ARTICLES

Determination of Tetracycline and Streptomycin in Mixed Fungicide Products by Capillary Zone Electrophoresis

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A method of capillary zone electrophoresis (CZE) was used to determine tetracycline and streptomycin content in commercial agriculture products. The results indicated that this method was capable of analyzing the mixed fungicide in formulated products with instrument detection limit (IDL) of 0.50 μg/mL and a method detection limit (MDL) of 0.52 μg/mL for tetracycline, and IDL of 1.00 μg/mL and MDL of 1.22 μg/mL for streptomycin. Precision expressed by relative standard deviation (RSD) ranged from 1.44 to 4.37% of tetracycline and 1.00 to 4.20% of streptomycin. Recoveries were in the region of 98.2−102.5% for tetracycline and 95.3−103.0% for streptomycin. The low detection limit, the low RSD values, and the high percentage of recovery confirmed that the CZE technique is a sensitive and selective method. And the CZE method can analyze both tetracycline and streptomycin at the same time without complicated extraction and further derivative reaction.

Keywords: *CZE; antibiotic fungicide; detection limit; recovery*

INTRODUCTION

Tetracycline [4*S*-(4α,4aα,5aα,6*β*,12aα)-4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide] (Figure 1A), is prepared by the catalytic reduction of chlorotetracycline (aureomycin) (*1*) which is the metabolite of the fungus *Streptomyces aureofciens*. Tetracycline is not only a clinically used compound, but also an important fungicide used in the preservation of harvested fruits and vegetables and extermination of insect pests, and as an animal feed supplement (*2*).

Streptomycin [*O*-2-deoxy-2-(methylamino)-α-L-glucopyranosyl-(1→2)-*O*-5-deoxy-3-*C*-formyl-α-L-lyxofuranosyl-(1→4)-*N*,*N*′-bis (aminoiminomethyl)-D-streptamine] (Figure 1B), is the metabolite of the fungus *Streptomyces griseus* (*3*). It is an aminoglycoside antibiotic used as a veterinary drug, especially in bee-keeping, for the treatment of bacterial diseases (*4*).

Several techniques have been employed to assay tetracycline: for example, microbiological assay (*2*, *5*, *6*), continuous-flow chemiluminometric method (*7*), spectrophotometry and flow injection method (*8*), flow injection with chemiluminescence detection (*9*), and metal chelate affinity chromatography (*10*). The bioassay method has its limitations in both selectivity and efficiency. It cannot distinguish the actual antibiotic from false products and is time-consuming (*11*, *12*).

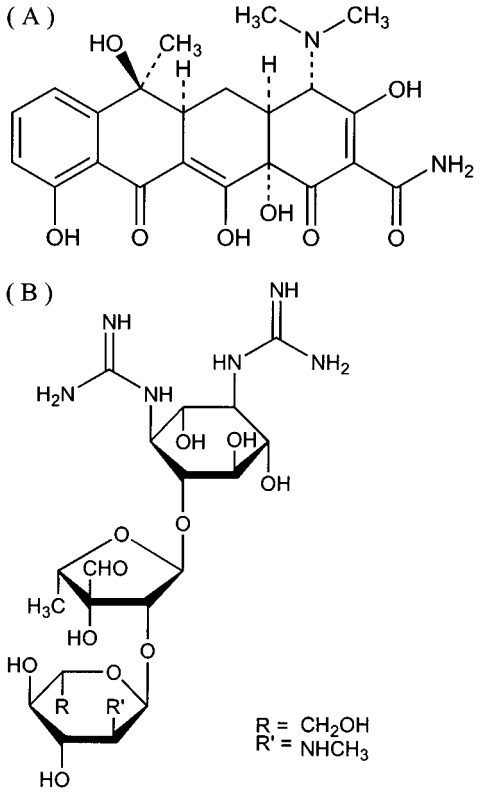

Figure 1. Chemical structures of tetracycline (A) and streptomycin (B).

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Liquid chromatographic methods for tetracycline determination have also been developed in recent years (*13*−*17*).

