A rapid and simple procedure was developed for the determination of copper, iron, manganese, and zinc in animal feeds using an identical flow injection spectrophotometric manifold but different chromogenic reagents and different detection wavelengths. Bis(cyclohexanone)oxalydihydrazone, formaldoxime, 1,10-phenanthroline, and xylenol orange were adopted as chromogenic reagents for Cu, Mn, Fe, and Zn, respectively. Detection conditions such as manifold parameters, buffer pH, reagent concentration, temperature, and acidity of sample solution were optimized. Analytical characteristics of the method and interference of metal ions commonly present in feeds were studied. By changing the reagents and detection wavelengths, which can be done quickly, the proposed low cost flow injection system can determine Cu, Fe, Mn, or Zn in the range of 0.5–10 mg/L with a sampling throughput of 120/h.

Flow injection spectrophotometric methods for determination of Cu have included adoption of (2-(2-thiazo)-4-methyl-5-sulfo-methyl-aminobenzoic acid) (3), dithiocarbamate (4), N,N-bis(2-hydroxy-5-sulfophenyl)-c-cyanoformazan (5), bathocuproine (6), 2,9-dimethyl-1,10-phenanthroline (7), and bis(cyclohexanone)oxalydihydrazone (Cuprizone; 8) as chromogenic reagents in aqueous solutions. Diethyl-dithiocarbamate (DDTC; 9–11) and bis(acetyl-acetone)ethylenediaminate (12) were also used to determine Cu after extraction into organic solvents. Sensitive methods based on the catalytic effect of Cu(II) on oxidation of hydroquinone by hydrogen peroxide (13), oxidative coupling of 3-methyl-2-benzothiaolinone with chromotropic acid (14), and oxidative coupling of N-phenyl-phenylenediamine with m-phenylenediamine (15) were also developed. Among these methods, the Cuprizone (8) method is desirable for its moderate reaction and high interference tolerance.

Spectrophotometric methods for determination of Mn were reviewed by Chriswell et al. (16) but only a few of them were applied in flow injection analysis (FIA). Mesquita et al. (17) reported a method based on permanganate formation with high selectivity in soil, plant, and rock analysis; however, the method needs high temperature (95°C), which is inconvenient in FIA. FI spectrophotometric methods based on Mn-catalyzed reactions (18–21) reportedly had higher sensitivity, but their reaction time was relatively long and their selectivity was not good. The formaldoxime method was applied to FI determination of Mn in plant digest (22) because of its high sensitivity and rapid formation of a stable colored complex.

Although many other reagents such as N-phenylbenzohydroxamic acid (23), bathophenanthroline (24), ferrozine (25, 26), and 2-(5-Nitro-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino)phenol (Nitro-PAPS; 27) have been adopted as color reagents, 1,10-phenanthroline was the
most widely used chromogenic reagent for FI determination of Fe because of its superiority (28) over the others. 

FI spectrophotometric methods for determination of Zn were developed on the basis of its color reaction with xylenol orange (29, 30), PAN (31), 5-Br-PAN (32), 8-(benzenesulfonamido)quinoline (33), and PAR (34); however, because of interference by metal ions, these methods required appropriate masking or preseparation of samples. Koupparis et al. (35) reported a method based on the zincon method with differential demasking of cyanide metal complexes with cyclohexanone, but cyanide, a very toxic agent, had to be used.

FI spectrophotometric methods for simultaneous determination of Cu and Fe (36), Cu and Zn (37, 38), and Fe and Zn (39) were reported, but their applicability to routine analyses was limited by the need for special instruments or date process procedures.

In the present study, a simple and practical FI spectrophotometric procedure was developed for determination of Cu, Mn, Fe, and Zn in feeds. The proposed method used an identical manifold but different detection wavelengths and color reagents. The chromogenic reagents adopted were Cuprizone, formaldoxime, 1,10-phenanthroline, and xylenol orange, respectively. By changing appropriate reagents and the detecting wavelength, which can be done quickly, the low cost FI system can detect the desired elements conveniently.

**Experimental**

**Reagents**

All chemicals were of analytical grade and distilled water was used throughout.

