High-Performance Liquid Chromatographic Determination of Carbadox, Olaquindox, Furazolidone, Nitrofurazone, and Nitrovin in Feed

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ABSTRACT

A high-performance liquid chromatography with gradient programming method was developed to determine the amount of carbadox (CBX), olaquindox (OLQ), furazolidone (FZ), nitrofurazone (NF), and nitrovin (NTV) in feed simultaneously. Complete separation of the drugs was obtained using a C_8 silica gel column with gradients of acetonitrile as mobile phase. The mobile phase used an acetonitrile gradient with an initial hold time of 1 min at 0% acetonitrile, followed by an increase to 50% acetonitrile over 10 min. The correlation coefficients (r) for calibration curves of the five feed additives were greater than 0.999. The relative standard deviations (RSDs) of peak areas from four injections for these drugs at three concentrations were less than 3.0%, although the RSD for NTV at 5 ppm was somewhat large (6.7%). The medicated feeds were extracted by pretreating with water, extracted with 95% dimethylformamide overnight at room temperature, and cleaned up on a column of alumina oxide. Recoveries of CBX, OLQ, FZ, NF, and NTV from low level spiked feed were 102.0, 94.6, 97.4, 110.6, and 66.0%, respectively, and from high level spiked feed, they were 114.09, 99.1, 97.3, 109.9, and 62.7%, respectively.

A project for promoting feed quality sponsored by the Council of Agriculture was carried out at this institute. The project dealt with the assay of drugs in feed, including carbadox (CBX), olaquindox (OLQ), furazolidone (FZ), nitrofurazone (NF), sulfamethazine (SMT), sulfathiazole, and pyrimethamine. Among these feed additives, CBX and OLQ are derivatives of quinoxaline-1.4-dioxide, whereas FZ and NF belong to the furan group. NTV is one of the furans allowed for use as a feed additive *(6)*. Although there are official (Chinese National Standard) methods for several of these drugs (Table 1), the methods for CBX and NF are spectrophotometric, which is time-consuming.

Most of the previously published methods for the determinations of OLQ, CBX, NF, FZ, and NTV in feed are concerned only with one (single) drug in feed *(1–4, 8, 9, 11, 14, 16–19)*, and a few experiments were conducted to simultaneously determine two *(5, 10, 12, 13, 20, 22)*, three *(15)*, or four drugs *(21)*. As for this work, there were no reports on the simultaneous determination of residual drugs of OLQ, CBX, NF, FZ, and NTV in feed by high-performance liquid chromatography (HPLC). To simplify the assay procedure, as well as to reduce the workload and increase accuracy, an HPLC with gradient program is presented here for quantitative and simultaneous determination of the above five drugs in feed.

MATERIALS AND METHODS

Apparatus: (i) liquid chromatograph. Analyses were performed with an HPLC system equipped with a Shodex degasser (Shodex, Tokyo, Japan), a Kratos Spectroflow 400 pump (Kratos, New Jersey, USA), a Kratos Spectroflow 491 injector (fix injection volume 20 μl), a Kratos Spectroflow 783 detector at wavelength 365 nm, and an SIC chromatogram processor 7000B integrator (SIC, Tokyo, Japan).

(ii) Chromatographic column. The chromatographic column used was the Kratos spheri-5, RP-8, 5 μm, 250 by 4.6 mm with Waters RCSS Guard-Pak C-18 guard column (Waters, Minnesota, USA).

LC conditions. Gradient elution was applied. The chromatographic column was washed in advance with 100% water for 1 min; then, the gradient concentration of acetonitrile was increased. The concentration of acetonitrile was gradually increased to 50% over 10 min, then maintained at 50% for 9 min. After that, the concentration of acetonitrile was decreased to 0 (100% of water) in 3 min and maintained for 2 min. The sample was not injected until equilibrium was achieved. The flow rate was 1 ml/min, the injection volume was 20 μl, and the detecting wavelength was 365 nm.

Organic solvents and reagents. Acetonitrile (LC grade), dimethylformamide (DMF), and alumina (basic, 1076) were obtained from E. Merck (Darmstadt, Germany). Water of LC grade was obtained from Kowei Chemical (Taipei, Taiwan).