In recent years, some capillary electrophoresis (CE) methods for tetracycline analysis have been reported (*18*−*21*). However, there is no complete qualitative and quantitative methodology for the determination of tetracycline in commercial agriculture formulated products.

For streptomycin analysis, nonchromatographic methods are not specific enough (*22*), such as spectrofluorometric assay (*23*) and the electrophoretic method (*24*). Immunological assays are very sensitive and can be used as screening tests, but results obtained with these assays must always be confirmed by a more selective method such as chromatography (*4*). Many liquid chromatographic methods have been developed for the determination of streptomycin in animal tissue (*25*), milk (*26*, *27*), and food (*4*).

The official method of analysis for tetracycline and streptomycin is based on a LC method, but the large amounts of volatile organic solvents used in the mobile phase are undesirable to most analysts. Thus, a fast and efficient method is desirable for routine analysis.

Use of CE methods to analyze streptomycin in different matrixes have been reported recently, such as in eggs yolk (*28*) and in distilled water (*29*). As with tetracycline, there is no complete report on the determination of streptomycin in commercial agriculture formulated products.

Capillary zone electrophoresis is an efficient separation technique in which charged solutes are differentially transported through open capillaries under the influence of an applied field (*30*). In our previous work the CZE technique has successfully separated the antibiotic fungicides blasticidin S (*11*) and kasugamycin (*12*). The physical and chemical properties of tetracycline and streptomycin, particularly their multiple ionic natures and water solubilities, make them suitable for CZE analysis. Therefore, this paper reports a simple CZE method for the determination of tetracycline and streptomycin in commercial agriculture formulated products.

MATERIALS AND METHODS

Standards and Samples. Standards of tetracycline, MW = 444.4 (hydrochloride salt, MW = 480.9, purity > 95%) and streptomycin, MW = 581.6 (sulfate salt, MW = 1457.3, purity > 99.9%) were purchased from Sigma (St. Louis, MO) and Riedel-deHaën (Germany), respectively. Commercial formulated samples were purchased from markets in different areas of Taiwan. All samples were in wettable powder formulation (WP) and contained 1.0% tetracycline and 9.0% streptomycin.

Solvents and Chemicals. All solvents and chemicals were analytical grade. Tetracycline contains three distinct functional groups, i.e., the tricarbonyl methane group with pK_a 3.30, the ammonium group with pK_a 7.68, and the phenolic diketone group with pK_a 9.69 (*31*); thus, an aqueous running phosphate buffer of pH 2.5 (0.1 M, catalog #148−5011) was used, and the running buffer and the wash solution (catalog #148−5022) were purchased from Bio-Rad (Hercules, CA). Potassium dihydrogen phosphate (KH_2PO_4) and disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$) were purchased from Riedel-deHaën. The dilution buffer was composed of 10 mM KH_2PO_4 and 10 mM Na_2HPO_4 (pH 7.0). All buffer solutions were filtered through a 0.45-μm nylon filter (Lida Manufacturing Corp., Kenosha, WI).

CZE Analysis. The CZE method was performed using a Biofocus 3000 automated capillary electrophoretic apparatus. A Biofocus cartridge capillary (30 cm × 50 μm i.d., coated) was employed. The column temperature was 20 °C. A regulated dc power supply delivering 18 kV was used to provide high voltage between the ends of the column filled with running buffer. The sample was introduced into the capillary vessel using pressure injection mode at 5 psi × sec, and the volume of sample introduced into the capillary vessel during pressure injection was calculated to be 11.75 nL (*11*, *32*). The elution of a solute was monitored by an on-column UV−vis detector (195 nm) at the negative pole (Figure 2).

Column efficiency is expressed in terms of theoretical plates (N) (*11*, *30*):

$$N = 5.5\,(t_r/W_{1/2})^2 \quad (1)$$

where t_r is the retention time of the peak, $W_{1/2}$ is the width at half peak height.

Capillary conditioning between runs was conducted by rinsing with wash solution (1 min), H_2O (1 min), and running buffer (1 min) at high-pressure mode.

The reproducibility of retention time (t_r), peak area, linearity, and detection limit were used to evaluate the selectivity, sensitivity, and the reliability of the CZE method.