(a) Standard solution.—A 1000 mg/L Cu, Mn, Fe, and Zn mixing standard stock solution was prepared by dissolving 1 g pure Cu powder in 20 mL 7M nitric acid with heating. Then, 8.5 mL H₂SO₄ was added and the solution was heated to produce white mist. After cooling, 3.0761 g manganese sulfate monohydrate (MnSO₄·H₂O), 7.0226 g ammonium ferrous sulfate hexahydrate [(NH₄)₂Fe(SO₄)₂·6H₂O], and 4.3983 g zinc sulfate heptahydrate (ZnSO₄·7H₂O) were added and dissolved in ca 500 mL water. The solution was then diluted to 1000 mL with water. Working standards were obtained by appropriately diluting stock solution with 0.15M H₂SO₄.

(b) Carrier solution.—0.15M H₂SO₄.

(c) Reagents for Cu determination.—R1 contains 5% mass/volume (m/v) ammonium citrate, 7% (m/v) ammonium chloride, 4.8% (v/v) concentrated ammonia, and 1.2% (m/v) NaOH; R2 contains 0.15% (m/v) bis(cyclohexanone)oxalyl(dihydrazone (Cuprizone) and 5% (v/v) ethanol.

(d) Reagents for Fe determination.—R1 contains 10% (m/v) ammonium acetate, 12.5% (v/v) acetic acid, and 1.2% (m/v) NaOH; R2 contains 0.2% (m/v) 1,10-phenanthroline and 1% (m/v) ascorbic acid.

(e) Reagents for Mn determination.—R1 contains 2.7% (m/v) ammonium chloride, 20% (v/v) concentrated ammonia solution, 1.2% (m/v) NaOH, and 4% (m/v) ascorbic acid. R2 contains 7% (m/v) hydroxylammonium chloride and 3.5% (v/v) formaldehyde solution.

(f) Reagents for Zn determination.—R1 contains 40% (m/v) sodium acetate, 4% (v/v) acetic acid, and 5% (m/v) sodium thiosulfate; R2 contains 0.015% (m/v) xylenol orange and 4% (m/v) ammonium fluoride.

**Apparatus**

The FI manifold constructed for consecutive determination of Cu, Fe, Mn, and Zn is shown in Figure 1. Solutions were pumped with a self-developed peristaltic pump and samples were injected with a 16-way injection valve; a Model 721 spectrophotometer (Shanghai Third Analytical Instruments Factory, Shanghai, China) equipped with a self-made Z-shaped 10 mm flow cell (18 μL) was used as detector. Flow lines were made from 0.8 mm PTFE tubing, and Tygon pump tubes were used throughout. The reaction coil was placed in a small water bath filled with 45 ± 0.2°C recycled water supplied by a thermostat. A 586 computer installed with

Figure 1. Optimized manifold for the consecutive determination of Cu, Fe, Mn, and Zn in animal feeds. C, carrier solution; R1, R2, reagents; S, sample solution; MC, mixing coil (20 cm x 0.8 mm id); RC, reaction coil (150 cm x 0.8 mm id); P, peristaltic pump; T, thermostatic bath (45°C); D, spectrophotometer; W, waste.
self-developed software was used to control operation of the pump and injection valve, acquire absorbance data from the detector, and process the data to obtain results.

**Sample Preparation**

Add ca 3 mL water to accurately weighed (0.2–1.0 g) portion of feed sample, grind through 0.5 mm screen using a mill, and place in 250 mL Kjeldahl flask. Add 30 mL concentrated nitric acid, 1 mL concentrated perchloric acid, and 0.8 mL concentrated sulfuric acid. Heat gently until frothing ceases, and boil briskly until white mist is produced and ca 1 mL solution remains. Cool, add 30 mL water, and heat to boiling for 3 min; filter into 100 mL volumetric flask and dilute to volume with water. If feeds with low content of analytes are being analyzed, treat 2.5 g sample as above, but use 2 mL perchloric acid and 0.4 mL concentrated sulfuric acid, and dilute to 50 mL. Dilute aliquots with 0.15M H_2SO_4 if necessary.

**Results and Discussion**

**Optimization of Manifold**

The optimum manifold shown in Figure 1 was a compromised result for consecutive determination of Cu, Fe, Mn, and Zn. Because this study is focused on developing a method suitable for routine analysis, optimization was not performed simply to obtain the greatest peak height; calibration range, sample frequency, and system stability were all taken into consideration.

To obtain a more stable flow system for routine analysis, approximately equal flow rates were adopted for each of 2 confluence flow lines, i.e., the flow rate of carrier (C) equals that of reagent (R1), and the total flow rate of these 2 lines is close to that of reagent (R2). Because the solubility of Cuprizone is limited, high flow rate of R2 is necessary; otherwise the sensitivity of Cu determination might not be as high as desired.