Standards and calibration curve. OLQ, CBX, and NTV were provided by Kowei Chemical, Pfizer (Taipei, Taiwan), and Cynamid Taiwan (Taipei, Taiwan), respectively. FZ and NF standards were from U.S.P.C. (Maryland, USA). SMT and sulfathiazole were provided by Kotech (Taipei, Taiwan). Oxytetracycline (OTC) and chlortetracycline (CTC) were obtained from China Chemical & Pharmaceutical (Taipei, Taiwan). Procaine penicillin-G (PC-G), arsanilic acid (ASA), pyrantel pamonte (PRT), and bacitracin zinc (BCT) were obtained from Meiji Seika Kaisha, Pharmaceutical Division (Tokyo, Japan); Virbac Laboratories (Carros, France); Pfizer Quality Control; and Apothekernes Laboratorium A. S. (Oslo, Norway), respectively.

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TABLE 1. *Chinese National Standard (CNS) methods for the determination of OLQ, CBX, NF, FZ, and NTV*

Drug	Sample	Method	Publication time	AOAC
OLQ	Feed	HPLC (254 nm/372 nm)	1984	
CBX	Feed	Spectrophotometry (520 nm)	1982	Same as CNS method
NF				Spectrophotometry (440 nm)
FZ	Feed	HPLC (365 nm)	1986	Same as NF
NTV	Premix	Spectrophotometry (495 nm)	1984	

Twenty milligrams each of OLQ, CBX, NF, FZ, and NTV standards were weighed into separate 20-ml volumetric flasks, then diluted to volume with DMF to prepare 1,000-ppm stock standard solutions. Sonication was applied to accelerate the dissolution when necessary.

An appropriate amount of individual standard stock solutions was mixed and diluted with 95% DMF (DMF containing 5% water) to prepare the mixed working standard solutions for the calibration curve. For OLQ, CBX, NF, and FZ, the concentrations of standards for the three-point calibration curve were 2, 4, and 8 ppm, whereas for NTV, the concentrations of the calibration curve were 5, 10, and 20 ppm. Each concentration of solution was injected into HPLC four times; then, peak areas were measured to prepare the calibration curve.

Feed extraction and cleanup. Carrier and blank feed for growing pigs were provided by Cynamid and Animal Products Research Institute of Taiwan Sugar (Miaoli, Taiwan), respectively.

A brown glass column of 1 by 30 cm constricted at one end to 4 cm with a Teflon switch was used to fill alumina for feed cleanup. According to the procedure of Williams *(23),* the alumina was washed with water, filtered, washed with methanol three times, and dried; then, 5 g was weighed into the brown glass column.

Ten grams of feed was added to 5 ml water, stirred, and allowed to stand for 5 min. After adding 50 ml of 95% DMF, the feeds were shaken for 15 s, placed in a dark place overnight, and filtered through Toyo 5C filtration paper (Toyo, Tokyo, Japan). Twenty milliliters of the extract was transferred onto the previously prepared brown glass column containing alumina for cleanup, and the first 5 ml of eluant was discarded. The remaining eluant was filtered through a 0.45-μm millipore membrane, and an aliquot of 20 μl was injected into the HPLC. Eluant from high level spiked feeds was diluted fivefold with 95% DMF, then injected into the HPLC.

Filtration efficiency of alumina. Twenty milliliters of mixed working standard solution containing 4 ppm of OLQ, CBX, FZ, and NF and 10 ppm of NTV was added to 2 ml water, then was poured into the alumina column. The first 5 ml of eluant was discarded, and the remaining eluant was collected for HPLC injection. The injection volume was 20 μl. The difference in peak areas obtained from solutions filtered or not filtered by alumina was compared.

Determination of possible interference. Seven drugs (Table 2) (SMT, PRT, OTC, CTC, ASA, BCT, and PC-G) that are allowed for use in combination with the analytes were chosen to test their possible interference.

Recovery test in spiked feed. Adequate amounts of OLQ, CBX, NF, FZ, and NTV were added to the carrier to prepare the high concentration premix. Blank feed for growing pigs was passed through a 20-mesh sieve in advance, then the premix was added according to the regulation of the Council of Agriculture, the Republic of China (Table 2). The spiked concentrations of the low level feeds were 15, 11, 50, 7.5, and 5 ppm for OLQ, CBX, NF, FZ, and NTV, respectively. The high level feed concentrations were 100, 55, 500, 300, and 30 ppm, respectively. The spiked feeds were extracted three times per concentration by the method described above.

The drug concentrations were calculated from the calibration curve and multiplied dilutions factor to obtain the real concentrations in feed. Ten grams of feed was extracted with 5 ml water and 50 ml DMF; therefore, the dilution factor was 5.5. For the high level spiked feeds, another fivefold dilution was made; therefore, the dilution factor was equal to 27.5.

