A liquid chromatographic (LC) method is described for determination of total vitamin B₆ in soy-based infant formula. Total vitamin B₆ is quantitated by using ion-pair LC after precolumn transformation of phosphorylated and free vitamers into pyridoxol. The limit of detection is 0.3 ng and the limit of quantitation is 1.0 ng on-column (injection volume = 100 μL). Linear response ranged from 39 to 616 ng/mL ($r^2 = 0.99986$). Analysis of a soy-based infant formula control fortified at 6 different concentration levels gave recoveries that averaged 104%. Assay of SRM 1846 gave results within the certified range ($8.6 \pm 0.086$ mg/kg versus the certified value of $8.4 \pm 1.0$ mg/kg). The method provides a rapid and specific assay for the analysis of total vitamin B₆ in fortified soy-based infant formula.

Accepted methods for the determination of vitamin B₆ in foods include microbiological assay and liquid chromatography (LC). For microbiological assay, *Saccharomyces uvarum* is the preferred microorganism, but inaccuracies introduced into the assay through differential growth response to pyridoxol, pyridoxal, and pyridoxamine are difficult to overcome. Studies by Parrish et al. (1, 2) in the initial research with *S. uvarum* showed that on a molar basis the growth response was equal with pyridoxol and pyridoxal but less for pyridoxamine. Chromatographic procedures were developed to fractionate and isolate the vitamin B₆ forms prior to microbiological analysis. This approach is directly applied in AOAC Method 961.15 “Vitamin B₆ (Pyridoxine, Pyridoxal, Pyridoxamine) in Food Extracts” (3). AOAC Official Method 985.32 (3) uses *S. uvarum* for the assay of vitamin B₆ in milk-based infant formulas containing predominately supplemental pyridoxol.

Because of inherent problems associated with the microbiological assay, LC has largely replaced the technique in laboratories that have the required instrumentation and expertise. In depth reviews of LC methods for vitamin B₆ analysis of foods include Eitenmiller and Landen (4), Ubbink (5), and Gregory (6). Extraction procedures prior to LC quantitation can be categorized in 1 of 4 approaches: (1) Hydrolysis of the phosphate esters followed by quantitation of pyridoxal, pyridoxol, and pyridoxamine; (2) conversion of all forms to pyridoxol with quantitation of total vitamin B₆ as pyridoxol; (3) extraction of all nonbound forms of vitamin B₆ with quantitation as free vitamin B₆; (4) preservation of the phosphorylated forms with quantitation of 6 biologically active forms, glycosylated forms (PN-glucoside), and metabolites such as 4-4 pyridoxic acid (4-PA; 4). Each of these approaches has been used extensively. Chromatographic resolution is possible by either ion-exchange or reversed-phase chromatography. Detection for most methods is accomplished by fluorescence. Due to low UV detection sensitivity, UV can only be applied to the analysis of highly fortified foods, supplements, or pharmaceutical preparations. Currently, AOAC INTERNATIONAL does not provide LC methodology for analysis of vitamin B₆ in any sample matrix. Further, no methodology is approved for assay of vitamin B₆ in soy-based infant formula.

Reitzer-Bergaentzle et al. (7) reported an improved procedure for the analysis of total vitamin B₆ in foods using ion-pair chromatography. The method incorporates a precolumn transformation of phosphorylated and free vitamin B₆ forms into pyridoxol. Acid phosphatase hydrolysis is used for dephosphorylation followed by desamination with glyoxylic acid in the presence of Fe$^{2+}$ to convert pyridoxamine into pyridoxal. Pyridoxal is then reduced by sodium borohydride to pyridoxol. The method provided recoveries between 90 and 95%, and a detection limit of 0.02 μg/g. Following initial reporting of the procedure, a collaborative study was completed that included 12 European laboratories (8). Analysis of 8 dif-
Table 1. Evaluation of peak purity for pyridoxol

<table>
<thead>
<tr>
<th>Wavelength ratio</th>
<th>Standard ratio&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sample ratio&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>280/290</td>
<td>0.67</td>
<td>0.68</td>
</tr>
<tr>
<td>300/290</td>
<td>0.69</td>
<td>0.69</td>
</tr>
</tbody>
</table>

<sup>a</sup> Emission wavelength was constant for pyridoxol (395 nm).

<sup>b</sup> Ratios are based on the average of triplicate injections.

Different samples gave RSD values of 12–13% for samples with labeled or reference vitamin B<sub>6</sub> contents and 30–35% for foods with low vitamin levels. Values from 4 laboratories that used microbiological analysis agreed with the LC results. The method is now under the approval process of the European Committee for Standardization (CEN) for the quantitation of vitamin B<sub>6</sub> in foods (9).

