INTRODUCTION

Cyanocobalamin (Vitamin B₁₂) belongs to the B vitamin group and prevents pernicious anemia, which is caused by Vitamin B₁₂ deficiency. Because plant products contain very little Vitamin B₁₂, vegetarians and people who do not eat red meat need to supplement their diet by taking multivitamin tablets and beverages supplemented with Vitamin B₁₂. As the excessive consumption of Vitamin B₁₂ may cause asthma and folic acid deficiency, therefore, typically only a low level of Vitamin B₁₂ (e.g., ng/g) is added to products, thus making direct analysis difficult. As a result, Vitamin B₁₂ analysis usually involves complicated sample preparation, which presents challenges for product quality control.

Reversed-phase high-performance liquid chromatography (HPLC) with fluorescence, mass spectrometry, or ultraviolet (UV) detection are good techniques for Vitamin B₁₂ determination. Extracting Vitamin B₁₂ from a larger amount of sample is simple and effective for some relatively large and solid samples, such as multivitamin tablets; however, this strategy is not always suitable for liquid samples (e.g., beverages). In order to have enough Vitamin B₁₂ to detect, the size of the direct large-volume injection required also introduces interfering compounds. Therefore, adsorption onto charcoal, anion-exchange chromatography, or solid-phase extraction (SPE) on reversed-phase cartridges are the techniques usually used for concentrating the analyte from liquid samples while eliminating most of the interfering compounds.

The Dionex UltiMate® 3000 ×2 Dual HPLC system has been used to successfully execute on-line SPE methods that, coupled with HPLC, determine phenols in drinking and bottled water and polycyclic aromatic hydrocarbons (PAHs) in edible oils and drinking water. The Acclaim® Polar Advantage II (PA2) is a polar-embedded column designed for enhanced hydrolytic stability within a wide range of pH values (pH 1.5 to 10). It is compatible with 100% aqueous mobile phases, overcoming the limitations of conventional C8 and C18 reversed-phase columns. Thus, the PA2 column is a good SPE choice for concentrating polar and non-polar components in large-volume water samples (e.g., tap water and beverages) without adding organic solvents.

The work shown here reports a simple, fast, and effective on-line SPE method, followed by HPLC with UV detection, performed on an UltiMate 3000 HPLC system including a dual-gradient pump, autosampler, and column oven equipped with one 2p to 6p valve for determination of trace amounts of Vitamin B₁₂ added to beverages. An additional 2p to 6p valve can be added to this system for convenient method development. A small change in the flow scheme of the traditional on-line SPE mode reverses the flush direction on the SPE column and creates an on-line SPE system with a dual function (i.e., trapping the analyte and partially separating it from interferences prior to the analytical column) that eliminates interferences much more efficiently. Using this on-line SPE system, Vitamin B₁₂ in the beverage is...
trapped on an Acclaim PA2 SPE column and subsequently separated on an Acclaim PA2 analytical column. The entire analysis process is completed within 16 min and offers the advantages of full automation, absence of operator influence, and strict process control, compared to the traditional off-line SPE method. The off-line SPE method requires ~ 3 h just for sample preparation and is inefficient in its elimination of interferences. Although use of a vacuum SPE device to handle many samples in parallel may decrease average per-sample analysis time, the procedure is still complex and, therefore, not suitable for fast real-time monitoring during manufacture.