CZE Calibration Curve. Standards of tetracycline and streptomycin (0.0100 g) were weighed separately into a 10-mL volumetric flask and diluted with dilution buffer to obtain stock standard solution of 1000 μg/mL. Both the stock standard solutions were diluted with dilution buffer in sequence to obtain the final working standard solutions of concentrations 10.0, 20.0, 30.0, 40.0, and 50.0 μg/mL for tetracycline and 50.0, 100.0, 150.0, 200.0, and 250.0 μg/mL for streptomycin. The different working standard solutions were used for the calibration curve. Three replications were conducted, and linear regression was used to determine the suitability of the CZE method.

Sample Preparation for CZE Analysis. A proper amount of sample was weighed into a 10-mL volumetric flask and diluted with dilution buffer. The mixture was mixed with a mixer (Thermolyne 37600 , Dubuque, IA) for 1 min and a proper aliquot was injected into an autosampler vial through a 0.45-μm nylon syringe filter for CZE analysis.

Recovery. Because the accurate compositions of different commercial formulations were unknown, the effects of formulations on the CZE approach were analyzed by recovery. The recoveries of tetracycline and streptomycin from formulated products were determined by pipetting 0.1-mL aliquots of tetracycline and streptomycin stock standard solutions (1000 μg/mL) into each of the formulated samples (spiked samples). Another portion of the formulated samples were served as blanks (nonspiked samples). Each spiked and nonspiked sample was then mixed separately (1 min) before analyzing, and the recoveries were calculated as the difference of the amount of active ingredient found in the spiked sample and the nonspiked sample, expressed as a percentage of the amount of active ingredient added.

Limit of Detection. The IDL was determined by injecting a low concentration of working standard solution to produce a signal that was about three times the signal-to-noise ratio (*33*). The concentration of working standard solution that corresponds to 5.0 times IDL is used to determine the MDL. Repeat CZE analysis (seven times) produced data for the standard deviation (SD); 3 SD was used as the MDL. Precision expressed by RSD was used in judging the acceptability of the method. Three replications were conducted in all analyses.

RESULTS AND DISCUSSION

Chromatogram of Tetracycline and Streptomycin Standard. Typical electropherograms of tetracycline standard, streptomycin standard, commercial products, and dilution buffer are shown in Figure 3. It was demonstrated that excellent selectivity could be obtained with phosphate buffer at pH 2.5. The pH buffer for the best separation of streptomycin by CE method