RESULTS

Gradient analysis. Different ratios of acetonitrile and water were used in the gradient programming. The chromatographic column was first prewashed with 100% water for 1 min. The concentration of acetonitrile was increased gradually at a rate of 5% per min and then kept at 50%. OLQ, CBX, NF, and FZ could be eluted in 10 min. To elute the NTV, the highest concentration of acetonitrile (50%) was maintained for 9 min. Therefore, the five drugs could be completely separated by the above gradient.

TABLE 2. *Recommended uses of OLQ, CBX, NF, FZ, and NTV*

Drug	Low level (ppm)	High level (ppm)	Drug in combination	Country
OLQ	15–50	50–100	—	The Republic of China
	10–25	10–50	—	Japan
CBX	11–28	50–55	SMT	The Republic of China
	10–25	50	PRT	USA
NF	50 (chickens)	500 (pigs)	—	USA
FZ	100 (pigs)	300 (pigs)	OTC, ASA	USA
	7.5–10 (chickens)	200 (chickens)	OTC, BCT, PC-G	USA
NTV	5–15	20–30	CTC, SMT	The Republic of China

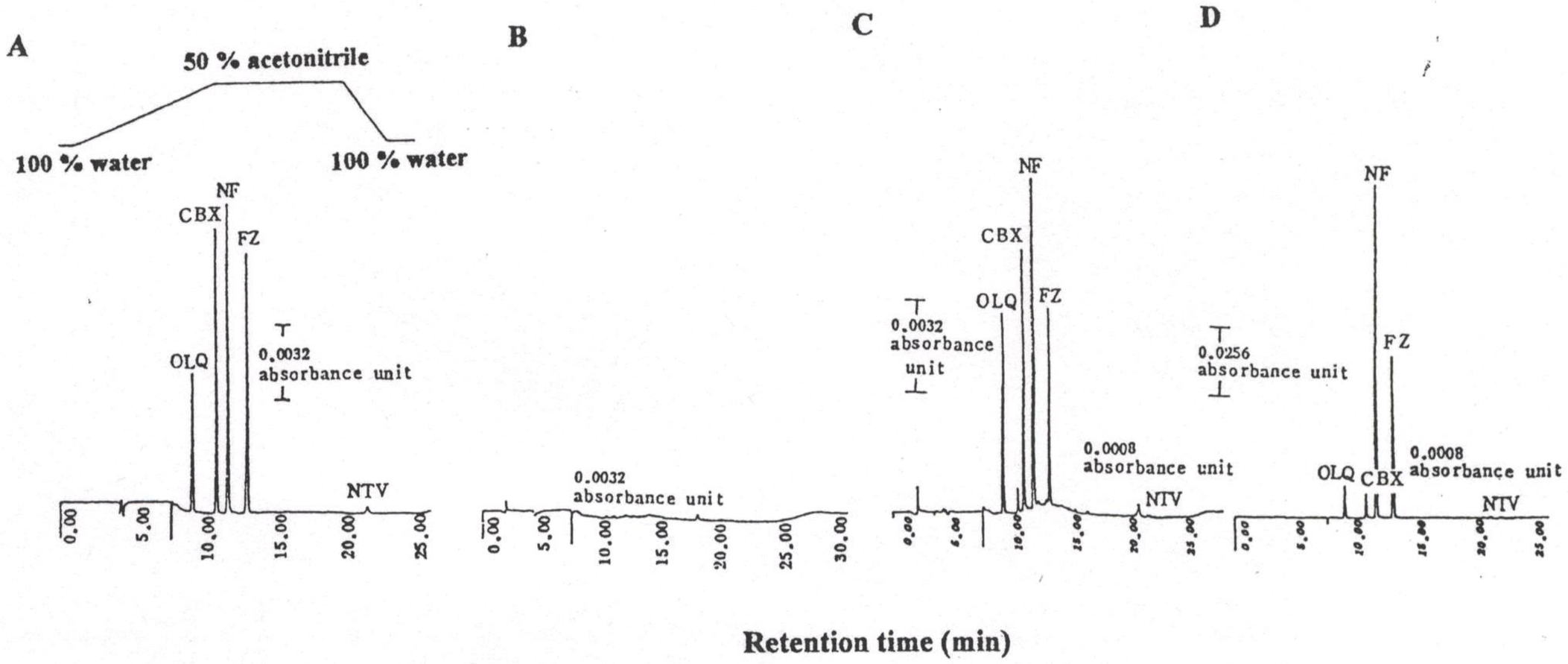

FIGURE 1. *Chromatograms of OLQ, CBX, NF, FZ, and NTV. (A) Gradient of acetonitrile and 2 ppm of OLQ, CBX, NF, and FZ, and 5 ppm of NTV standards. (B) Blank feed. (C) Blank feed spiked with 15, 11, 50, 7.5, and 5 ppm of OLQ, CBX, NF, FZ, and NTV, respectively. (D) Blank feed spiked with 100, 55, 500, 300, and 30 ppm of OLQ, CBX, NF, FZ, and NTV, respectively.*

Calibration curves. Linear responses were obtained for the five standards. The equations of the calibration curves of OLQ, CBX, NF, FZ, and NTV were $y = -890 + 6298x$, $y = -414 + 11975x$, $y = -2420 + 14559x$, $y = -0.9 + 10652x$, and $y = 7.0 + 117x$, respectively, with correlation coefficients all greater than 0.999. The RSDs of peak areas of four injections for OLQ, CBX, NF, FZ, and NTV at three concentrations (2, 4, and 8 ppm for OLQ, CBX, FZ, and NF; 5, 10, and 20 ppm for NTV) were 0.7 to 3.0%, 0.5 to 1.1%, 0.9 to 1.2%, 0.8 to 1.6%, and 1.3 to 6.7%, respectively.

Filtration efficiency of alumina. The filtration efficiencies of alumina for OLQ, CBX, NF, FZ, and NTV were 96, 98, 89, 99, and 106%, respectively. Except for a slight retention of NF, the other four drugs showed satisfactory filtration efficiencies.

