Animal Diets

by

W. Jeffrey Hurst and Robert A. Martin, Jr.  
Hershey Foods Corporation Technical Center  
1025 Reese Avenue  
Hershey, Pennsylvania 17033

An HPLC method is described for the analysis of added caffeine and theobromine in animal diets using HPLC with samples extracted in  $\text{CHCl}_3$  and interferences eliminated with a Sep-pak<sup>TM</sup>. This method has good accuracy and precision but is not suitable for matrixes where the methylxanthines exist as an integral part of the matrix (e.g., foods).

The HPLC Analysis of Caffeine and  
Theobromine in Animal Diets

An animal diet is a complex matrix of many components consisting of various amount of protein, fat, carbohydrate vitamins and minerals used as a carrier for a wide variety of drugs and food components. The matrix by virtue of its complexity presents a formidable analytical challenge for the analyst. HPLC allows the analysis of a large variety of potential components with great accuracy and precision in a short analysis time.

A method is described for the rapid accurate analysis of the pure added methylxanthines) caffeine and theobromine in animal diet using HPLC with sample cleanup accomplished using a commercially available Sep-pak<sup>TM</sup>.

### Methods and Materials

Extraction of caffeine and theobromine. Place 2.5 g  $\pm$  0.1 (weighed to 0.01 g) of animal diet into a flask add 100 ml of  $\text{CHCl}_3$  and heat the solution to 60°C for 30 min. Cool extract to room temperature and bring up to weight with  $\text{CHCl}_3$ . Withdraw 10 ml of  $\text{CHCl}_3$  extract and run through a Silica Sep-pak<sup>TM</sup>. Elute caffeine or theobromine with 15 ml of  $\text{CH}_3\text{OH}$ . Depending on the level of the compound of interest the sample can be injected directly from the  $\text{CH}_3\text{OH}$  or concentrated to an appropriate volume.

The analysis was accomplished by HPLC using a Waters' Radial Compression Module (RCM) with a  $\text{C}_{18}$  (Radial Pak A) cartridge. The mobile phase was 74/25/1  $\text{H}_2\text{O}/\text{CH}_3\text{OH}/\text{HOAc}$  at a flow rate of 3.0 ml/min. Detection was at 280 nm. Typical analysis for both compounds was less than 10 minutes. Standard concentrations were 0.5  $\mu\text{g}/\mu\text{l}$  for theobromine and .025  $\mu\text{g}/\mu\text{l}$  for caffeine. Figures 1 and 2 show sample chromatograms for standards and samples respectively.

### Results

Precision studies of the method show a %Cv of less than 1% for standards (n = 10) and less than 2% for sample (n = 10). Recovery studies of additions to diet are summarized in Table 1.

An additional study of multiple extractions (n = 4) of a spiked sample (60 mg/100 g theobromine) gave excellent data with data showing differences to be not significant at a 95% confidence level.

An examination of two rabbit diets with added theobromine gave data that can be seen in Table 2.

This method provides a fast accurate analysis of added pure methylxanthines in animal diets. Although it is not suitable for use in systems where the methylxanthine is in a bound form (i.e. like a food system) because the energy input from the  $\text{CHCl}_3$  is not sufficiently high as to extract