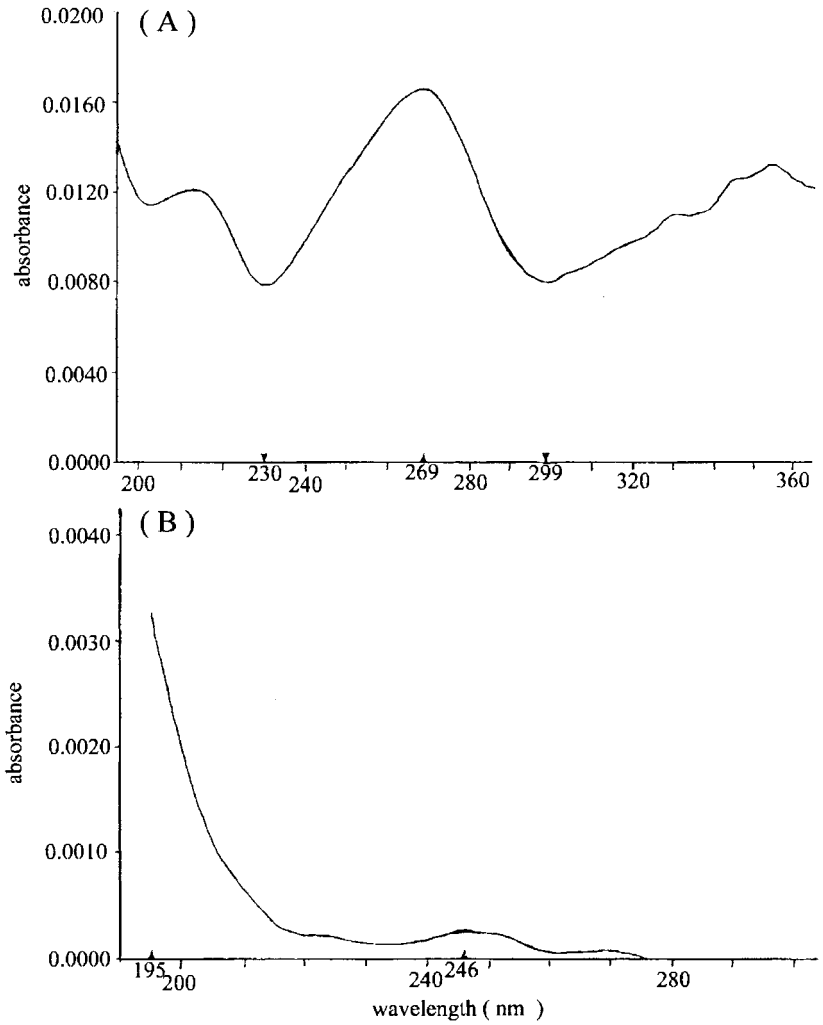

Figure 2. The UV spectra of tetracycline (A) and streptomycin (B).

was reported using phosphate−borate buffer solution at pH 6.35 (*28*), and the pH buffer for the best separation of tetracycline by CE method was reported using sodium carbonate−EDTA buffer at pH 10.75 (*20*). These reports indicated that an acid buffer was used for streptomycin separation, and an alkaline buffer was required for tetracycline separation. However, these two different buffers would make the simultaneous analysis of streptomycin and tetracycline difficult to conduct. Results achieved in this study showed that the simultaneous analysis of streptomycin and tetracycline would be possible if phosphate buffer of pH 2.5 was used. The retention times of tetracycline standards were consistent ranging from 6.15 to 6.20 min with RSD values ranging from 0.10 to 0.63% (Table 1). The retention times of streptomycin standards were also consistent ranging from 2.76 to 2.83 min with RSD values ranging from 0.21 to 1.36% (Table 1). The data indicated that the retention time was not influenced by the concentration selected from 10.0 to 50.0 μg/mL for tetracycline and 50.0 to 250.0 μg/mL for streptomycin (Table 1).

Column Efficiency. The retention time (t_r) and the peak width ($W_{1/2}$) were used to calculate the column efficiency expressed in terms of theoretical plates (N) (eq 1), and the column efficiencies of tetracycline and streptomycin were calculated to be 9306 ($t_r = 6.17$, $W_{1/2} = 0.15$), and 12065 ($t_r = 2.81$, $W_{1/2} = 0.06$), respectively. The results indicated that the column efficiency of streptomycin was higher than that of tetracycline. This may be explained by the chemical structure: the structure of an aminoglycoside antibiotic being more easily protonated than the structure of a fused four-member ring. The column efficiencies of different antibiotic fungicides previously analyzed by the CE method were 8078 for blasticidin S (*11*), 16589 for kasugamycin, (*12*), and 8378 for validamycin A (*12*).

Sensitivity of the CZE Method. The IDL, defined as 3 times the baseline noise, was estimated at 0.50 μg/mL (or 5.88 pg/injection) for tetracycline and 1.0 μg/mL (11.75 pg/injection) for streptomycin. The MDL was calculated to be 0.52 μg/mL for tetracycline and 1.22 μg/mL for streptomycin. The lower MDL of streptomycin than that of tetracycline could be explained by the lower ultraviolet adsorption efficiency of streptomycin than that of tetracycline at 195 nm selected (Figure 2). According to the Lambert−Beer law, the relationship between concentration (b), length of the light path (l), and the light absorbance (A) of a particular substance is expressed as $A = abl$, where a = extinction coefficient. If the concentration is given in g/mL, a becomes the specific extinction coefficient (a_s), and the molar extinction coefficient (a_m) is equal to a_s times molecular weight of that particular substance. Therefore, the specific extinction coefficient (a_s) and molar extinction coefficient (a_m) of tetracycline and streptomycin at 195 nm would be 28 and 12443 ($A = 0.014$, $b = 100$ μg/mL, $l = 50$ μm)