**EQUIPMENT**
Dionex UltiMate 3000 HPLC system including:
- DGP-3600A pump with SRD-3600 solvent rack with degasser
- WPS-3000TSL semiprep autosampler (with 2.5 mL sample loop)
- TCC-3200 Thermostatted Column Compartment equipped with two 2p–6p valves
- VWD-3400RS UV-vis Detector
- Chromeleon® Chromatography Data System (CDS) software Version 6.80 SR7
- Orion 420A+ pH meter, Thermo Scientific

**Conversion of WPS-3000TSL Autosampler for Large-Volume Injection for On-Line SPE**

The WPS-3000TSL autosampler has up to 45 positions for 10 mL vials that can accommodate the 2500 µL (and even 7500 µL) injection volume. By default, it features 15 positions in the triangular 5-position holders. In addition, the autosampler may be equipped with up to three 10-position, 10 mL trays (P/N 6820.4086) to increase the sample capacity for such applications. Because a sample volume of at least 2500 µL needs to be injected, the semipreparative version of the WPS-3000 autosampler is required (P/N 5822.0028 with temperature control, or 5822.0018 without temperature control). It is possible to convert an analytical WPS-3000(T)SL to the semipreparative variant by installing the Semipreparative Conversion Kit (P/N 6822.2450).

**REAGENTS**
- Deionized water, Milli-Q® Gradient A10, Millipore Corporation
- Acetonitrile (CH₃CN), HPLC grade, Fisher
- Potassium dihydrogen phosphate (KH₂PO₄), phosphoric acid (H₃PO₄), analytical grade, SCRC, China

**STANDARD**
Cyanocobalamin (Vitamin B₁₂) standard (≥ 97%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), China. Prepare a stock standard solution with concentration of 1.0 mg/mL by accurately weighing 10 to 20 mg of Vitamin B₁₂ powder and adding DI water to 10 to 20 g.

**SAMPLE PREPARATION**
Four types of beverages with different flavors were purchased from two Shanghai, China supermarkets. Prepare spiked samples by adding the proper volume of the stock standard solution to 100 mL of beverage. Filter samples through a 0.45 µm membrane (Millex®-HN) before analysis.

**CONDITIONS**

**On-Line SPE**
- Column: Acclaim PA2, 3 µm, 3.0 × 33 mm (P/N 066276) or 4.6 × 50 mm (P/N 063819)
- Analytical Column: Acclaim PA2, 3 µm, 3.0 × 150 mm (P/N 063705)
- Column Temperature: 25 °C
- Mobile Phase: For both SPE and separation pumps
  - A: 25 mM Phosphate buffer (dissolve ~ 3.4 g KH₂PO₄ in 1 L water and adjust pH to 3.2 with H₃PO₄)
  - B: CH₃CN
- In gradient (Table 1 for using the 3.3 × 30 mm of SPE column, Table 2 for using the 4.6 × 50 mm SPE column)
- Flow Rate: Tables 1 and 2
- Injection Volume: 2500 µL on the SPE column
- UV Detection: Absorbance at 361 nm
### Table 1. Gradients and Valve Switching for the Developed On-Line SPE Mode
(Using the Acclaim PA2 Column, 3 μm, 3.0 × 33 mm) and Separation

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Right Pump (for Separation)</th>
<th>Left Pump (for On-Line SPE)</th>
<th>Valve Switching</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flow Rate (mL/min)</td>
<td>Solvent A Buffer (% Vol.)</td>
<td>Solvent B CH(_3)CN (% Vol.)</td>
</tr>
<tr>
<td>0.00</td>
<td>0.6</td>
<td>90 10</td>
<td>97 3.0</td>
</tr>
<tr>
<td>6.00</td>
<td>9.00</td>
<td>97 3.0</td>
<td>77 23</td>
</tr>
<tr>
<td>9.00</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>9.45</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>11.00</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>11.10</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>13.00</td>
<td>58 42</td>
<td>20 80</td>
<td>97 3.0</td>
</tr>
<tr>
<td>13.50</td>
<td>20 80</td>
<td>97 3.0</td>
<td>97 3.0</td>
</tr>
<tr>
<td>14.00</td>
<td>90 10</td>
<td>97 3.0</td>
<td>97 3.0</td>
</tr>
<tr>
<td>14.60</td>
<td>90 10</td>
<td>97 3.0</td>
<td>97 3.0</td>
</tr>
<tr>
<td>16.00</td>
<td>90 10</td>
<td>97 3.0</td>
<td>97 3.0</td>
</tr>
</tbody>
</table>