In this study, the reaction coil (RC) length was optimized in the range of 100–250 cm. Results showed that the highest sensitivity was obtained at 150–200 cm for Cu determination, whereas there was no significant difference between peak heights in the range of 100–200 cm when Fe, Mn, or Zn was analyzed. Consequently, a reaction coil 150 cm long was used in this study. Injection volume was optimized from 25 to 250 µL, and results show that peak heights increased sharply to 150 µL, and then increased gradually. Broad peaks were produced above 150 µL, resulting in low sampling rate; therefore, a 150 µL injection volume was used.

**Optimization of Chemical Parameters**

Using the above optimized manifold and univariant method, chemical parameters such as reagent concentration, pH of buffer solution, acidity of sample solution, and temperature were optimized for each studied element (Table 1).

**Reagent Concentration**

For each determination, a solution of 0.15M H_2SO_4, which is equal to the sulfuric acid concentration in prepared sample solutions, was adopted as carrier to prevent refractive index difference between carrier and digested sample solutions. High concentration (capacity) buffer solutions were adopted to compensate for acid concentration variation during sample digestion, and 0.3M NaOH was added to the buffer solution to neutralize the 0.15M H_2SO_4 present in carrier and sample solutions.

The concentrations of Cuprizone and 1,10-phenanthroline were optimized in the range of 0.05–0.2%. Although peak heights of 10 mg/L Cu and Fe increase with up to 0.10% of color reagent, and then remain unchanged, 0.15% Cuprizone and 0.20% 1,10-phenanthroline were adopted in this study because of reports that higher concentrations increase interference tolerance. Maintaining mass concentration ratio of hydroxylammonium chloride and formaldehyde at 2:1, the

| Table 1. Optimal detection conditions and analytical characteristics of the methods |
|----------------------------------------|----------------|----------------|----------------|----------------|
| Method | Cu | Fe | Mn | Zn |
| Color reagent | Cuprizone | Ferroin | Formaldoxime | Xylenol orange |
| Detect wavelength (nm) | 600 | 510 | 460 | 570 |
| Buffer pH | 9.0 | 4.8 | 10.5 | 6.0 |
| Temperature (°C) | 45 | 45 | 45 | 45 |
| Linear range (mg/L) | 0–10 | 0–10 | 0–10 | Nonlinear |
| Regression equation | Slope (Abs/mg/L) | 0.0639 | 0.05002 | 0.05094 | — |
| | Intercept (Abs) | -0.001842 | 0.008863 | 0.01120 | — |
| | Correlation coefficient | 0.9994 | 0.9998 | 0.9998 | — |
| | Detection limit (mg/L) | 0.2 | 0.01 | 0.04 | — |
| | Determination range (mg/L) | 0.5–10 | 0.5–10 | 0.5–10 | 0.5–10 |
| | RSD (n = 11; %) | 0.5 | 0.5 | 1.0 | 0.9 |
| | Sample throughput/h | 120 | 120 | 120 | 120 |
formaldehyde concentration was optimized from 1 to 7%, and peak heights of 10 mg/L Mn increased up to 3.0% formaldehyde, then remained unchanged. Consequently, 3.5% formaldehyde and 7% hydroxylammonium were adopted. Experiments have shown that peak heights of 10 mg/L Zn increase as xylenol orange concentration increases from 0.005 to 0.012% and decrease above 0.012%; thus, 0.012% xylenol orange was used in the following studies.

**Buffer pH**

To investigate the effect of buffer pH on the determination of each analyte, a series of pH 4.0–7.0 acetate buffer and pH 8.5–11.0 ammonium buffer solutions was used, and 1.2% (m/v) NaOH was added to each prepared buffer solution to neutralize the 0.15M H₂SO₄ present in carrier and sample solutions. Figure 2 demonstrates that the peak heights of 5 mg/L Cu(II) increased as pH increased to 9.0, then decreased sharply, whereas peak heights of Mn(II) increased sharply as buffer pH increased to 10.0, then decreased very gradually. Consequently, pH 9.0 and pH 10.5 ammonium buffer solutions were adopted for determination of Cu and Mn, respectively.

The influence of buffer pH on determination of 5 mg/L Zn(II) was investigated in the range of pH 5.2–6.3. As shown in Figure 2, the Zn(II) determination is very pH dependent. A high concentration of pH 6.0 acetate buffer solution was used in this study.