Determination of possible interference. Seven drugs allowed for use in combination with the analytes were tested for possible interference. The chromatogram showed no interfering peaks from OTC, CTC, BCT, or PC-G (eluted for 25 min). The retention times for PRT, ASA, and SMT were 9.73, 11.10, and 12.32 min, respectively. Therefore, PRT, ASA, and SMT did not interfere with the detection of the five drugs.

Recovery test of spiked feeds. Most of the interfering matrix could be removed by the above-mentioned extraction method, and a stable LC baseline was obtained (Fig. 1B).

The recovery rates for OLQ, CBX, NF, FZ, and NTV from spiked feed at the low levels were 102.0, 94.6, 97.4, 110.6, and 66.0%, respectively, whereas those at high levels were 114.0, 99.1, 97.3, 109.9, and 62.7%, respectively. NTV was calculated by peak height because of the low spike level and small peak (Fig. 1C and 1D). The RSDs for OLQ and NTV were slightly higher, whether spiked at low or high levels (Tables 3 and 4).

DISCUSSION

The difference in polarity between OLQ and NTV was too great to be analyzed by isocratic HPLC. When the concentration of acetonitrile was 20% (mixed with water), peaks of OLQ and solvent appeared at the same time, and NTV could not be eluted for 28 min, revealing that NTV could be retained in the chromatographic column for a long time. The

TABLE 3. *Recovery of OLQ, CBX, NF, FZ, and NTV in feed spiked at low level*

	OLQ (15 ppm)	CBX (11 ppm)	NF (50 ppm)	FZ (7.5 ppm)	NTV (5 ppm)[a]
1	15.2	10.3	47.9	8.4	3.4
2	14.8	10.7	49.6	8.5	3.5
3	14.2	10.1	47.6	8.0	3.1
4	17.1	10.4	49.5	8.4	3.0
Mean	15.3	10.4	48.7	8.3	3.3
(RSD)	(8.2%)	(2.4%)	(2.2%)	(2.7%)	(7.2%)
Recovery	102.0%	94.6%	97.4%	110.6%	66.0%

[a] Calculated by peak height.

TABLE 4. *Recovery of OLQ, CBX, NF, FZ, and NTV in feed spiked at high level*

	OLQ (100 ppm)	CBX (55 ppm)	NF (500 ppm)	FZ (300 ppm)	NTV (30 ppm)[a]
1	119.9	55.0	482.6	331.9	17.4
2	112.8	53.6	487.6	322.3	24.1
3	111.4	54.7	489.2	333.0	19.3
4	111.9	54.2	485.9	331.1	14.5
Mean	114.0	54.4	486.3	329.6	18.8
(RSD)	(3.5%)	(1.1%)	(0.6%)	(1.5%)	(21.5%)
Recovery	114%	99.1%	97.3%	119.9%	62.7%

[a] Calculated by peak height.

peaks for CBX, NF, and FZ overlapped. When the concentration of acetonitrile was increased to 40%, peaks of OLQ, CBX, NF, and FZ overlapped even more significantly; NTV eluted at about 9 min. At 60% acetonitrile, NTV eluted at about 4 min; peaks of the other four drugs still overlapped with solvent peaks. Therefore, the gradient program was needed to simultaneously determine these five drugs.