### Table 2. Gradients and Valve Switching for the Developed On-Line SPE Mode
(Using the Acclaim PA2 Column, 3 μm, 4.6 × 50 mm) and Separation

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Right Pump (for Separation)</th>
<th>Left Pump (for On-Line SPE)</th>
<th>Valve Switching</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flow Rate (mL/min)</td>
<td>Solvent A Buffer (% Vol.)</td>
<td>Solvent B CH(_3)CN (% Vol.)</td>
</tr>
<tr>
<td>0.00</td>
<td>0.5</td>
<td>90 10</td>
<td>97 3.0</td>
</tr>
<tr>
<td>7.0</td>
<td>11.4</td>
<td>97 3.0</td>
<td>70 30</td>
</tr>
<tr>
<td>11.4</td>
<td>11.65</td>
<td>97 3.0</td>
<td>97 3.0</td>
</tr>
<tr>
<td>12.0</td>
<td>12.15</td>
<td>97 3.0</td>
<td>97 3.0</td>
</tr>
<tr>
<td>12.5</td>
<td>14.0</td>
<td>97 3.0</td>
<td>97 3.0</td>
</tr>
<tr>
<td>14.0</td>
<td>14.1</td>
<td>97 3.0</td>
<td>97 3.0</td>
</tr>
<tr>
<td>14.1</td>
<td>16.0</td>
<td>97 3.0</td>
<td>97 3.0</td>
</tr>
<tr>
<td>16.0</td>
<td>16.5</td>
<td>97 3.0</td>
<td>97 3.0</td>
</tr>
<tr>
<td>16.5</td>
<td>19.0</td>
<td>97 3.0</td>
<td>97 3.0</td>
</tr>
<tr>
<td>19.0</td>
<td>19.1</td>
<td>97 3.0</td>
<td>97 3.0</td>
</tr>
<tr>
<td>19.1</td>
<td>19.5</td>
<td>97 3.0</td>
<td>97 3.0</td>
</tr>
<tr>
<td>19.5</td>
<td>20.0</td>
<td>97 3.0</td>
<td>97 3.0</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

Evaluation of Direct Large-Volume Injection

After a large volume of filtered aqueous sample solution (2500 μL) was drawn into the loop, the loop was switched into the flow path. The sample solution in the loop was delivered as a continuous sample (no mixing with the mobile phase) to the analytical column, and the analytes were bound to the front-end of the analytical column, due to the very weak elution ability of the aqueous sample solution. The analytes were then eluted as the mobile phase containing organic solvent passed through the column. The analytical column has both SPE and separation functions. Although a low concentration (1 ng/mL, Figure 1A) of Vitamin B₁₂ standard may be successfully analyzed using this injection mode, the determination had significant interference when applied to real sample analysis (Figure 1C), demonstrating that it cannot be applied at this concentration range.

Evaluation of Traditional On-Line SPE

The commonly used on-line SPE flow scheme (shown in Figure 2) was directly coupled to the analytical HPLC column using one six-port (2p to 6p) column valve. The filtered sample was injected directly onto the system and delivered to the SPE column for enrichment (1-2 position) using the left pump; the analytical column was simultaneously equilibrated with the right pump. After the analytes were bound to the SPE column and impurities washed out, the SPE column was switched into the analytical flow path to elute the bound analytes (6-1 position). Then the analytes were separated on the analytical column and detected by the UV detector.

Column: Acclaim PA2, 5 μm, 4.6 × 150 mm
Eluents: CH₃CN – 25 mM Phosphate buffer (dissolve ~ 3.4 g KH₂PO₄ in 1 L water, and adjust pH to 3.2 with H₃PO₄) in gradient: CH₃CN, 0 – 2 min, 0.0%; 9 – 10 min, 90%; 10.1 min, 0.0%
Column Temp.: 30 °C
Flow Rate: 1.0 mL/min
Injection Vol.: 2500 μL
Detection: UV at 361 nm
Chromatograms: A. 1 ng/mL of Vitamin B₁₂
B. 2 μg/mL of Vitamin B₁₂
C. A beverage sample

Figure 1. Chromatograms using the direct, large injection volume mode.