Although the Fe(II)-1,10-phenanthroline method was reported to be pH independent in the range of pH 2–9, a pH 4.8 acetate buffer solution was adopted in this study because the method reportedly has a higher interference tolerance at this pH.

**Acidity of Sample Digest Solution**

During sample digestion, acid concentration may vary, depending on the amount of sulfuric acid necessary to digest organic matter and loss through evaporation. Experiments were therefore conducted to determine the effect of various concentrations of sulfuric acid in sample solution. Figure 3 indicates that for Cu, Fe, and Mn determination no significant differences were shown in the range of 0.1–0.18M H₂SO₄, but peak heights of Zn decreased as sulfuric acid concentration increased in this range. The peak heights of 5 mg/L Zn(II) in 0.14 and 0.16M H₂SO₄ were 108 and 98% of that in 0.15M H₂SO₄, respectively.

**Temperature**

Temperature was optimized in the range of 25–55°C. Figure 4 illustrates that peak heights of Fe, Mn, and Zn were not significantly influenced by temperature, but the copper method is temperature-dependent. The peak heights of 5 mg/L Cu(II) increased significantly from 25 to 45°C, and then gradually at above 45°C. Because air bubbles were produced in the flow system above 50°C, a temperature of 45°C was adopted in this study.
Analytical Characteristics

Analytical characteristics such as sampling rate, detection range, regression equation, correlation coefficient, and relative standard deviation (RSD) of each method were determined for the above optimized conditions (Table 1). Sampling rates and RSDs were determined by repeated injection \( (n = 11) \) of a sample containing 5 mg/L Cu(II), Mn(II), Fe(II), or Zn(II). Regression equations and coefficients were obtained by determining 5 standards covering the detection range. Detection limits were calculated as 3 \( F \) above the blank value, where \( F \) is the SD \( (n = 11) \) for a blank solution.

Under the above optimized conditions, the Zn calibration is nonlinear in the range of 4–10 mg/L Zn, but linear in the range of about 0–4 mg/L Zn. A linear range of 0–10 mg/L Zn could be obtained if the xylene orange concentration were increased to 0.03%; however, the sensitivity decreased, and even worse, the interference tolerance to Mn(II) and Fe(II) decreased significantly. Therefore, nonlinear calibration was adopted for Zn determination.

Interference Study

The interference of several major ions present in animal feeds was studied by using a solution containing 5 mg/L Cu(II), Fe(II), Mn(II), or Zn(II) and adding various concentrations of interfering agents. Any deviation of ±5% or more from the standard absorbance value was taken as interference.

Table 2 indicates that the Cu and Fe methods had relatively higher interference tolerance, but the Mn and Zn methods had serious interference from certain metal ions. Fortunately, the coexisting concentrations of these certain metal ions in animal feeds are generally lower than the permissible concentrations.

The interference of Fe(II) was not investigated because it is oxidized to Fe(III) after sample digestion. Interfering agents such as Cr, Cd, Ni, Pb, and Hg were not studied because they occur at very low concentrations in animal feeds (40).

Sample Analysis

Eight samples of trace mineral premix were analyzed by the proposed FI spectrophotometric methods; the results obtained by the sample presenter, who used the standard AAS methods (41) were adopted as reference.

Table 3 illustrates that relative errors between these 2 methods were <13%, i.e., relative deviations between these 2 methods were 6%. Results of the paired \( t \)-test are shown in Table 3. All \( t \) values were \( t_{0.05} \) (7) = 2.36, which means that there are no significant differences between the proposed FI spectrophotometric method and the standard AAS method.

Further study demonstrated that the difference between these 2 methods results mainly from sample preparation, i.e., samples were digested in the proposed FI spectrophotometric procedure but incinerated in the standard AAS procedure. Very

<table>
<thead>
<tr>
<th>Interfering agent</th>
<th>Cu</th>
<th>Fe</th>
<th>Mn</th>
<th>Zn</th>
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<tr>
<td>Ca(II)</td>
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<td>&gt;500</td>
<td>500</td>
<td>250</td>
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<tr>
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<td>&gt;500</td>
<td>500</td>
<td>50</td>
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<td>&gt;100</td>
<td>50</td>
<td>25</td>
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<tr>
<td>Fe(III)</td>
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<td>—</td>
<td>25</td>
<td>50</td>
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<td>Mn(II)</td>
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<td>&gt;100</td>
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<td>—</td>
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<tr>
<td>Co(II)</td>
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<td>&gt;100</td>
<td>10</td>
<td>0.5</td>
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</tbody>
</table>

