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Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 85–93
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

# Assay and purity control of tetracycline, chlortetracycline and oxytetracycline in animal feeds and premixes by TLC densitometry with fluorescence detection

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Received 16 January 2003; received in revised form 18 March 2003; accepted 18 March 2003

**Abstract**

Methods using TLC densitometry with fluorescence detection are described for the assay and purity control of tetracycline (TC), chlortetracycline (CTC), and oxytetracycline (OTC) in animal feeds and premixes. With a silica gel layer previously sprayed with 10% (m/v) sodium EDTA solution adjusted to pH 8.0 or 9.0, all the major impurities were separated from the main components and from each other. The mobile phase consisted of dichloromethane, methanol, and water. After development, the plate was dipped in a 30% (v/v) solution of liquid paraffin in hexane. Quantitation was realized by fluorescence densitometry at 400 nm. The limit of quantitation (LOQ) for tetracycline impurities is 0.8 μg/g, corresponding to 0.2% of the label claimed tetracycline (400 μg/g). The LOQ for impurities of tetracycline and chlortetracycline in premixes is 0.2% of the label-claimed TC (40 mg/g) and CTC (200 or 400 mg/g). The LOQ for impurities of oxytetracycline in a premix is 0.1% of the label claimed OTC (100 mg/g).


*Keywords:* TLC densitometry; Fluorescence; Tetracyclines; Animal feeds; Premixes; Assay; Purity control

## 1. Introduction

Tetracycline antibiotics such as tetracycline (TC), chlortetracycline (CTC), and oxytetracycline (OTC) are commonly used in animal feeds to improve growth rates and to prevent diseases. Veterinary therapeutical products also contain these antibiotics in higher amounts to treat various infections. The chemical structures of TC, CTC, OTC, and their impurities are shown in Fig. 1. The bulk samples of tetracyclines used to prepare animal feeds and premixes may contain various fermentation and degradation impurities.

4-Epitetracycline (ETC), anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC) are the major degradation impurities of TC [1]. For CTC, 4-epichlortetracycline (ECTC) is the major degradation impurity [2]. TC and demeclocycline (DMCTC) are the major fermentation impurities

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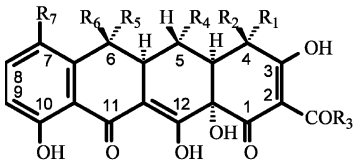
doi:10.1016/S0731-7085(03)00153-5



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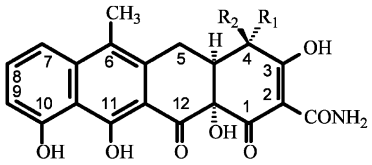

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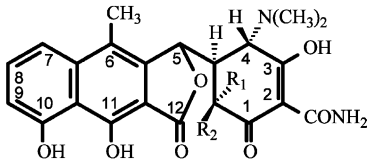

III

| Structure | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ | $R_7$ | Compound |
|---|---|---|---|---|---|---|---|---|
| I | $N(CH_3)_2$ | H | $NH_2$ | H | $CH_3$ | OH | H | TC |
| | H | $N(CH_3)_2$ | $NH_2$ | H | $CH_3$ | OH | H | 4-ETC |
| | $N(CH_3)_2$ | H | $CH_3$ | H | $CH_3$ | OH | H | ADTC |
| | $N(CH_3)_2$ | H | $NH_2$ | OH | $CH_3$ | OH | H | OTC |
| | H | $N(CH_3)_2$ | $NH_2$ | OH | $CH_3$ | OH | H | 4-EOTC |
| | $N(CH_3)_2$ | H | $CH_3$ | OH | $CH_3$ | OH | H | ADOTC |
| | $N(CH_3)_2$ | H | $NH_2$ | H | $CH_3$ | OH | Cl | CTC |
| | H | $N(CH_3)_2$ | $NH_2$ | H | $CH_3$ | OH | Cl | 4-ECTC |
| | $N(CH_3)_2$ | H | $CH_3$ | H | $CH_3$ | OH | Cl | ADCTC |
| | $N(CH_3)_2$ | H | $NH_2$ | H | H | OH | Cl | DMCTC |
| | $N(CH_3)_2$ | H | $NH_2$ | OH | $=CH_2$ | - | H | MTC |
| II | $N(CH_3)_2$ | H | | | | | | ATC |
| | H | $N(CH_3)_2$ | | | | | | 4-EATC |
| III | H | OH | | | | | | α-APOTC |
| | OH | H | | | | | | β-APOTC |