Figure 2. Flow schematic for traditional on-line SPE mode for sample preparation and analysis.
As shown in Figure 3, compared to using the direct large-volume injection mode, there was a better analysis result for the same sample; however, the elimination of interferences was not efficient, and the determination of Vitamin B₁₂ still was compromised. Table 3 lists the gradient and valve-switching times.

**Development of On-Line SPE Mode with Dual Function**

A small change in the flow scheme of the traditional on-line SPE mode reverses the flush direction on the SPE column, as shown in Figure 4, and creates an on-line SPE system on which the SPE step can have a dual function to more efficiently eliminate interferences. The SPE process in the developed mode is different from that described in the traditional one. The bound analyte on the SPE column is more selectively eluted from the SPE column using a mobile phase gradient similar to the first dimension of a two-dimensional chromatography system. As the SPE process runs (1-2 position), the analytical column equilibrates. Just before the front portion of the analyte peak elutes from the SPE column, the SPE column switches into the analytical flow path (6-1 position). As soon as the analyte is completely eluted from the SPE column, the SPE column switches out of the analytical flow path and back to the SPE flow path (1-2 position). Therefore, only those interferences co-eluting with analytes enter the analytical column, and more interferences are removed. The volume of analyte cut from the SPE column is separated on the analytical column and detected by the UV detector.
This newly developed on-line SPE mode with a dual function (i.e., analyte capture and partial separation) runs automatically, controlled by Chromeleon software.

Setting the valve-switching time is the key to success of this on-line SPE mode. The following equation may be used to estimate the valve-switching time:

\[ t_{\text{valve-switching}}^2 = t_{\text{valve-switching}}^1 + \left( \frac{v_1}{v_2} \right) \times w_h \]

The notation \( t_{\text{valve-switching}}^1 \) represents the first valve-switching time when the front shoulder of the analyte peak is just eluting from the SPE column at the flow rate for SPE; \( t_{\text{valve-switching}}^2 \) represents the second valve-switching time when the SPE column is switched out of the analytical flow path; \( v_1 \) and \( v_2 \) represent the flow rates for SPE and separation, respectively; and \( w_h \) represents baseline peak width (min) of Vitamin B\(_{12}\) on the SPE column.

Therefore, during method development, a UV detector is required after the SPE column to monitor the elution of analyte on the column in order to determine the valve-switching time. As shown in Figure 5, the front shoulder of the Vitamin B\(_{12}\) peak eluting from the SPE column at 1.0 mL/min (\( v_1 \)) appears at 11.75 min (\( t_{\text{valve-switching}}^1 \)). The peak is detected by the UV detector and the baseline peak width of Vitamin B\(_{12}\) on the SPE column is 0.25 min (\( w_h \)). When the flow rate for the separation on the analytical column is 0.5 mL/min (\( v_2 \), the second valve-switching time of 12.25 min (\( t_{\text{valve-switching}}^2 \)) is calculated using the equation.

Actually, timing must be set shortly before this determined \( t_{\text{valve-switching}}^1 \) because 12.25 min is the time point when Vitamin B\(_{12}\) arrives at the detector. Hence, the switching needs to occur 0.05 min earlier to make sure that the Vitamin B\(_{12}\) is still completely contained within the SPE column. This time difference is estimated based on the volume of the capillary from the column to the detector and the applied flow rate.

