DETECTION OF CARBADOX IN FEED BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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The public health problem resulted from the commonly use of the carbadox (CBX) in swine feed has spurred so many scholars trying to develop a simply and precisely detecting method. The most difficult problem was the preparation of feed sample. We extracted the CBX from the feed by pretreating with water, extracting with 95% dimethylformamide overnight at room temperature, cleaning up on a columm of alumina and filtering through a 0.45u millipore filter membrane. Injected 20 ul filterate into high performance liquid chromatography. The mobile phase was acetonitrile mixed with double distilled water (20+80) and its flow rate was 2.5 ml/min. The chromatogram showed excellent baseline resolution, and no interfering peak. Recoveries from medicated feed were 91.2 % for 50 ppm, 93.8% for 10 ppm and 92.5% totally. This method was capable of detecting from 2 to 0.2 ppm for CBX standard concentration. Its repeatabilities were 96.7% for peak area and 99.5% for retention time. The wavelength of maximum absorbance of CBX was 313 nm according to spectrophotometry. These results suggested the possibility of a simple and precise method for detecting the CBX in feed.

Carbadox (Fig. 1) (methyl 3-(2- quinoxalinylmethylene) carbazate N^1, N^4-dioxide) is used in swine feed at various levels to increase weight gain and to improve feed efficiency. It is also useful for the prevention of swine dysentery and bacterial enteritis. An official spectrophotometric method, involving prior complex extract procedures, has been described for determination in feed by Horwitz in U. S. A.[4]

Instrumental conditions for determining CBX by HPLC have been well documented in the literature[3,6,7,8,10,12] The increasing use of HPLC in the determination of drugs and additives in feeds, and the possible wildspread use of this technique in feed-industry routine control laboratories, has led to the development of a fast, accurate and reproducible HPLC method of analysis for CBX in such samples[8,9,12]

The purposes of the present studies are to verify and extend the HPLC methods made by others. Particular attention is placed on the preparation of samples.

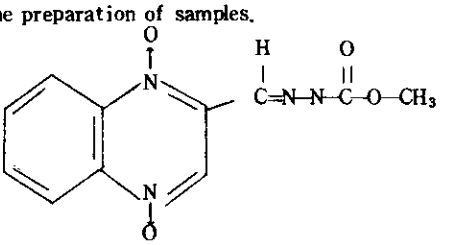

Fig. 1 Structure of carbadox

MATERIALS AND METHOD

1. Spectrophotometer : Hitachi Model 200–20 and Model 056–1001 recorder[a]. Scanning speed 60 mm/min, Chart Speed 20 mm/min, Range : 1.
2. High performance liquid chromatograph : Model 450 (variable wavelength etector) equipped with U6K injection system and Data Module integrator[b]. General operating conditions : detector 313 nm, flow rates 2.5 ml/min, chart speed 0.5 cm/min, injection volume 20 ul.
3. Chromatographic column : 5 cm × 3.9 mm id stainless steel guard column containing uBondapak C_{18}/Corasil followed by 10 cm × 8 mm id Radial-Pak cartridge column containing uBondapak C_{18}. The latter column was compressed by Z-Module™ Radial compression separation system[b]
4. Mobile phase : Acetonitrile[c]–double distilled water (20+80). Mix and filter through a 0.45 um filter[d] then degas.
5. Sample extraction solvent : Dimethylform-amide (DMF)[e] —double distilled water (95 + 5).
6. Alumina : Merck 70 — 230 mesh, neutral. Treat alumina as in 42.031(a) (13th ed.)[4].
7. Chromatographic tubes : 1 x 30 cm glass column contricted at one end to about 4 mm.
8. Carbadox stock standard solutions : 500 ug/ml. Dry the carbadox reference standard[f] at 105 ℃ for 2 hours[5]. Weigh exactly 5 mg and place into 25 ml volumetric flask. Dilute to 10 ml with extracting solvent.
9. Working standard : Dilute stock solutions with exactly solvent so that final concentration (ug/ml) is 10, 5, 2, 1, 0.5, 0.2.
10. Feedstuff : Unmedicated feed (blank feed)[g].
11. Preparation of sample extract : Ground coarse or pelleted feeds to pass 20 mesh seive. Weighed exactly 10 g sample into 100 ml glass stopper volumetric flask. Swirled while adding exactly 5 ml water, and let stand 5 min. Added exactly 50 ml 95% DMF, stoppered tightly, shaked vigorously 15 sec, and let stand overnight in dark at room temperature. Filtered through rapid paper and transfered about 15 ml onto chromatographic column containing about 5 g alumina held in column with glass-wool plug. Discarded first several milliliters of eluate and collected remaining eluate. The eluate was filtered through prefilter and 0.45 u millipore-membrane filter for HPLC injections. Quantiated drugs by external standard method — Comparing peak area ratios of feed extracts to working standards of about the same concentration using identical injection volumes. Repeated if integration start, stop marks and width of peak were not well.