Fig. 1. Structures of TC, CTC, OTC, and their impurities.

of CTC. 4-Epioxytetracycline (EOTC), α- and β-apooxytetracycline (α-APOTC and β-APOTC) are the main degradation impurities of OTC while TC is the main fermentation impurity [3]. 2-Acetyl-2-decarboxamide derivatives of the tetracyclines (ADTC, ADCTC, and ADOTC) are also present as fermentation impurities in commercial samples [1–3]. Upon storage, tetracyclines in animal feeds and premixes may also be subject to extensive degradation.

It is known that the degradation compounds of tetracyclines are almost inactive or even toxic. EATC has been identified as an agent causing Fanconi-type syndrome [4]. To ensure the safety of the animals, a good analytical method is required for the quality control of tetracyclines in animal feeds and premixes. Toxic degradation compounds should be quantified. Liquid chromatography with mass spectrometric detection (LC–MS) methods for the simultaneous analysis of TC, CTC, OTC and their major degradation compounds in animal feeds have been described [5,6]. However, the LC–MS instruments are still quite expensive and are not readily available for chemists in testing laboratories. Several other reversed-phase LC methods using conventional UV or fluorescence detection have also been described for the determination of TC, CTC or OTC in animal feeds and premixes [7–12] as well as in milk and eggs [13]. For all the LC methods, however, anomalous peak distortions for CTC were observed because of the rapid on-column formation of the keto-tautomers of CTC and its epimer ECTC. The keto-enol transformation of tetracyclines has been thor-

oughly investigated in our laboratory [14]. To extract tetracyclines from the animal feeds and premixes, an aqueous McBuffer was used to extract tetracyclines [7–12]. However, it has been reported that severe epimerisation degradation of tetracyclines can occur in aqueous McBuffer solution [12].

We have previously reported on a semi-quantitative TLC method with fluorescence detection for the purity control of six tetracyclines in bulk sample [15]. The natural fluorescence of tetracyclines and their impurities in alkaline media was used for semi-quantitation. The visual detection limit was 1 ng. Based on this experience, a quantitative TLC method using fluorescence densitometry was developed for the assay and purity control of TC, CTC, and OTC in animal feeds and premixes.

## 2. Experimental

### 2.1. Chemicals

Methanol was obtained from Merck-Belgolabo (Overijse, Belgium) and redistilled in glass apparatus. Dichloromethane and hexane were from Acros Organics (Geel, Belgium). Other reagents were of analytical reagent quality (E. Merck, Darmstadt, Germany). Demineralized water was freshly distilled in glass apparatus.

### 2.2. Reference substances and samples

Reference substances for TC·HCl, ETC·HCl, ATC·HCl, EATC·HCl, CTC·HCl, ECTC·HCl, OTC, EOTC, α-APOTC and β-APOTC were available from Acros. House standards of DMCTC·HCl and metacycline hydrochloride (MTC·HCl) were available in the laboratory [16,17]. ADTC was kindly made available by Pfizer (Groton, CT, USA). Small amounts of ADCTC and ADOTC were isolated from CTC·HCl and OTC commercial samples using a described TLC method [18]. TC·HCl sub-standard in Kieselgur (50 mg/g) was prepared by mixing 0.500 g of TC·HCl reference standard with 9.500 g of Kieselgur (E. Merck).

Premixes of TC·HCl (40 mg/g), CTC·HCl (100 and 200 mg/g) and OTC (100 mg/g) as well as animal feeds containing TC·HCl (400 μg/g) were obtained from the Belgian market. These samples were stored in a cool, dry and dark place. With the exception of the label claim of tetracyclines, the composition of other ingredients in these samples was unknown to us.