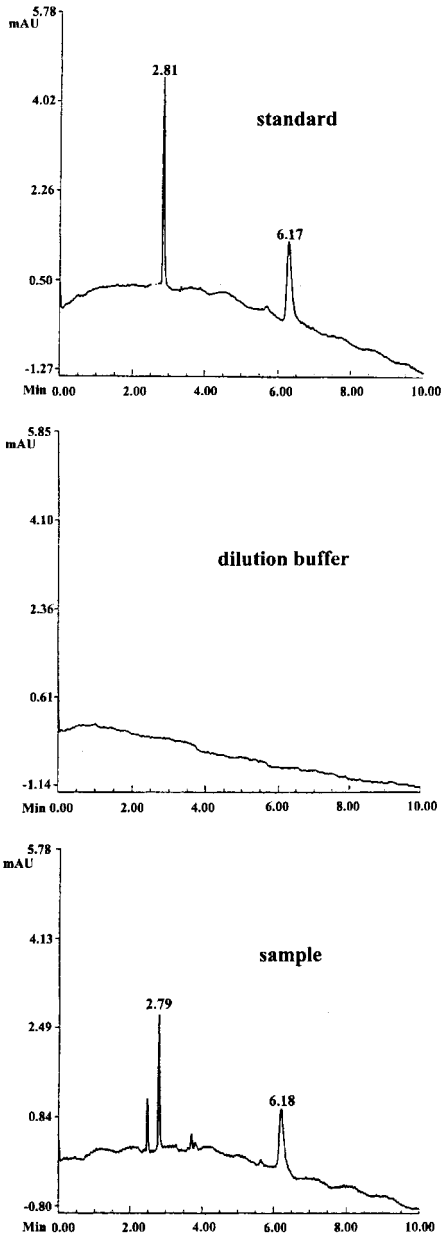

Figure 3. Typical electropherograms of tetracycline standard (t_r = 6.17, 588 pg), streptomycin standard (t_r = 2.81, 2938 pg), dilution buffer, and commercial products.

Table 1. Precision of Retention Time of Tetracycline Standard and Streptomycin Standard Analyzed by CZE Method

concentration (μg/mL)	retention time[a] (min)	precision (%, RSD)
	Tetracycline Standard	
10.0	6.20	0.10
20.0	6.20	0.10
30.0	6.17	0.38
40.0	6.16	0.38
50.0	6.15	0.63
	Streptomycin Standard	
50.0	2.83	0.61
100.0	2.77	0.21
150.0	2.79	1.36
200.0	2.76	1.16
250.0	2.76	1.05

[a] Mean of three measurements.

and 6.6 and 3838 (A = 0.0033, b = 100 μg/mL, l = 50 μm), respectively.

Table 2. Determination of Tetracycline and Streptomycin Content in Commercial Products (10% WP) by CZE Method

commercial product[a] (% w/w, claimed)	CZE method (% w/w[b], RSD)	tolerance (%)
	Brand A. 10% WP	
tetracycline (1.0%)	0.9, 1.44	0.6−1.2
streptomycin (9.0%)	3.5, 1.00	7.2−10.8
	Brand B. 10% WP	
tetracycline (1.0%)	1.1, 4.31	0.6−1.2
streptomycin (9.0%)	3.8, 4.20	7.2−10.8
	Brand C. 10% WP	
tetracycline (1.0%)	0.8, 4.37	0.6−1.2
streptomycin (9.0%)	3.5, 1.22	7.2−10.8

[a] 10% WP contained tetracycline 1.0%, and streptomycin 9.0%. A, B, and C were products of different brands. [b] Mean of three measurements.