The objective of this study was to evaluate the Reitzer-Bergaentzle method for the analysis of vitamin B<sub>6</sub> in soy-based infant formula. The method potentially can be incorporated into regulatory use in the United States and its application expanded as a simplified approach for analysis of total vitamin B<sub>6</sub> in foods.

**METHOD**

**Apparatus**

(a) **Liquid chromatograph.**—Hewlett Packard Series 1100 LC system (Avondale, PA), which includes a quaternary solvent pump, auto-injector, and “Chem-Station” data acquisition system.

(b) **Column.**—Luna 5 μ Phenyl-Hexyl Column, 4.6 × 250 mm, Part No. 00G-4257-RO (Phenomenex, Inc., Torrance, CA).

(c) **Fluorescence detector.**—Model 1046A programmable fluorescence detector (Hewlett Packard) or equivalent.

(d) **Controller environment incubator shaker.**—New Brunswick Scientific Co. (Edison, NJ).

(e) **Ultrasonic cleaner.**—5.2 gallon ultrasonic cleaner (Thomas Scientific, Swedesboro, NJ).

**Reagents**

(a) **Glyoxylic acid monohydrate.**—Cat. No. G-4627, pyridoxamine–2HCl, pyridoxal–HCl, pyridoxol–HCl, sodium borohydride, 1-heptanesulfonic, sodium salt (Sigma Chemical, St. Louis, MO).

(b) **Mobile phase.**—MeOH-0.01M H<sub>2</sub>PO<sub>4</sub> (28 + 72). Dissolve 1.14 g ortho phosphoric acid and 0.5 g 1-heptanesulfonic acid, and sodium salt in 1000 mL distilled water. Filter through 0.45 μm Nylon filter. Mix in the ratio 28:72 at the pump.

(c) **Acid phosphatase solution, 20 mg/mL.**—Dissolve 20 mg/mL in 0.05M sodium acetate solution (pH previously adjusted to 4.5).

(d) **Glyoxylic acid solution, 1M.**—Dissolve 4.70 g in ca 30 mL 2.5M sodium acetate solution. Adjust pH to 4.5 with 6M potassium hydroxide solution. Dilute to 50 mL with distilled water.

(e) **Ferrous sulfate solution, 10 g/L.**—Dissolve 0.50 g ferrous sulfate heptahydrate in 50 mL 0.05N sodium acetate solution (pH previous adjusted to 4.5).

(f) **Stock standard solutions.**—Prepare individual stock standards for the 3 vitamins as follows: Dissolve 0.386 g of the respective vitamin in 1000 mL distilled water. Intermediate standards are prepared by diluting 25.0 mL of the respective stock standard to 250 mL with distilled water.

(g) **Sodium acetate solution, 0.625M.**—Dissolve 85 g sodium acetate trihydrate in ca 900 mL distilled water. Adjust to pH 4.5 with glacial acetic acid. Dilute to 1000 mL with distilled water.

Other required reagents and solutions can be obtained from the original method (7, 8).

**Sample Description and Preparation**

A nonfortified soy-based infant formula developed by Chase et al. (10) was used. The infant formula was made from all the ingredients of a typical soy-based formula minus the added vitamins. After mixing the ingredients, the mixture was pasteurized, spray dried, and blended. Reconstitution of the nonfortified infant formula and other powdered commercial soy-based infant formulas for precision studies was accomplished by weighing ca 10 g powder and combining it with 50 g hot (60°C) distilled water. After thorough mixing on a magnetic stir plate, the reconstituted sample was sonicated for 15 min and homogenized with a Polytron.

**Sample Extraction**

For ready-to-feed, concentrate, and powdered infant formula, weigh 25, 13, and 2.5 g amounts, respectively, into 50 mL Erlenmeyer flasks. Add distilled water (60°C) to bring the initial volume to ca 25 mL. Sonicate samples for 5 to 10 min to adequately disperse the sample. Add in order the following reagents: 2 mL 0.625M sodium acetate solution, 2.5 mL 1M glyoxylic acid reagent, 0.8 mL 10 g/L ferrous sulfate solution, and 1.0 mL 20 mg/mL acid phosphatase solution. Add four 6 mm glass beads to help ensure good mixing during incubation. Prepare the working standards in the same way after pipetting 0, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 mL intermediate pyridoxol standard solution into separate 50 mL Erlenmeyer flasks and adding 25 mL distilled water to each flask. Then add all of the above mentioned reagents, except for the acid phosphatase reagent. Place all of these samples and working standards in an orbital shaker bath at 37°C overnight (or at least 12 h) to ensure complete dephosphorylation of the samples. Remove the incubated extracts from the shaker, then, after cooling, make up to 50.0 mL volume with distilled water and mix well. Filter to remove the precipitated proteins and other solids. Transfer 5.0 mL clear filtrate to a
small flask or beaker; add 4.5 mL 0.1M sodium borohydride solution. After shaking, add 0.5 mL glacial acetic acid and mix well by swirling gently. After the effervescence has subsided, filter ca 2 mL of the final extract through a 0.45 μm Nylon syringe filter for analysis by LC.

