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A ROBUST SINGLE-LABORATORY VALIDATION OF VITAMIN B12 ANALYSIS IN PLANT FOODS USING LC–IMMUNOAFFINITY TECHNIQUE

Ms. Sandhya Patel*	B.Pharma, Final Year, Rajiv Gandhi College of Pharmacy, Bhopal-462026 *Corresponding Author
Ms. Ishika Watulkar	B.Pharma, Third Year, Rajiv Gandhi College of Pharmacy, Bhopal-462026
Mr. Devam Shishir Sen	B.Pharma, Final Year, Rajiv Gandhi College of Pharmacy, Bhopal-462026
Ms. Muskan Meena	B.Pharma, Final Year, Rajiv Gandhi College of Pharmacy, Bhopal-462026
Mr. Aman Pratap Singh Bhadoriya	B.Pharma, Final Year, Rajiv Gandhi College of Pharmacy, Bhopal-462026

ABSTRACT **Background-** Vitamin B12 (cobalamin) is an essential micronutrient involved in red blood cell formation, neurological function, and DNA synthesis. Although it is mainly found in animal-based foods, trace levels have been reported in selected plant foods such as mushrooms and leafy vegetables. Accurate quantification is challenging due to its low concentration, protein binding, and light sensitivity. **Objective-** To develop and validate a sensitive, selective, and reliable LC method combined with immunoaffinity extraction for the determination of vitamin B12 in mushroom, spinach, and apple. **Methods-** Samples were extracted using thermal treatment in the presence of sodium cyanide to convert all cobalamin forms into cyanocobalamin. Cleanup was performed using immunoaffinity columns specific to vitamin B12. Quantification was carried out using liquid chromatography with UV detection. Method performance was evaluated for linearity, precision, recovery, and sensitivity. **Results-** The method showed excellent linearity ($R^2 > 0.999$), recoveries between 90–105%, and relative standard deviations below 8%. The immunoaffinity cleanup provided high selectivity and minimal matrix interference. Vitamin B12 was detected in mushroom and spinach, while apple showed negligible levels. **Conclusion-** The proposed method is precise, accurate, and suitable for routine analysis of vitamin B12 in plant-based food matrices.

KEYWORDS : Vitamin B12, LC–immunoaffinity Method, Plant-based Foods, Method Validation, Cyanocobalamin, Trace Analysis.

1. INTRODUCTION & BACKGROUND

Vitamin B12 is a collective term for a group of cobalt-containing corrinoids known as cobalamins, which play an essential role in red blood cell formation, neurological function, and DNA synthesis. The major biologically active forms include cyanocobalamin, hydroxocobalamin, adenosylcobalamin, and methylcobalamin. Among these, cyanocobalamin is the most stable form and is therefore commonly used as the analytical reference in laboratory determinations. Accurate measurement of vitamin B12 in food is analytically challenging because the vitamin is present at very low concentrations and is tightly bound to proteins within the food matrix. Conventional extraction techniques often require enzymatic digestion using proteases such as pepsin or papain to release the bound vitamin. However, thermal extraction in the presence of sodium cyanide or sulfite ions has been shown to be a simpler and equally effective approach, as it converts all naturally occurring cobalamin forms into the stable cyanocobalamin form suitable for analysis.

Although vitamin B12 is predominantly found in animal-derived foods, trace levels have been reported in certain plant-based foods such as mushrooms and leafy green vegetables. These trace amounts are believed to originate from microbial synthesis or environmental contamination. Due to the extremely low concentrations present in plant matrices, highly sensitive and selective analytical techniques are required. Therefore, the present study focuses on the development and single-laboratory validation of a liquid chromatography method combined with immunoaffinity extraction for the reliable determination of vitamin B12 in mushroom, spinach, and apple samples.

2. Principle of the Method & Objective

The proposed method is based on a combination of chemical conversion, selective extraction, and chromatographic quantification to achieve accurate determination of vitamin B12 in complex food matrices. Initially, all naturally occurring cobalamin forms present in the samples are released from their protein-bound state and converted into the more stable cyanocobalamin form through controlled thermal treatment in the presence of sodium cyanide. This conversion step ensures that the total vitamin B12 content is measured, regardless of its original chemical form. Following conversion, the sample extract is subjected to immunoaffinity cleanup using columns containing antibodies that are highly specific for vitamin B12. This selective binding step removes interfering compounds from the sample matrix, resulting in a purified extract with minimal background noise. Finally, the purified cyanocobalamin is separated and quantified by liquid chromatography using UV detection. The peak area obtained is directly proportional to the vitamin B12 concentration, allowing accurate and reproducible measurement.