Table 3. Recovery of Tetracycline and Streptomycin in Formulated Products by CZE Method

formulation[a] (% w/w)	recovery (%)		
	range	average[b]	RSD
	Brand A. 10% WP		
tetracycline (1.0%)	95.5−101.1	98.2	2.90
streptomycin (9.0%)	102.3−103.7	103.0	0.95
	Brand B. 10% WP		
tetracycline (1.0%)	102.3−102.6	102.5	0.21
streptomycin (9.0%)	94.1−102.5	99.2	4.49
	Brand C. 10% WP		
tetracycline (1.0%)	98.0−103.9	101.0	2.92
streptomycin (9.0%)	93.7−96.8	95.3	2.33

[a] 10% WP contained tetracycline 1.0%, and streptomycin 9.0%. A, B, and C were products of different brands. [b] Mean of three measurements. [c] Mean of three measurements

Determination of Tetracycline and Streptomycin Content in Commercial Formulated Products. The official tolerance for commercial product is +20% to −40% for an active ingredient within 0.1% to 1.0% concentration, and +20% to −20% for an active ingredient within 1.0% to 10.0% concentration. Thus, the tolerance for 1.0% tetracycline ranged from 1.2 to 0.6 and the tolerance for 9.0% streptomycin ranged from 10.8 to 7.2%. The CZE analyses showed that the contents of tetracycline in all three samples were within the official tolerance levels (Table 2), but the contents of streptomycin in all three samples were lower than the limit of tolerance. The typical electropherograms of standards, commercial products, and dilution buffer are shown in Figure 3.

Precision of the CZE Method. The precision of the CZE method in the analysis of tetracycline and streptomycin in the mixed fungicides was determined by the RSD values (Table 2). The results indicated that the RSD values ranged from 1.44 to 4.37% for tetracycline and 1.00 to 4.20% for streptomycin (Table 2). Similarly, all the RSD values were less than 10%, indicating that the precision of the CZE method was excellent (RSD less than 15%). Precision of the assay for streptomycin in formulated products was better than the precision of the assay for streptomycin in egg yolks previously reported (*28*), because the RSDs in egg yolks ranged from 2.7% to 13.8% and were higher than the RSDs in the commercial products. The method therefore can be used for tetracycline and streptomycin analysis.

Influence of Formulations on the CZE Performance. The influence of formulation on the CZE method was validated by the recovery. It was found that

Table 4. Corrected Concentration of Tetracycline and Streptomycin in Formulated Products Analyzed by the CZE Method

formulation[a] (% w/w)	concentration (%)		
	calculated[b]	corrected[c]	difference[d]
	Brand A. 10% WP		
tetracycline (1.0%)	0.87	0.89	−2.30
streptomycin (9.0%)	3.46	3.35	3.18
	Brand B. 10% WP		
tetracycline (1.0%)	1.12	1.09	2.68
streptomycin (9.0%)	3.84	3.88	−1.04
	Brand C. 10% WP		
tetracycline (1.0%)	0.78	0.77	1.28
streptomycin (9.0%)	3.55	3.72	−4.79

[a] 10% WP contained 1.0% tetracycline and 9.0% streptomycin. A, B, and C were products of different brands. [b] Concentration calculated from calibration curve. [c] Corrected concentration = (calculated concentration/average recovery) × 100%. [d] Difference = [(calculated concentration − corrected concentration)/calculated concentration] × 100%.

the average recoveries for tetracycline ranged from 98.2% for sample A to 102.5% for sample B, with RSD values in the region of 0.21−2.92%; whereas the average recoveries for streptomycin ranged from 95.3% for sample C to 103.0% for sample A, with RSD values in the region of 0.95−4.49% (Table 3). The data indicated that there was no matrix interference.

The recoveries of streptomycin from commercial products were also better than the recoveries of streptomycin from egg yolks previously reported (*28*), and the average recovery of streptomycin from egg yolks was 71.8%. The concentration of each sample corrected for recovery showed that the contents of tetracycline was in the official tolerance level, but the contents of streptomycin for all three products were below the tolerance level (Table 4). The results were also confirmed by the HPLC method (data not shown).

Conclusion. The new CZE method is a good method with high precision, accuracy, linearity, and sensitivity. There was no matrix interference observed. In addition, this CZE method is practical because it not only provides a direct analysis procedure without extraction, but also offers the opportunity to save the labor and the chemicals. Another advantage is that the CZE method can analyze tetracycline and streptomycin in one run.

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