**Chromatographic Conditions**

(a) *Instrument parameters.*—Injection volume, 100 μL; flow rate 1.0 mL/min.

(b) *Fluorescence detector parameters.*—Excitation wavelength (E<sub>x</sub> = 290 nm); emission wavelength (E<sub>x</sub> = 395 nm); gain = 16.

(c) *LC configuration.*—First, inject the working standards to establish linearity (r = 0.999) allowing for a 20 min run time per injection. Upon completion of the standard injections, inject the samples while interspersing with alternate standard injections.

**Calculation**

The concentrations (ng/mL) of total vitamin B<sub>6</sub> in the sample extracts are calculated by linear regression using the pyridoxol calibration standards. In this method, all vitamin B<sub>6</sub> is converted to the pyridoxol form. Thus, the total vitamin B<sub>6</sub> content of the sample is obtained from the linear regression plot.

**Results and Discussion**

*Spectral Purity*

The purity of the pyridoxol peak was established by a ratioing technique developed by Haroon et al. (11). While the emission wavelength was kept constant at 395 nm for the analytes, the fluorescence intensity was determined at the excitation wavelengths of 280, 290, and 300 nm. The response ratios were calculated for 280/290 and 300/290 nm. The ratios were compared for the standard and the commercial infant formula extract (Table 1), indicating spectral purity of the peak.

Using calibration standards for each of the forms of vitamin B<sub>6</sub> ranging from 39 to 616 ng/mL, a linear plot with correlation coefficient of 0.999 or better was obtained. [The range of the standard plot covers the range of 0.5 to 7 times the minimum concentration permitted by the Infant Formula Act of 1980, 35 μg/100 Kcal (12).] A typical calibration plot (least squares fit) of pyridoxol had the following parameters: the intercept, slope, and correlation coefficient were 10.581,
922.76, and 0.99981 (r^2 = 0.99986), respectively. The limit of detection (3σ) and limit of quantitation (10σ) were calculated to be 0.3 and 1.0 ng, respectively (13).

When determining the LC parameters, mixed standard solutions of pyridoxal, pyridoxol, and pyridoxamine were chromatographed in order to ascertain the resolution of the column with respect to the different vitamers. An example of the chromatogram of the mixed standard is shown in Figure 1. The retention times were 9.6, 10.7, and 17.3 min for pyridoxal, pyridoxol, and pyridoxamine, respectively, at a flow rate of 1.0 mL/min.

The amount of ferrous sulfate heptahydrate solution (10 g/L) required to complete the conversion of pyridoxamine to pyridoxal was determined by assaying pyridoxamine at levels representing up to 8 times the minimum level of vitamin B₆ required by the Infant Formula Act (12). After addition of 0.10, 0.40, 0.80, 1.2, and 1.6 mL of the ferrous sulfate solution to the reaction flask, 0.80 mL was found to convert 100% of the pyridoxamine to pyridoxal. Therefore, this amount of ferrous sulfate solution was used throughout the study and will ensure complete conversion of exceedingly high levels of vitamin B₆ that might be present in infant formulas.

In addition, a nonfortified soy-based infant formula blank material was spiked with a 0.46 μg/mL pyridoxamine standard. The recoveries were found to be 100%. These same standards and the infant formula were used to test the conversion efficiency of the various transformation steps. Conversions of

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![Figure 2](image-url)  
**Figure 2.** Chromatograms of commercial infant formula (a) before spiking and (b) after spiking with pyridoxamine standard.
pyridoxamine to pyridoxal with glyoxylic acid, then pyridoxal to pyridoxol with NaBH₄ were found to be 100% complete. An example of chromatograms of a commercially prepared infant formula before and after spiking with a pyridoxamine standard is shown in Figure 2. The small broad peak occurring at elution time of 15–17 min might represent a trace amount of pyridoxamine that was not reduced to pyridoxal and other late eluting compounds originating from the sample. After correcting for the formula weight difference between pyridoxamine and pyridoxol, the recovery was calculated to be 100%.