(a) : Hitachi, Ltd. Tokyo, Japan.
(b) : Waters Associates, Inc, Milford, MA, USA
(c) : Lichrosolv, Merck & Co.
(d) : Millipore Corp, Bedford, MA, USA.
(e) : Reagent grade, Merck & Co.
(f) : Pfizer Limited Agricultural Division, Tamsui, Taiwan.
(g) : Feedstuff Factory, Taiwan Provincial Farmers Association, Kaohsiung.

Calculated amount of drug in feed as follows :

ppm drug = peak area sample x ppm std / peak area std x 5.5

Calculated the confidence level (C. L.) follows [1] :

$$C.L. = \pm S\ t/N$$

S = standard error

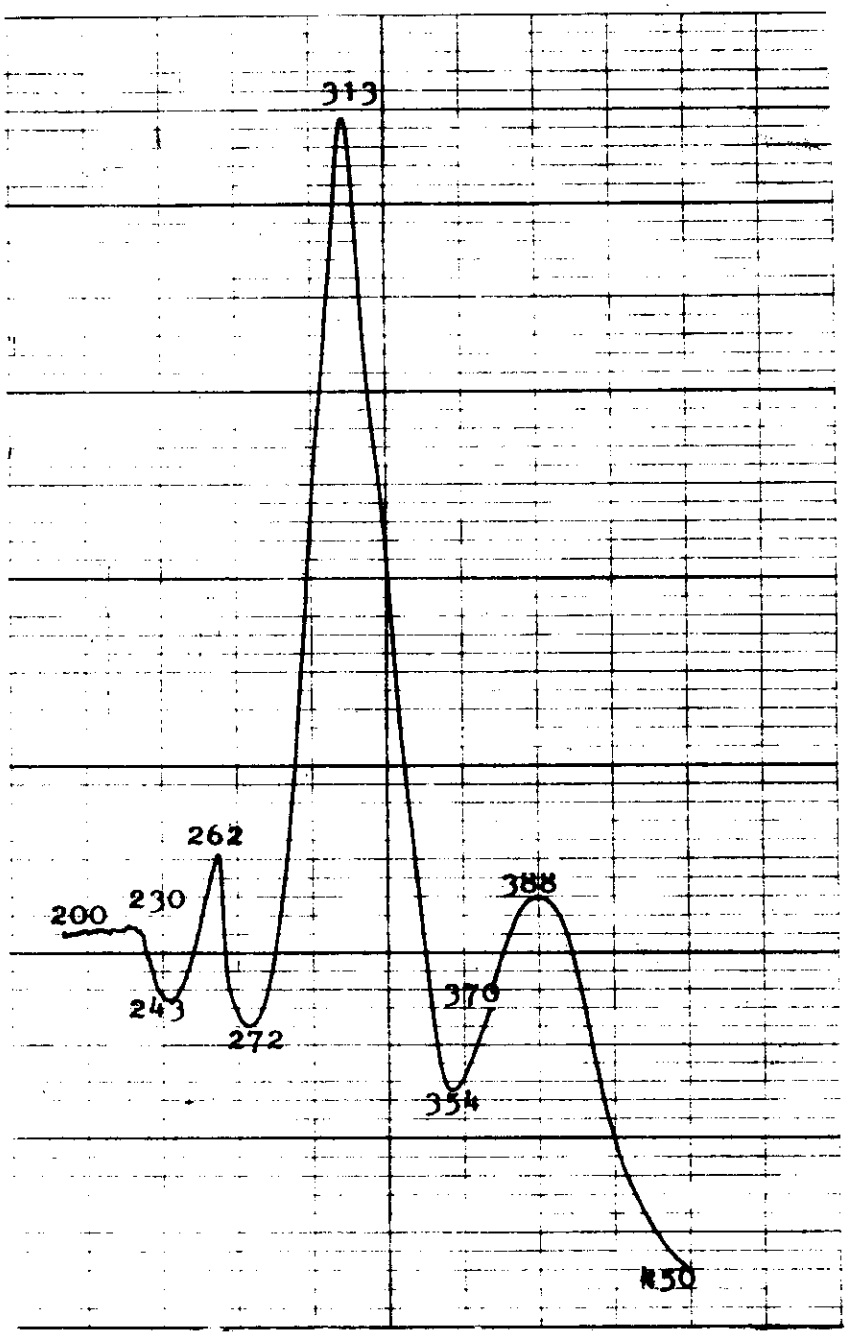

Fig.2 UV–Vis–absorptionspectrum of 5ppm carbadox

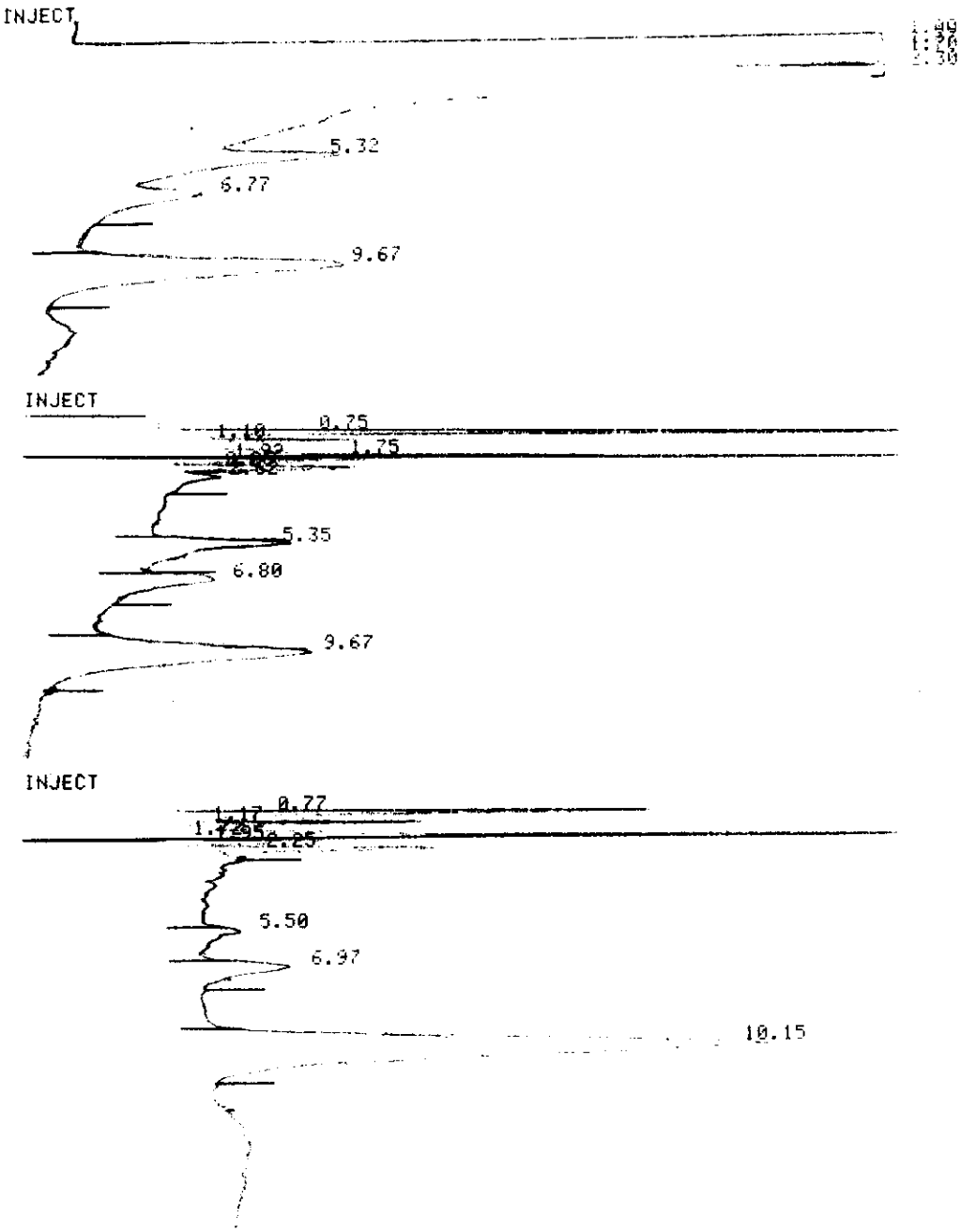

Fig.3 The chromatogram of blank feeds with or without passing through chromatographic tubes packed with alumina.
Upper : without passing through it
Middle : the same feed with passing through it
Lower : another feed with passing through it
The retention time of carbadox is 4 : 20.