### 2.3. Sample preparation of premixes

To 2.5 g of premixes (claimed content 40–200 mg/g), 100 ml of acidified methanol (methanol+1 M HCl, 99/1, v/v) was added. The compounds of interest were extracted by ultrasonication for 15 min. The extracts were filtered through a Whatman No 2 paper. The first 5-ml of filtrate was discarded. The filtrate was then collected and diluted with methanol to a concentration of tetracyclines corresponding to 50 μg/ml. The final solution also contained 50 μg/ml of internal standard (IS), i.e. β-APOTC for the analysis of TC·HCl and CTC·HCl premixes, and MTC for the analysis of OTC premixes. The sample solution was stable for at least 12 h at 4 °C.

### 2.4. Sample preparation of animal feeds

Three aliquots of 12.5 g of animal feeds (claimed content 0.4 mg TC·HCl/g) were accurately weighed. Two aliquots were fortified with 50.0 and 100.0 mg, respectively, of laboratory-prepared TC·HCl sub-standard in Kieselgur (50 mg/g). To each of these three aliquots, 12.5 g of Kieselgur was added and the samples were mixed well mechanically. The mixed powder was transferred into a glass column (2 cm, i.d.). Acidified methanol (90 ml) was added on the column and the extract was collected in a 100-ml volumetric flask in an ice bath. The flow was not restricted. IS, i.e. 10 ml of a 0.5 mg/ml solution of β-APOTC in methanol, was added to the flask and the sample was diluted to 100 ml with methanol. The sample solution was then filtered through a Whatman No 2 filter paper. The first 5-ml of filtrate was discarded and a following fraction was collected. The final sample solution contained 50 μg/ml of TC·HCl and IS. For the fortified samples, the IS concentration

remained to be 50 μg/ml but the TC·HCl concentration was increased from 50 to 75 μg/ml and 100 μg/ml, corresponding to 50 and 100% increase of the label claimed TC·HCl concentration, respectively. The sample solutions were kept at 4 °C and applied onto the TLC plate within 2 h.

### 2.5. Reference solution

Reference solution for the determination of TC·HCl in premixes and animal feeds contained 50 μg/ml of TC·HCl and 5 μg/ml of each of the TC impurities (ETC·HCl, EATC·HCl and ATC·HCl) in methanol. This solution also contained 50 μg/ml of β-APOTC as the IS. Reference solution for the determination of CTC·HCl in premixes contained 50 μg/ml of CTC·HCl and 5 μg/ml of each of the CTC impurities (ECTC·HCl, TC·HCl and DMCTC·HCl) in methanol. The same solution contained 50 μg/ml of β-APOTC as the IS. Reference solution for the determination of OTC in premixes contained 50 μg/ml of OTC and 5 μg/ml of each of the OTC impurities (EOTC, TC and β-APOTC) in methanol. MTC (50 μg/ml) was added to this solution as the IS. The reference solution was stable for at least 12 h at 4 °C.

### 2.6. TLC

Precoated silica gel layer on glass (20 cm × 20 cm) was purchased from Macherey-Nagel (Düren, Germany, No 809013). Before use the silica gel plates were sprayed with a 10% (m/v) solution of sodium edetate pH 8.0 (for TC and CTC) or 9.0 (for OTC). The plates were dried in a horizontal position for at least 1 h at room temperature and then in an oven at 110 °C for 1 h shortly before use [15].

Aliquots (2 μl) of the sample and reference solutions (corresponding to 100 ng for TC, CTC, and OTC as well as 10 ng for the impurities) were applied to the plate with a microsyringe (Hamilton, Bonaduz, Switzerland). On each plate six replicates of the sample solutions and reference solutions were applied.