Table 2. Tolerance of some interfering agent concentrations (mg/L) in determination of 5 mg/L Cu(II), Mn(II), Fe(II), and Zn(II)

<table>
<thead>
<tr>
<th>Interfering agent</th>
<th>Cu</th>
<th>Fe</th>
<th>Mn</th>
<th>Zn</th>
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<tr>
<td>Ca(II)</td>
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<td>250</td>
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<tr>
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<td>500</td>
<td>50</td>
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<td>25</td>
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<tr>
<td>Fe(III)</td>
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<td>—</td>
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<td>50</td>
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<tr>
<td>Mn(II)</td>
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<td>&gt;100</td>
<td>—</td>
<td>50</td>
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<td>—</td>
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<tr>
<td>Co(II)</td>
<td>10</td>
<td>&gt;100</td>
<td>10</td>
<td>0.5</td>
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</tbody>
</table>

Table 3. Comparison of Cu, Fe, Mn, and Zn concentrations in feeds obtained by proposed FI and standard method (41)

<table>
<thead>
<tr>
<th>Sample</th>
<th>FI a</th>
<th>AAS b</th>
<th>Er (%) c</th>
<th>Mn, g/kg</th>
<th>FI a</th>
<th>AAS b</th>
<th>Er (%) c</th>
<th>Fe, g/kg</th>
<th>FI a</th>
<th>AAS b</th>
<th>Er (%) c</th>
<th>Zn, g/kg</th>
<th>FI a</th>
<th>AAS b</th>
<th>Er (%) c</th>
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<td>47.4</td>
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<td>9.70</td>
<td>8.0</td>
<td>60.6</td>
<td>62.9</td>
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<td>35.0</td>
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<td>2</td>
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<td>6.9</td>
<td>13.8</td>
<td>14.6</td>
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<td>42.0</td>
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<td>8.70</td>
<td>2.5</td>
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<td>54.6</td>
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<td>40.0</td>
<td>7.0</td>
<td>7.70</td>
<td>7.90</td>
<td>2.5</td>
<td>51.6</td>
<td>51.2</td>
<td>0.8</td>
<td>28.6</td>
<td>30.0</td>
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<tr>
<td></td>
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<td>0.095</td>
<td>8.4</td>
<td>7.90</td>
<td>7.20</td>
<td>9.7</td>
<td>3.17</td>
<td>3.00</td>
<td>5.7</td>
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<td>2.41</td>
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<td></td>
<td>7</td>
<td>0.148</td>
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<td>13.0</td>
<td>36.1</td>
<td>40.0</td>
<td>9.8</td>
<td>10.7</td>
<td>12.0</td>
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<tr>
<td></td>
<td>8</td>
<td>0.150</td>
<td>0.148</td>
<td>1.4</td>
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<td>0.314</td>
<td>0.322</td>
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</tbody>
</table>

\( a \) Mean values of 2 determinations.

\( b \) Determined by the sample presenter using the standard AAS methods.

\( c \) Error (%).

\( d \) Trace mineral premix.
close results were obtained if the same sample solution was analyzed by these 2 methods. Because the proposed digestion procedure saves time and fulfills the relative deviation requirement (≤15% for 2 parallel determinations of a sample) in the China National Standard Method GB/T13885-92 (41), the proposed FI spectrophotometric method is applicable for determination of Cu, Fe, Mn, and Zn in trace mineral premix.

Conclusion

A simple, rapid, low cost FI spectrophotometric procedure was developed for consecutive determination of Cu, Fe, Mn, and Zn using an identical manifold. The method is applicable for routine analysis of these 4 elements in animal feeds. The study demonstrated that by selecting appropriate color methods, many FI spectrophotometric methods can be conducted with an identical manifold, which is very helpful for routine analysis. Further study is being conducted in this laboratory on developing an FI spectrophotometric procedure for routine analysis of crude protein, phosphorus, calcium, chloride, Cu, Fe, Mn, and Zn based on a common manifold.

Acknowledgments

The authors thank Shuwen Cui, director of technical office of China National Center for Quality Supervision & Test of Feed, for presenting samples with AAS results. This work was supported by National Natural Science Foundation of China under contract Nos. 29825114 and RCEES9902.

References