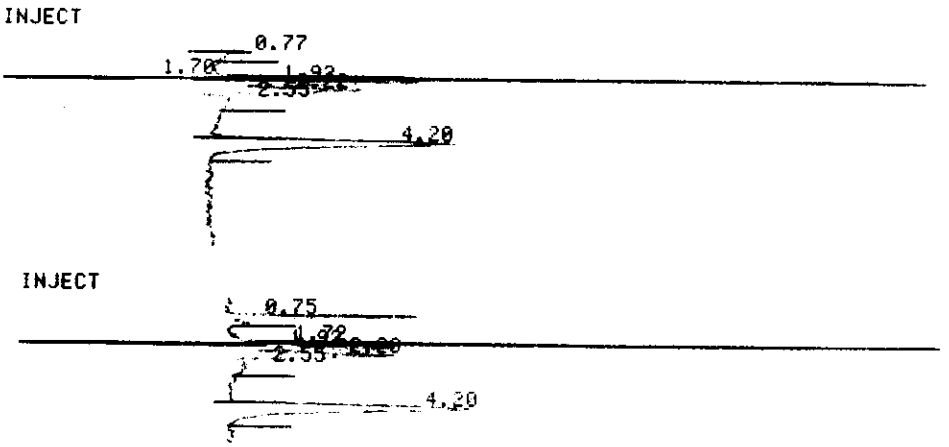

Fig. 4 The chromatogram of 1 ppm carbadox standard solution with or without passing through chromatographic tubes packed with alumina
Upper : without passing through it
peak area – 286103 microvolt-sec
Lower : with passing through it
peak area – 284827 microvolt-sec

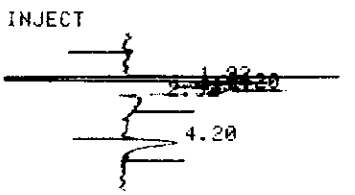

Fig. 5 The chromatogram of 0.2 ppm carbadox standard solution.
Peak area – 59175 microvolt-sec