The chromatographic chamber was lined with paper and equilibrated with a mobile phase of dichloromethane–methanol–water (59:35:6, v/v/v, for TC, 60:35:5, v/v/v, for CTC, and 58:35:7, v/v/v, for OTC) for at least 1 h prior to use. The plate was developed at room temperature over a distance of 15 cm. The developed plate was dried in an oven at 105 °C for 2 min and was then dipped vertically for 1 s in a tank containing liquid paraffin in hexane (30:70, v/v). The spots were measured with a CS-930 TLC densitometer (Shimadzu, Kyoto, Japan) using the following parameters: linear scanning; scan step in the y-direction = 0.1 mm; beam size = 1.2 mm × 6 mm; fluorescence mode with $\lambda = 400$ nm; filter No = 3; linearizer = off; background correction = on; drift line integration = 0.5.

### 2.7. Calculation

For the animal feeds, standard addition method was used to calculate the contents of tetracycline and impurities. TC·HCl (%) in the animal feeds was calculated as follows: TC·HCl (%) = [A/B]/C × 100 (%), where A was the ratio of TC·HCl response versus IS response in the animal feeds; B was TC·HCl response versus IS response in the reference solution; and C was the mean recovery of adding 50 and 100% of labelled claimed amount of TC·HCl. A similar equation was also used to calculate the content of the impurities, assuming the impurities having the same recovery as TC·HCl.

For the premixes, calculation was made based on direct comparison of analytes of interest in the samples with those in the reference solution. The results were not corrected for the recovery since virtually 100% recovery was achieved.

## 3. Results and discussion

### 3.1. Development of the TLC method

Quantitative TLC methods suitable for assay and purity control of TC, CTC and OTC in commercial bulk samples had been developed in our laboratory [19–22]. The results obtained by UV TLC-densitometry were well agreeable with those obtained by LC methods [1–3]. Later, we also reported on a simple semi-quantitative TLC

Table 1
Calibration curves for TC, CTC, OTC and their impurities, as obtained with the TLC method

| | Intercept | Slope | $r$ | $S_{y,x}$ | $R$ (ng)[a] | $n$[b] |
|---|---|---|---|---|---|---|
| TC·HCl | −1073 | 142 800 | 0.9999 | 71 | 0.2–100 | 5 × 3 |
| ETC·HCl | −1100 | 144 500 | 0.9954 | 163 | 0.2–10 | 3 × 3 |
| ATC·HCl | −1210 | 154 000 | 0.9903 | 202 | 0.2–10 | 3 × 3 |
| EATC·HCl | −1200 | 172 100 | 0.9917 | 134 | 0.2–10 | 3 × 3 |
| CTC·HCl | −289 | 153 900 | 0.9995 | 170 | 0.2–100 | 5 × 3 |
| ECTC·HCl | 883 | 126 600 | 0.9912 | 211 | 0.2–10 | 3 × 3 |
| DMCTC·HCl | −104 | 292 100 | 0.9936 | 214 | 0.2–10 | 3 × 3 |
| TC·HCl | −423 | 134 600 | 0.9920 | 106 | 0.2–10 | 3 × 3 |
| OTC | 964 | 217 800 | 0.9926 | 451 | 0.1–100 | 5 × 2 |
| EOTC | 1210 | 272 000 | 0.9922 | 120 | 0.1–10 | 3 × 2 |
| β-APOTC | 1620 | 279 600 | 0.9891 | 554 | 0.1–10 | 3 × 2 |
| TC | 1310 | 371 100 | 0.9904 | 723 | 0.1–10 | 3 × 2 |

[a] Range (ng on the plate) examined.
[b] Levels examined × number of tracks.

method with fluorescence detection suitable for the rapid and simple purity control of tetracyclines [15]. The natural fluorescence of tetracyclines and their impurities in alkaline media was used for semi-quantitation. Based on these experiences, a quantitative TLC method using fluorescence densitometry was developed for the assay and purity control of TC, CTC, and OTC in premixes and animal feeds. Fluorescence densitometry was chosen because it is a more sensitive and also a more selective detector than UV densitometry, as it is more suitable to exclude interference arising from the premixes and animal feeds matrices.