A number of chromatographic columns with gradient program and flow rate effect were tested. The results demonstrated that although NTV could be eluted (at about 20 min), CBX and NF still could not be completely separated.

Chromatographic columns tested included Kratos RP-300 (5 mm by 25 cm), Kratos OD-300 (5 mm by 25 cm), Kratos C_{18} (5 mm by 22 cm), Finepak C_1 (5 mm by 30 cm), Merck select B (7 mm by 12.5 cm), Spheris C_8 (5 mm by 15 cm), Nucleosil C_8 (5 mm by 10 cm), and Kratos C_{18} (22 mm by 18 cm). Different gradient programs were tested 8 to 12 times for each column. The best result and complete separation was obtained from Kratos RP-8 (5 mm by 25 cm) (Fig. 1A).

NTV has the lowest absorbance (Fig. 1A). The Association of Official Analytical Chemists method uses spectrometry (495 nm) to detect NTV in feed. However, absorbance of NTV at 365 nm was only about 15% lower than that at 500 nm (or 480 nm). To detect the other four drugs simultaneously, we still used 365 nm as the detection wavelength. Although the peak height of NTV was low, the repeatability of peak area was satisfactory, and the correlation coefficient of the calibration curve was also high.

To investigate the effect of alumina on the filtration efficiency of OLQ, CBX, NF, FZ, and NTV during the extraction procedure, drug standards mixed with water (same volume as the extracted fluid) were filtered through the alumina. The result suggested that only NF was slightly retained (filtration efficiency, 89%), while the other four drugs could be filtered almost completely.

Recoveries of these five drugs in feed were satisfactory, except for NTV. Recovery of OLQ and FZ was higher than 100% (Tables 3 and 4); this phenomenon might be due to the error from the feed preparation (since the spiked level was quite low). However, the recovery did not exceed 100% by more than 10%. According to the Food and Drug Administration *(7)*, the assay limits for the detection of drugs in feed range from +5 to +50%, depending on the analytical methods. For example, the assay limits for CBX in feed determined by spectrometric method is +25%. The recovery for CBX was close to that of a previous report at 91.2 to 93.8% *(9)*.

The recommended dose for NTV in feed is low (Table 2) and even worse is the low absorbance (Fig. 1A). The chromatogram of NTV in feed showed a small peak. A short peak was observed after fourfold amplification; therefore, the peak height was used for quantification of NTV. Since not only the recovery of NTV was poor but also the deviation was high (Tables 3 and 4), the extraction method for NTV needs further improvement.