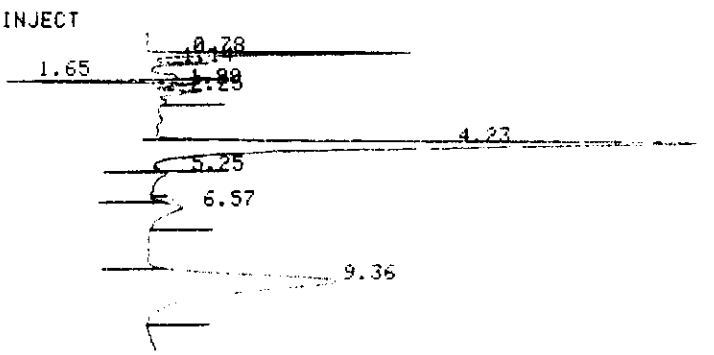

Fig. 6 The chromatogram of swine feed spiked with 50 ppm carbadox.
Detected amount – 48.5 ppm

Table. 1 The repeatabilities of carbadox at different standard solutions

Concentration	Injection Number 1	2	3	4	5	Mean ± S.E.	Relative error %	Repeatability %
2ppm								
P.A.@	662498	692960	638557	643968	659314	659459±21263	3.2	96.8
R.T.#	255	255	255	255	253.2	254.6 ±0.80	0.3	99.7
1ppm								
P.A.	130811	314179	323235	333741	316782	319750±9050	2.8	97.2
R.T.	253.2	252	253.2	252	252	252.5 ±0.66	0.3	99.7
0.5ppm								
P.A.	150040	150107	153950	147880	150775	150550±2191	1.5	98.5
R.T.	250.2	252	252	252	252	251.6 ±0.81	0.3	99.7
0.2ppm								
P.A.	59008	64762	63569	56315	59175	60566±3502	5.8	94.2
R.T.	2252	252	252	252	252	252±0.00	0.0	100
Total								
P.A.							3.3	96.7
R.T.						252.7 ±1.34	0.5	99.5

* 0.01 A.U.F.S.

@Peak Area — Microvolt — Sec

Retention Time — Sec.

Table 2. Recoveries of carbadox from same feed spiked with different levels

Spiked mount of feed	Recovery amount ppm	Mean ± S.E. ppm	Recovery %
50 ppm		45.38 ± 3.80	91.2 ± 7.6
No.1	48.5		
No.2	42.2		
No.3	42.4		
No.4	49.2		
10 ppm		9.38 ± 0.42	93.8 ± 4.2
No.1	9.1		
No.2	9.2		
No.3	10.0		
No.4	9.2		
Total			92.5 ± 1.8

* 0.04 A.U.F.S.

t = C.L. limit factor
N = test number

RESULTS

The UV-absorptionspectrum of carbadox showed that the wavelength of maximum absorbance was 313 nm (Fig. 2). We used it for detection.

The purification of sample extracts by passing through alumina showed no interfering peak (Fig. 6) and getting excellent baseline resolution (Fig. 3). We used four different feed and obtained the same results. The baseline resolution was bad without passing through alumina and would obtain a skewed peak (rider) if spiked with CBX. The alumina would not retain any CBX and eluted completely (Fig. 4).

The repeatabilities of carbadox by HPLC were 96.7% for peak area 99.5% for retention time (Table 1). The plot of peak area vs concentration indicated the response to carbadox was linear from 0.2ppm to 2ppm at 0.01 AUFS and the regression equation was Y (peak area) = 334,675 x (concentration) − 11,993. The peak height of 0.2ppm carbadox was about 1 cm, so 0.1ppm CBX could be detected easily (Fig. 5).

Recoveries from medicated feed were 91.2% for 50ppm, 93.8% for 10ppm and 92.5% totally (Table 2). The confidence interval was between 91% and 94% when the confidence level limit was 95%, between 90.3% and 94.7% when the confidence level limit was 99%.

DISCUSSION

We used 313 nm for detection to obtain maximum absorbance according to UV-aborptionspectrum of CBX (Fig. 2). But many authors used different wavelengths e.g. 254nm[6], 280nm[7], 306nm[10], 365[3 8 11]. This condition might involve the background (the property of solvent) or the components of sample extract. The maximum absorbance was 303nm when its background was water according to the Merck Index[13].