The IS used in the quantitative UV densitometry methods, i.e. minocycline (MC) which has minimal natural fluorescence, was less suitable as an IS for the quantitative fluorescence densitometry. It was found that MTC was an appropriate IS for the assay and purity control of OTC in premixes and animal feeds. MTC is not used to prepare medicated animal feeds and is not present in OTC samples. β-APOTC is not present in TC and CTC samples and, therefore, can be used as the IS.

In order to enhance the fluorescence of the tetracyclines, the developed TLC plates were dipped into a mixture of paraffin and hexane. The influence of the paraffin concentration on the fluorescence of TC, CTC, OTC and their impurities was studied. By dipping in a mixture of 20 to 40% paraffin in hexane, an optimal and stable enhancement was achieved. Sensitivity improvement was 2 to 5-fold. With more than 60% paraffin in hexane, the silica gel layer became apparently transparent and was no longer suitable for fluorescence densitometry. A mixture of 30% paraffin in hexane was used for further studies. The dipping time period was less critical: no noticeable difference on fluorescence intensity was observed between 1 and 3 s dipping.

The fluorescence stability of TC, CTC, OTC and their impurities on the TLC plate was also studied. During the first 10 min after introduction in the densitometer a moderate increase of the fluorescence intensity (up to 150%) was observed and then a stable fluorescence was achieved. This phenomenon was probably due to the warming-up effect of the plate after being inserted into the densitometer since it was observed on all the plates stored at room temperature no matter how long the plate had been dipped with paraffin. On a slightly warmed-up plate in an oven at 40 °C, stable fluorescence was obtained from the beginning. In order to improve the method repeatability, scanning the whole plate was not initialised until stabilised signals were obtained by repeatedly scanning one track.

Results for the calibration curves are summarised in Table 1. Mean peak area from ratio analyte: IS from the indicated number of tracks was used to construct the slope, intercept, correla-

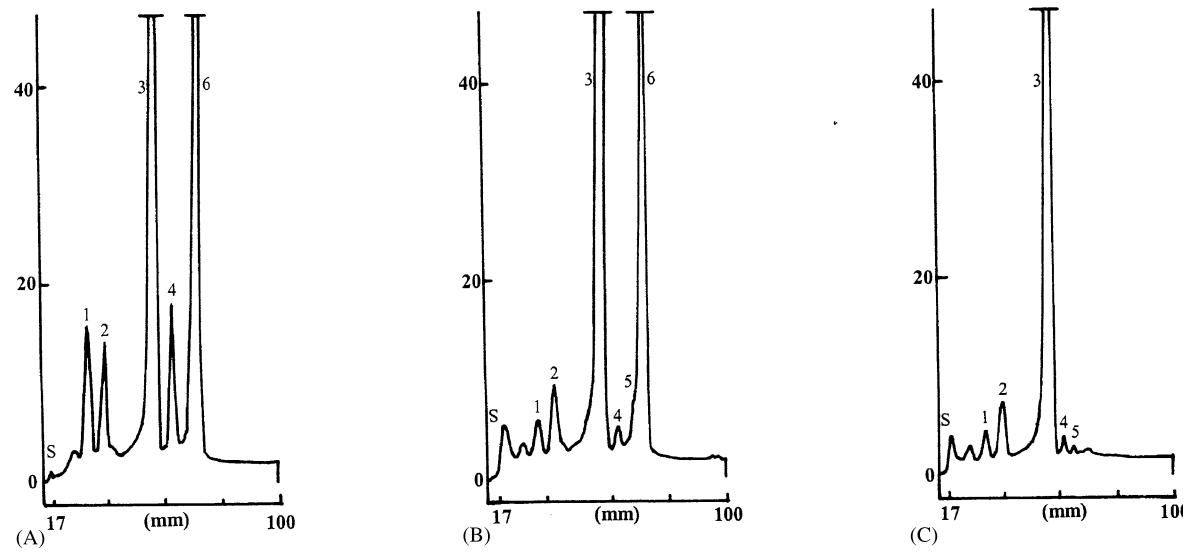


Fig. 2. Typical chromatograms obtained by the proposed TLC method for the assay and purity control of tetracycline in animal feeds. (A) Reference solution for the assay and purity control, (B) sample solution with internal standard (IS), (C) sample solution. See Section 2 for conditions. Sample: TLC·HCl-S3. Peak identity: 1 = ETC, 2 = EATC, 3 = TC, 4 = ATC, 5 = ADTC, 6 = β-APOTC (IS). S, starting point.