**Sample:** Xanthine Standards  
(20  $\mu$ l injection size)

**Column:** Radial Compression Module  
(Radial Pak-A)

**Mobile Phase:** 74/25/1 ( $H_2O/CH_3OH/HOAc$ )  
@ 3.0 ml/min

**Detector:** 254 nm @ 0.1 AUFS

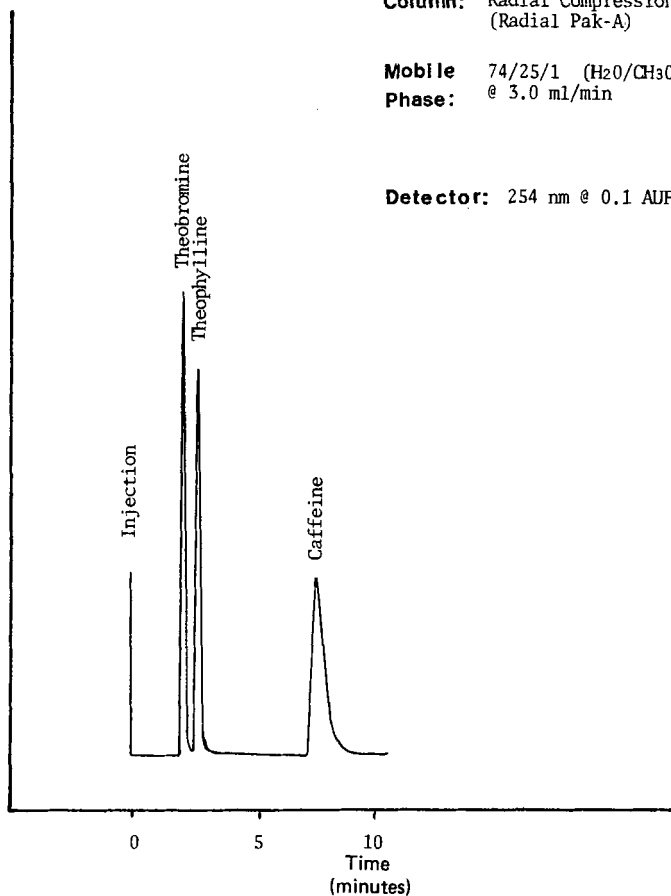


Figure 1. Analysis of Xanthine Standards, 20  $\mu$ l inj. size; Column: Radial Compression Module, Rad-Pak-A; Mobile Phase: 74/25/1 ( $H_2O/CH_3OH/HOAc$ ) @ 3.0 ml/min; Detector: UV/254 nm @ 0.1AUFS.

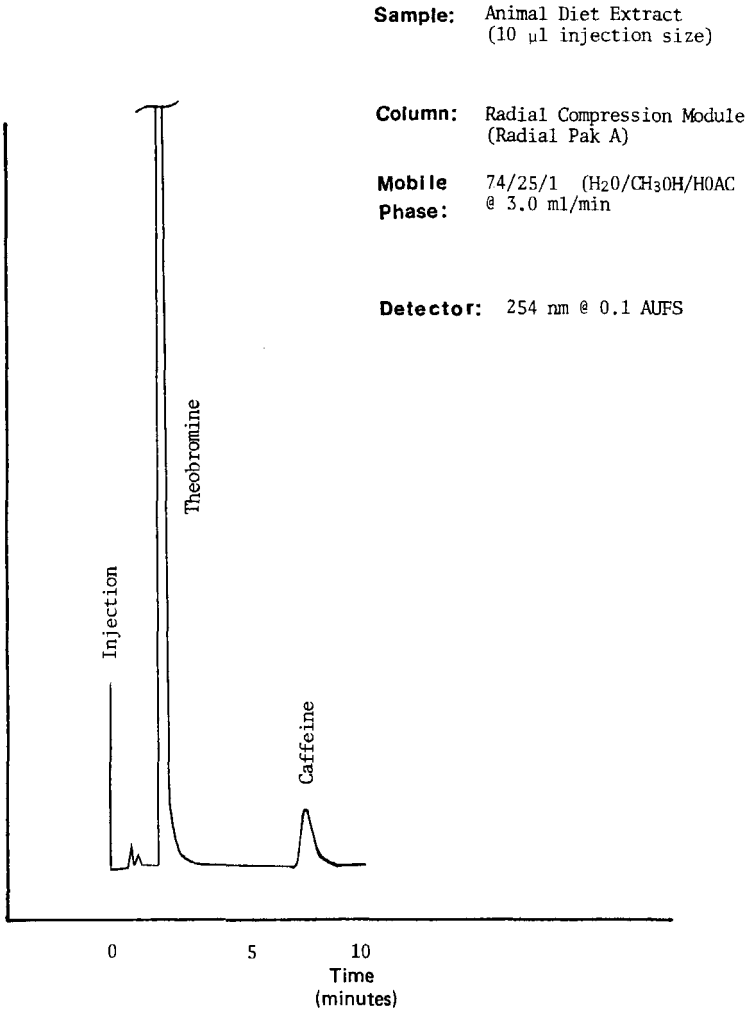


Figure 2. Analysis of Animal Diet Extract, 10  $\mu$ l inj. size;  
Operating conditions as in Figure 1.

Table 1  
Recovery Study of Added Caffeine and  
Theobromine to Animal Diets

n = 2

<u>% Theobromine Added</u>	<u>% Theobromine Rec.</u>	<u>% Recovery</u>
+.027	.025	95.6
+.068	.067	98.6
+.135	.134	<u>99.3</u>
		x = 97.8

<u>% Caffeine Added</u>	<u>% Caffeine Rec.</u>	<u>% Recovery</u>
+.002	.0019	95.0
+.004	.003875	93.8
+.008	.00795	<u>99.4</u>
		x = 96.1

Table 2

<u>Amount Added Theobromine</u>	<u>Amount Analyzed Theobromine</u>
60 mg/100 g	57.6 mg/100 mg
120 mg/100 g	114 mg/100 mg

all of the bound xanthines. This method will extract 70-75% of the total compounds in the food system.

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