A delay of \( t_{\text{valve-switching}}^2 \) in Vitamin B\(_{12}\) analysis is applied to verify the accuracy of the equation. Comparison of the peak area when \( t_{\text{valve-switching}}^1 = 11.70 \text{ min} \) and \( t_{\text{valve-switching}}^2 = 12.25 \text{ min} \) (0.8056), to when \( t_{\text{valve-switching}}^1 = 11.65 \text{ min} \) and \( t_{\text{valve-switching}}^2 = 12.30 \text{ min} \) (0.8065), shows that the equation is accurate for estimating valve-switching time in this on-line SPE mode. In addition, a slightly earlier \( t_{\text{valve-switching}}^1 \) and/or delay in \( t_{\text{valve-switching}}^2 \) are used to avoid losing analytes in real sample analysis, although this allows more interferences to enter the analytical column.

In practice, an additional 2p to 6p valve may be used to construct a two-valve system for convenient method development. The flow schematic of the two-valve setup is shown in Figure 6. The left valve can be used to switch the SPE column or separation column into the flow path of the detector.

<table>
<thead>
<tr>
<th>Position of Left Valve</th>
<th>Position of Right Valve</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>1-2</td>
<td>Determine switching time of right valve during method development</td>
</tr>
<tr>
<td>6-1</td>
<td>1-2</td>
<td>Load sample and analysis</td>
</tr>
<tr>
<td>6-1</td>
<td>6-1</td>
<td>Transfer analytes from SPE column to analytical column</td>
</tr>
</tbody>
</table>

Figure 6. Flow schematic with two 2p to 6p valves as a new mode for development of on-line SPE methods.
Discussion of the SPE Method

The effects of the size of the SPE column on the elimination of impurities using the developed on-line SPE mode were investigated. Three Acclaim PA2 columns with different sizes, 4.6 × 50 mm, 3.0 × 33 mm, and 4.3 × 10 mm (guard column), were used for SPE. As shown in Figure 7, the elimination of impurities on the larger column was more efficient, which can be attributed to the separation being more efficient on the larger column than on the smaller one, thereby allowing fewer impurities to enter the analytical flow path. However, the larger SPE column required more time to run the on-line SPE method. Therefore, the selection of the 3.0 × 33 mm column size was a compromise between elimination of impurities and analysis time.

In addition, different SPE columns will use different concentrations of organic solvents to elute Vitamin B₁₂. This may cause a change in the retention time of Vitamin B₁₂ on the separation column; therefore, if a different SPE column is used, the separation conditions on the analytical column also may need to change.

A selectivity difference between the separation column and the SPE column can be used to achieve better separation of Vitamin B₁₂ and to minimize interferences from the SPE column. This may also involve a difference in the mobile phase between the SPE and separation steps.

**Figure 7. Chromatograms of a beverage sample spiked with 1.5 ng/mL of Vitamin B₁₂ standard extracted on different SPE columns using the developed on-line SPE mode.**
Reproducibility, Linearity, and Detection Limit

Method reproducibility was estimated by making eight consecutive injections (injection volume 2500 μL) of a beverage sample spiked with 0.5 ng/mL of Vitamin B12 standard. Retention time reproducibility was 0.027% (RSD) and peak area reproducibility was 2.2% (RSD). Figure 8 shows an overlay of chromatograms for the eight consecutive injections.

Calibration linearity for Vitamin B12 was investigated by using five standard concentrations (0.1, 0.2, 0.5, 1.0, and 5.0 ng/mL). The external standard method was used to establish the calibration curve and to quantify Vitamin B12 in samples. Excellent linearity was observed from 0.1 to 5.0 ng/mL when plotting the concentration versus the peak area.

The linearity equation of Vitamin B12 is as follows:

\[ A = 0.0686c - 0.0006 \]

The symbol A represents peak area and c represents Vitamin B12 concentration (ng/mL). The correlation coefficient (r) is 0.9999.