Table 2
Assay and purity control of TC in animal feeds by TLC with fluorescence densitometry

| Sample | Claimed content (TC·HCl μg/g) | (%) Recovery | | (%) Against claimed TC·HCl (400 μg/g) | | | |
|---|---|---|---|---|---|---|---|
| | | At 600 μg/g (+50%) | At 800 μg/g (+100%) | ETC·HCl | EATC·HCl | TC·HCl | ATC·HCl |
| TC·HCl-S1 | 400 | 78.1 (3.6) | 72.6 (5.0) | 6.0 | < 0.2 | 89.0 (1.6) | 0.3 |
| TC·HCl-S2 | 400 | 74.6 (5.0) | 73.1 (4.2) | 4.0 | 1.3 | 109.4 (1.0) | 0.8 |
| TC·HCl-S3 | 400 | 75.4 (1.0) | 80.1 (5.6) | 3.3 | 5.5 | 96.4 (4.5) | 0.8 |

The values reported are the means of total 12 determinations (two independent extracts × six spots for each extract). Relative standard deviation (R.S.D.) is mentioned in parentheses.

tion coefficient and standard error of estimate. Good linearity was obtained in the range examined. The limit of quantitation (LOQ) was 0.2 ng per spot for TC, CTC and their impurities, which corresponded to 0.2% of the sample load. For OTC and its impurities, the LOQ was 0.1 ng per spot, corresponding to 0.1% of the sample load. The fluorescence of the tetracyclines is stronger at pH 9.0 than at pH 8.0.

### 3.2. Assay and purity control of TC in animal feeds

Since blank control animal feeds of the same composition were not available to us, the determination of TC·HCl in animal feeds was performed using a standard addition method. Known amounts of TC·HCl were fortified into the animal feeds and recovery was determined. Thanks to the compactness of the spots, obtained for tetracy-