In order to establish precision of the analysis at the lower quantitation limit, 5 replicates of the nonfortified soy-based infant formula were analyzed. A mean value of 14.1 ± 0.83 µg/100 Kcalorie (n = 5) was obtained. The mean value of 14.1 is less than 50% of the minimum fortification level of infant formula (35 µg/100 Kcal). Moreover, commercial infant formulas typically have a label declaration of 60 µg vitamin B₆/100 Kcal and are often found to contain about 150% of label claim (14). Thus, the nonfortified soy-based infant formula has a sufficiently low level of vitamin B₆ to evaluate the precision of the method at vitamin B₆ levels well below the minimum fortification level for infant formulas.

Figure 3. Chromatograms of nonfortified soy-based infant formula (a) before spiking and (b) after spiking with pyridoxamine standard.
Furthermore, an additional aliquot of the nonfortified infant formula was spiked with a 0.31 mg/mL pyridoxamine standard, resulting in 100.5% recovery. An example of the chromatograms is shown in Figure 3.

Five replicate sample aliquots of the nonfortified infant formula were fortified with up to 245 mg/100 Kcal pyridoxamine. The recoveries and standard deviations at each fortification level are shown in Table 2. The average recovery was 104%. The recoveries of vitamin B₆ are fairly consistent throughout the concentration range used (17.5 to 245 mg/100 Kcal).

There is no certified reference material for soy-based infant formula available; however, SRM 1846, Standard Reference material for milk-based infant formula, was available for comparative analysis. Therefore, 10 replicate aliquots of SRM 1846 were assayed by the proposed method. A mean value of 8.60 ± 0.086 mg/kg vitamin B₆ was obtained. This value compares favorably with the certified value of 8.40–1.0 mg/kg vitamin B₆. The AOAC microbiological assay gave a mean value of 7.57–0.80 mg/kg vitamin B₆ (n = 20).

Analysis of 2 commercial soy-based infant formulas was performed by the proposed method and by AOAC Official Method 985.32. The results of both methods were comparable (Table 3). The somewhat lower results of the AOAC method may be, in part, attributed to the variable response of the organism to the vitamin B₆ isomers. The coefficient of variation (CV) of 5.3% is comparable with CVs of 6% reported in the original study of products containing > 5 μg/g pyridoxal. It should be noted that soy-based infant formulas are likely to contain some naturally occurring vitamin B₆ and that glycosylated pyridoxine forms are present (15). Ink et al. (16) showed that the bioavailability of PN-glucoside is poor for humans.

**Conclusions**

This method provides a simple and rapid technique to assay total vitamin B₆ in soy-based infant formula. An experienced analyst can easily analyze 10–15 samples in 2 ½ working days with overnight injections. The method would be applicable to most food matrixes.

**References**


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**Table 2. Recovery of pyridoxamine from soy-based infant formula**

<table>
<thead>
<tr>
<th>Spiking level a</th>
<th>Recovery, % b</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>7×</td>
<td>103 ± 0.89</td>
<td>0.86</td>
</tr>
<tr>
<td>3.5×</td>
<td>104 ± 1.10</td>
<td>1.1</td>
</tr>
<tr>
<td>2×</td>
<td>105 ± 3.40</td>
<td>3.3</td>
</tr>
<tr>
<td>1×</td>
<td>105 ± 4.00</td>
<td>3.8</td>
</tr>
<tr>
<td>½×</td>
<td>105 ± 5.60</td>
<td>5.4</td>
</tr>
</tbody>
</table>

a Five replicates were assayed at each spiking level (2 injections per sample). × is equivalent to 35 μg/100 Kcal.
b Values are the mean % recovery ± standard deviation.

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**Table 3. Determination of total vitamin B₆ in infant formula by LC and AOAC methods**

<table>
<thead>
<tr>
<th>Infant formula a</th>
<th>Declared, μg/100 Kcal</th>
<th>LC method b, μg/100 Kcal</th>
<th>AOAC microbiological method c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ready-to-feed</td>
<td>60</td>
<td>135 ± 7.26 (5.3%)</td>
<td>121</td>
</tr>
<tr>
<td>Soy powder</td>
<td>60</td>
<td>157 ± 8.27 (5.3%)</td>
<td>148</td>
</tr>
</tbody>
</table>

a Soy-based commercial infant formulas.
b % RSD in parenthesis, n = 10.
c AOAC Official Method 985.32.