The detection limit for Vitamin B12 was calculated using the equation:

\[ \text{Detection limit} = S_t \left( n - 1, 1 - \alpha = 0.99 \right) \]

The symbol S represents Standard Deviation (SD) of replicate analyses, n represents number of replicates, \( t_{n - 1, 1 - \alpha = 0.99} \) represents Student’s value for the 99% confidence level with n – 1 degrees of freedom.

Using eight consecutive injections of a beverage sample spiked with 0.5 ng/mL of Vitamin B12 standard to determine the S value, the estimated method detection limit (MDL) was 0.046 ng/mL.

Sample Analysis

Four types of beverages with different flavors and different product batch numbers were analyzed and the results summarized in Table 4.

### Table 4. Analysis Results for Beverages

<table>
<thead>
<tr>
<th>Sample</th>
<th>Batch Number</th>
<th>Detected (μg/100 mL)</th>
<th>Labeled (μg/100 mL)</th>
<th>Added (μg/100 mL)</th>
<th>Found (μg/100 mL)</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Orange flavor</td>
<td>H22333</td>
<td>0.21</td>
<td>—</td>
<td>0.045</td>
<td>0.049</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>H22335</td>
<td>0.20</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2 Litchi flavor</td>
<td>F10014</td>
<td>0.27</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>F10632</td>
<td>0.30</td>
<td>—</td>
<td>0.045</td>
<td>0.046</td>
<td>102</td>
</tr>
<tr>
<td>3 Kaffir lime flavor</td>
<td>F10522</td>
<td>0.38</td>
<td>0.045</td>
<td>0.048</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F12203</td>
<td>0.37</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4 Peach flavor</td>
<td>A31601</td>
<td>0.1</td>
<td>—</td>
<td>0.045</td>
<td>0.041</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>A41020</td>
<td>0.36</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>C10553</td>
<td>0.0076</td>
<td>0.06–0.18</td>
<td>0.050</td>
<td>0.052</td>
<td>104</td>
</tr>
</tbody>
</table>

Note: 1. Two injections were made for each.
2. Found = Measured value of spiked sample – Measured value of sample.
Figure 9 shows an overlay of chromatograms of samples #1 and #4, plus the same sample spiked with 0.45 ng/mL of Vitamin B₁₂ standard. Figure 10 shows chromatograms of samples with different batch numbers.
Evaluation of a Larger Injection Volume

Typically, a larger-volume sample injection improves the detection sensitivity in on-line SPE; however, more interferences also will be bound to the SPE column, which may result in an overload of the SPE column. So the effects of a larger injection volume (> 2500 μL) on this on-line SPE method were estimated. The ratio of peak area was 2.97 and was obtained by injecting 7500 μL (three consecutive injections of 2500 μL using User Defined Program injection mode) of sample spiked with Vitamin B₁₂ standard, compared to injection of 2500 μL of sample spiked with Vitamin B₁₂ standard. This ratio of 2.97 was very close to the theoretical value of 3.0.

A comparison of the two injection volumes is shown in Figure 11. There is no evident increase of interferences entering the analytical flow path when using this on-line SPE mode with injection volume 7500 μL. This demonstrates that a larger injection volume of sample may be applied to achieve higher sensitivity when using this on-line SPE method and this particular sample. This type of study must be done with each sample when larger injections are considered because the effect of injection volume on the final chromatogram is likely to depend strongly on the individual sample matrix composition.

CONCLUSION

This work describes a new and simple design that requires just a small change in the flow scheme of the traditional on-line SPE mode in order to create a full gradient on the SPE column to pre-separate target analytes and then transfer them to the separation column. This new design fully uses the separation power of both columns (rather than performing just enrichment and pre-treatment on the first stationary phase) and may eliminate interferences much more efficiently. The additional dual-valve design is easy to use and convenient for method development. The Dionex UltiMate 3000 ×2 Dual HPLC system provides an efficient platform for this new design and is more convenient than the heart-cut column-switching technique.¹¹ Sub-ppb concentrations of Vitamin B₁₂ in beverages were determined with satisfactory results.
REFERENCES