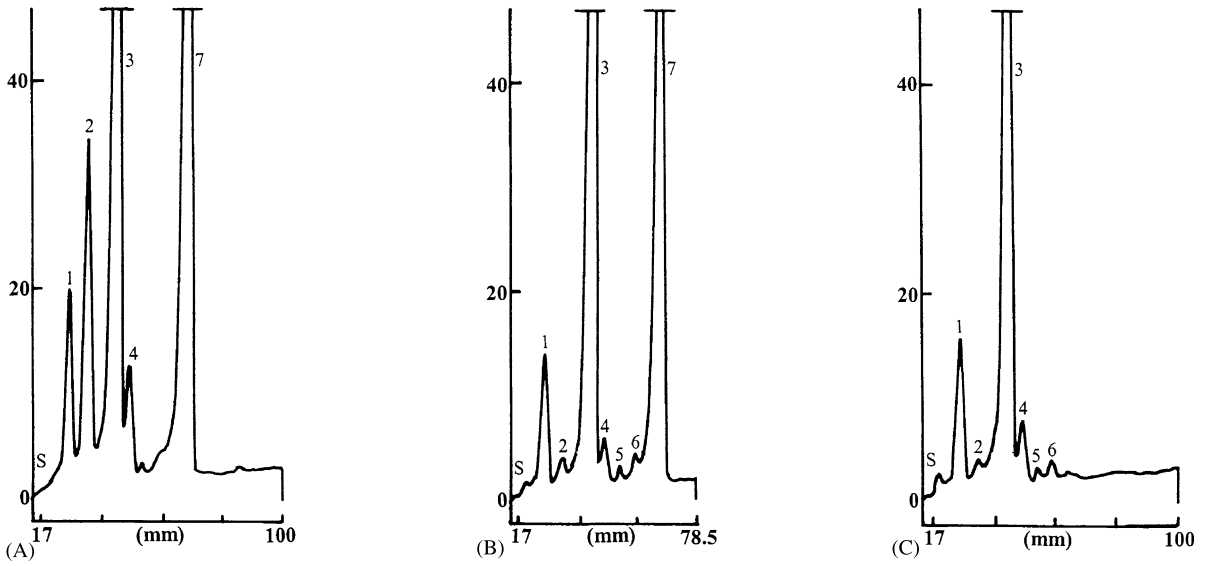


Fig. 3. Typical chromatograms obtained by the proposed TLC method for the assay and purity control of CTC in premix. (A) Reference solution for the assay and purity control, (B) sample solution with internal standard (IS), (C) sample solution. See Section 2 for conditions. Sample: CTC·HCl-S2. Peak identity: 1 = ECTC, 2 = DMCTC, 3 = CTC, 4 = TC, 5 = ADCTC, 6 = ADTC, 7 = β-APOTC (IS). S, starting point.

clines and their impurities, they were well separated from each other. The separation was very similar to what we had described for the semi-quantitative analysis of bulk tetracyclines where the photography of the TLC spots was shown [15]. Fig. 2 shows a typical chromatogram for the determination of TC·HCl in animal feeds. The identity of the spots was confirmed by comparison of their $R_f$ values and colours with those of reference substances. Spots of TC and ETC showed yellowish green fluorescence while ATC and EATC spots had orange fluorescence. When added to the animal feeds, TC, ETC, ATC and EATC coeluted with the spots to be identified. No other unidentified fluorescence spots were observed.

Results for the determination of TC in three animal feeds are reported in Table 2. The recovery of 70–80% for TC was comparable to results (71–100%) in literature, where the McBuffer was used to extract TC [11]. Although the McBuffer may give slightly better recovery, the aqueous nature of the McBuffer buffer and its incompatibility with the TLC prevented us from using it for the extraction. The extraction solution used here, acidified methanol, was easily evaporated once applied onto the TLC plate and, therefore, was more suitable for the TLC application. The mean recovery of adding 50 and 100% of the label claimed amount of TC·HCl was used to calculate the TC·HCl content. Since the exact composition of the feeds is not always known to the testing laboratory, possible matrix mismatches should be carefully evaluated, when the extracts are measured against reference solutions. Determination of recovery for each sample (standard addition method) was, therefore, the approach of choice to alleviate the potential matrix mismatch issue. Recoveries for the impurities were not determined and the calculation was based on the mean recovery of TC. TC stability during the extraction process was indicated by the identical amounts of ETC, EATC and ATC found in the original sample and in the samples fortified with 50 and 100% of the label claimed amount of TC·HCl.

From Table 2, it is obvious that degradation products of TC were present in various but significant amounts in the animal feeds. ETC·HCl ranges from 3.3 to 6% in those animal feeds containing 0.4 mg/g TC·HCl. EATC seems to be a