REFERENCES

1. Aerts, M. M., W. M. Beek, and U. A. Brinkman. 1990. On-line combination of dialysis and column-switching liquid chromatography as a fully automated sample preparation technique for biological samples. Determination of nitrofuran residues in edible products. J. Chromatogr. 500:453–468.
2. Aerts, M. M., W. M. Beek, H. J. Keukens, and U. A. Brinkman. 1988. Determination of residues of carbadox and some of its metabolites in swine tissues by high-performance liquid chromatography using on-line pre-column enrichment and post-column derivatization with UV-VIS detection. J. Chromatogr. 456:105–119.
3. Aerts, R. M., and G. A. Werdmuller. 1988. Liquid chromatographic determination of carbadox in complete feeds, premixes, and concentrates: interlaboratory study. J. Assoc. Off. Anal. Chem. 71:484–490.
4. Bories, G. F. 1979. High-performance liquid chromatographic determination of olaquindox in feeds. J. Chromatogr. 172:505–508.
5. Cieri, U. R. 1979. High pressure liquid chromatographic detection and estimation of furazolidone and nitrofurazone in animal feeds. J. Assoc. Off. Anal. Chem. 62:168–170.
6. Council of Agriculture. 1998. Feed additives regulation. Council of Agriculture, Taipei, Taiwan, Republic of China.
7. Food and Drug Administration. 1988. Additives and their uses. *In* Feed additive compendium. The Miller Publishing Company, Minnetonka, Minn.
8. Gliddon, M. J., C. Cordon, and G. M. Parnham. 1983. Determination of nitrovin in animal feedingstuffs by high-performance liquid chromatography. Analyst 108:116–119.
9. Lin, S. Y., T. F. Chiou, and C. S. Shen. 1983. Detection of carbadox in feed by high performance liquid chromatography. Taiwan J. Vet. Med. Anim. Husb. 42:15–23.
10. Lowie, D. M., R. Y. Teague, Jr., F. E. Quick, and C. L. Foster. 1983. High pressure liquid chromatographic determination of carbadox and pyrantel tartrate in swine feed and supplements. J. Assoc. Off. Anal. Chem. 66:602–605.
11. Luchtefeld, R. G. 1977. Detection of low-level carbadox residues in animal feeds by high pressure liquid chromatography. J. Assoc. Off. Anal. Chem. 60:279–283.
12. Malisch, R. 1986. Multi-method for determining residues of chemotherapeutics, antiparasitics and growth promotors in foods of animal origin. 2. Determination of nitrofurans and nicarbazin. Z. Lebensm. Unters. Forsch. 183:253–266.
13. McGary, E. D. 1986. Quantitative determination of sulphamethazine and carbadox in animal feeds by paired ion high-performance liquid. Analyst 111:1341–1342.
14. Nagata, T., and M. Saeki. 1987. Determination of olaquindox residues in swine tissues by liquid chromatography. J. Assoc. Off. Anal. Chem. 70:706–707.
15. Rihs, V. T., and J. Schveider. 1980. Die bestimmung von carbadox, furazolidon und olaquindox in futtermitteln mittels hochdruck-fliissigkeits chromatographie. Schweiz. Landwirtsch. Monatsh. 58:135–138.
16. Rose, M. D., J. Bygrave, and J. A. Tarbin. 1995. Determination of quinoxaline carboxylic acid (metabolite of carbadox) in animal tissue by HPLC. Food Addit. Contam. 12:177–183.
17. Roybal, J. E., R. K. Munns, and W. Shimoda. 1985. Liquid chromatographic determination of carbadox residues in animal feed. J. Assoc. Off. Anal. Chem. 68:653–657.
18. Smallidge, R. L., N. W. Rowe, N. D. Wadgaonkar, and R. W. Stringham. 1981. High performance liquid chromatographic determination of furazolidone in feed and feed premixes. J. Assoc. Off. Anal. Chem. 64:1100–1104.
19. Spierenburg, T. J., H. van Lenthe, G. de Graaf, and L. P. Jager. 1988. Liquid chromatographic determination of olaquindox in medicated feeds and in contents of porcine gastrointestinal tract. J. Assoc. Off. Anal. Chem. 71:1106–1109.
20. Sugden, E. A., A. I. MacIntosh, and A. B. Vilim. 1983. High pressure liquid chromatographic determination of nitrofurazone and furazolidone in chicken and pork tissues. J. Assoc. Off. Anal. Chem. 66:874–880.
21. Thorpe, V. A. 1980. Sample preparation of carbadox, furazolidone, nitrofurazone, and ethopabate in medicated feeds for high pressure liquid chromatography. J. Assoc. Off. Anal. Chem. 63:981–984.
22. Thorpe, V. A. 1988. A collaborative study: high-pressure liquid chromatographic determination of carbadox and pyrantel tartrate in animal feeds. J. Chromatogr. Sci. 26:545–550.
23. Williams, S. 1984. Official methods of analysis of the Association of Official Analytical Chemists. AOAC, Philadelphia, Pa.

飼料內卡巴得、歐來金德、富來頓、乃挫夫喃頓及乃託文之高效液相色層分析法

林士鈺*　鄭秀蓮

摘要　爲同時分析飼料內卡巴得、歐來金德、富來頓、乃挫夫喃頓及乃託文，利用C_8矽膠層析管在梯度分析條件下，可得完全分離之層析圖。梯度分析主要以每min5% acetonitrile之線性梯度。五種飼料添加物檢量線之相關係數(Y)均在0.999以上且相對標準偏差在0.3%以內，僅5ppm乃託文大些(6.7%)。飼料先加水處理後，以95% dimethylformamide 於室溫中過夜萃取，再通過三氧化二鋁管柱。低濃度飼料回收率分別爲102.0, 94.6, 97.4, 110.6及66.0%，高濃度則爲114.0, 99.1, 109.9及62.7%。

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