The mobile phase, acetonitril in water, was the same[3,6,7,8,10,12]. But its percentage was a little different.

We could detect 0.2ppm CBX easily and the sensitivity might have been higher because the peak height of 0.2ppm CBX chromatograph was about 1cm. Luchtefeld could detect 0.024 ppm but its peak height was unknown[8].

The extracting solvent, DMF was formulated with 5% water for two reasons. First, pelleted feeds that contained drugs generally required a water pretreatment to release the drug, which was absorbed on feed ingredients during the pelleting process. If DMF was added to a watertreated feed, the heat of reaction resulted in a volume change. In using additive volumes, this change was undesirable and was minimized by a prior addition of water to the DMF. Second, small amounts of water in a DMF extract of carbadox caused elution of the drug when the extract was put on an alumina column[12]. Thorpe's research showed that the incomplete recoveries as determined by HPLC, used no water pretreatment, for overnight extraction at room temperature with 100% DMF, extraction with 95% DMF, and extraction in a water bath 1 hour at 50 ℃[12]. He found that with water pretreatment, HPLC results compared favorably with official method[4] results when the overnight extraction was used.

Blank feed or spiked feed was extracted and passed through an alumina column[10,12]. In all cases, 100% of the drug was eluted and interferances for detection by HPLC were retained on the column, as shown by comparisons of peak height (or peak area) or equivalent

concentration of drugs before and after the addition to blank feed carried through the extraction and cleanup procedures (Fig. 3 and 4). The same results were reported by Rihs et al[10] and Thorpe[12] The cleanup method obtained excellent baseline resolution (Fig. 3 and 5). But Luchtefeld used celite for cleanup [8]. If further interferences, influenced by the composition of the feed, a standard addition or spike must be analyzed along with the feed sample in order to determine the recovery of CBX from that particular type of feed. The spike could be analyzed by adding a known amount of CBX to a duplicate size sample. This recovery could then be used to calculate the amount of CBX present. It also might be resolved by changing percentage of mobile phase[8,12].

We could obtain 92.5% recovery (Table 2), under the addition of 10ppm and 50ppm. Luchtefeld got 90% for 0.96ppm, 80% for 0.48ppm, 74% for 0.24ppm, 54% for 0.12 ppm, 44% for 0.06ppm, 33% for 0.024ppm. Rih et al found the recovery was between 85% and 95% under addition of 30-100ppm[10]. The recovery of 50ppm olaquindox by Bories' research was 96%[2].

To conclude, a sample extraction and cleanup technique to detect CBX in feed was performed in our laboratory. The technique was superior to the official method[4,12], because extraction techniques were easier and recovery was good. This method was available and has become the tendency to detect CBX in feed[9]

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以高性能液相層析法測定飼料卡巴得

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中文摘要

由於卡巴得經常被使用於猪飼料中而造成公共衛生上的問題，所以許多學者在研究一個簡便而又準確的測定方法。這些測定方法中最困難的部分便是飼料樣品的處理。我們萃取方法是先加水處理後，以 95% Dimethylformamide 於室溫中過夜萃取，再通過矽土管柱後經 0.45μ微孔濾膜過濾而得。取 20μl 濾液注入高性能液相層析儀內，以 Acetonitrile+水（ 20+80 ）當作移動相，每分鐘 2.5ml 的流速。由層析圖上顯示良好的基線分離率，且沒有干擾波峯出現。飼料內卡巴得之回收率，50ppm時爲 91.2%，10ppm時爲 93.8%，總平均爲 92.5%。以卡巴得標準溶液測之，可測出 2 至 0.2ppm，以波峯面積而言，其複驗性達 96.7；以滯留時間而言，則達 99.5%。經分光光度計掃描得知，卡巴得最大吸收波長爲 313 nm。因此，我們能用一個簡便而又準確的方法來測定飼料內之卡巴得。