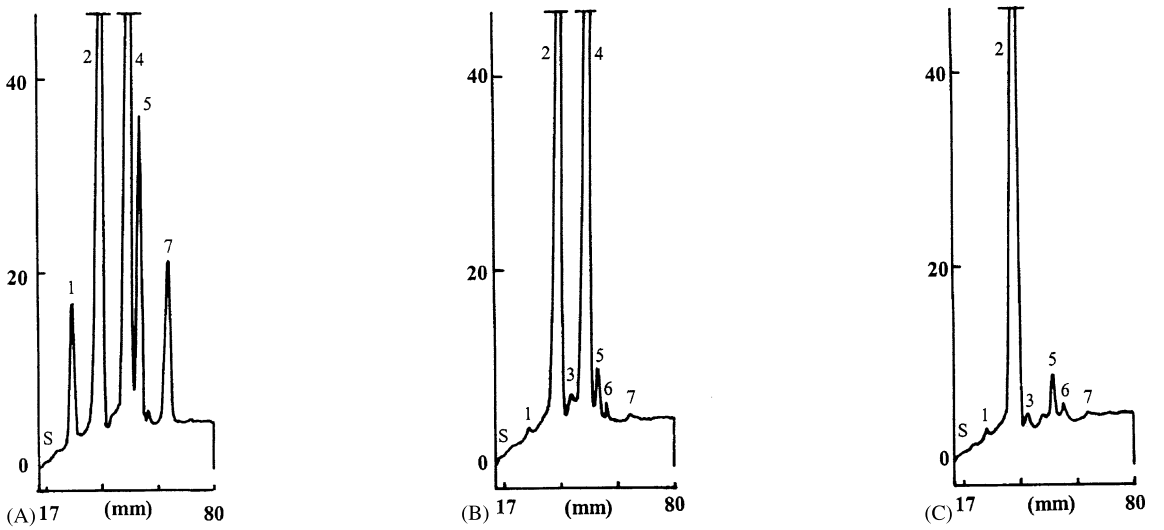


Fig. 4. Typical chromatograms obtained by the proposed TLC method for the assay and purity control of OTC in premix. (A) Reference solution for the assay and purity control, (B) sample solution with internal standard (IS), (C) sample solution. See Section 2 for conditions. Sample: OTC-S1. Peak identity: 1 = EOTC, 2 = OTC, 3 = α-APOTC, 4 = MTC (IS), 5 = ADOTC, 6 = β-APOTC. S, starting point.

Table 3
Assay and purity control of TC, CTC and OTC in premixes by TLC with fluorescence densitometry

| Sample | Claimed content (mg/g) | (%) Against claimed | | | |
|---|---|---|---|---|---|
| | | ETC·HCl | EATC·HCl | TC·HCl | ATC·HCl |
| TC·HCl-S4 | 40 | 3.0<br>ECTC·HCl | <0.2<br>DMCTC·HCl | 79.2 (2.7)<br>CTC·HCl | <0.2<br>TC·HCl |
| CTC·HCl-S1 | 200 | 2.6 | 0.7 | 99.2 (3.9) | 2.6 |
| CTC·HCl-S2 | 100 | 5.1<br>EOTC | 0.4<br>OTC | 87.2 (2.8)<br>TC | 4.1<br>APOTC[a] |
| OTC-S1 | 100 | 0.4 | 97.3 (3.5) | 0.4 | 0.3 |

The values reported are the means of total 12 determinations (two independent extracts × six spots for each extract). R.S.D. is mentioned in parentheses.

[a] Includes both α-APOTC and β-APOTC.

more important impurity in TC·HCl animal feeds than ATC. Up to 5.5% of EATC·HCl was found in one sample.

### 3.3. Determination of TC, CTC and OTC in premixes

All tetracyclines were sufficiently extracted from premixes by ultrasonication with acidified methanol. Complete recovery (>98%) was achieved for the samples spiked with 50 or 100% of tetracyclines against label. Therefore, measurement of tetracyclines using standard addition method was unnecessary. The analytes in the premixes were directly calculated against those in the reference solutions. Figs. 3 and 4 show typical chromatograms for the determination of CTC·HCl and OTC in premixes, respectively. Under these TLC

conditions, no keto-enol transformation was observed and, therefore, nice, compact spots were observed for both CTC and ECTC. The use of acidified methanol also prevented the degradation of CTC into ECTC. This degradation was at a very significant magnitude (about 30%) when a McBuffer was used to extract CTC [12]. A chromatogram very similar to that in Fig. 2 was obtained for TC·HCl in premixes. The identity of the spots was confirmed by comparing their $R_f$ values and colours with those of reference substances. Results for the assay and purity control of TC·HCl, CTC·HCl, and OTC in premixes are shown in Table 3. The amount of TC·HCl in the premix was only 79.2% against the claimed content. The low TC·HCl content is probably not due to the degradation upon storage since only very low amounts of impurities were present in this sample. Coincidentally, using a different method, the amount of tylosin in this premix was also found to be only 78% against its label.

## 4. Conclusion

A TLC densitometric method with fluorescence detection has been developed for the assay and purity control of TC, CTC and OTC in animal feeds and premixes. This method is sensitive and easy to use.

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