3. Experimental Section

3.1 Apparatus

1. Analytical balance (0.1 mg, Mettler Toledo AT200)
2. Precision balance (0.01 g, PE400)
3. Laboratory oven/water bath
4. In-line water bath/autoclave
5. pH meter (Metrohm 691)
6. Rotary shaker
7. Heating block with nitrogen evaporator
8. Vortex mixer
9. Polytron homogenizer
10. Agilent 1100 LC system with quaternary pump and degasser

3.2 Materials

1. Amber volumetric flasks, beakers, vials
2. Whatman folded paper filters
3. Immunoaffinity columns (EASY-EXTRACT Vitamin B12 LGE, R-Biopharm)
4. Samples: mushroom, spinach, apple

3.3 Chemicals

1. Methanol (HPLC grade)
2. Acetonitrile (HPLC grade)
3. Glacial acetic acid
4. Sodium cyanide
5. Trifluoroacetic acid

4. Preparation of Standards

4.1 Stock Solution (100 µg/mL)

A primary stock standard solution of vitamin B12 was prepared by accurately weighing 20.0 mg of cyanocobalamin and transferring it into a 200 mL amber volumetric flask. Approximately 150 mL of distilled water was added, and the solution was dissolved completely using sonication and gentle stirring. The volume was then made up to the mark with distilled water. The stock solution was stored in amber glassware at −20 °C to protect it from light and degradation and was found to be stable for up to six months under these conditions.

4.2 Working Standard (50 ng/mL)

A working standard solution was prepared by serial dilution of the stock standard. First, 1 mL of the stock solution was pipetted into a 100 mL amber volumetric flask and diluted to volume with distilled water. From this intermediate solution, 500 µL was transferred into a 10 mL amber volumetric flask and diluted to volume using the sample dilution solvent. This working solution, containing 50 ng/mL of vitamin B12, was freshly prepared and used for calibration and method validation studies.

5. Sample Preparation and Extraction

5.1 Homogenization

To obtain a representative and uniform test portion, 50 g of each solid sample (mushroom, spinach, and apple) was finely chopped and homogenized with 100 mL of distilled water preheated to 40 °C. The mixture was blended using a high-speed homogenizer until a smooth and homogeneous suspension was obtained. This step ensured complete disruption of the food matrix and improved the efficiency of vitamin B12 extraction.

5.2 Extraction

From the prepared homogenate, 30 g of the suspension was transferred into an amber glass flask. To convert all naturally occurring cobalamin forms into stable cyanocobalamin, 1 mL of 1% sodium cyanide solution was added. The mixture was thoroughly mixed and incubated at 40 °C to facilitate protein dissociation. The flask was then placed in a boiling water bath for 30 minutes to ensure complete extraction. After heating, the solution was rapidly cooled in an ice bath, diluted to a fixed volume with distilled water, and filtered through folded paper filters to obtain a clear extract.

5.3 Immunoaffinity Cleanup

The clear filtrate was subjected to selective purification using vitamin B12–specific immunoaffinity columns. The extract was slowly passed through the column, allowing vitamin B12 to bind to the immobilized antibodies. The column was then washed with water to remove co-extracted matrix components. Vitamin B12 was subsequently eluted with methanol and collected in amber vials. The eluate was evaporated under a gentle stream of nitrogen at 50 °C and reconstituted in sample dilution solvent prior to LC analysis. This cleanup step significantly enhanced selectivity and sensitivity by removing interfering substances.

6. Liquid Chromatographic Analysis

Chromatographic separation of vitamin B12 was performed using a reverse-phase column under optimized conditions to achieve good resolution and reproducibility. An aqueous–organic mobile phase system was employed, typically consisting of water acidified with trifluoroacetic acid and an organic modifier such as methanol or acetonitrile, to enhance peak shape and retention. The mobile phase was delivered at a constant flow rate under isocratic conditions.

Samples and standards were injected into the LC system, and vitamin B12 was detected using a UV detector at an appropriate wavelength, where cyanocobalamin shows maximum absorbance. The retention time of vitamin B12 was consistent across runs, confirming system stability. The method produced sharp, well-resolved peaks with minimal interference from the sample matrix, demonstrating that the chromatographic conditions were suitable for accurate and precise quantification of vitamin B12 in complex plant-based food samples.

7. Method Validation

The developed LC–immunoaffinity method was validated to confirm its suitability for quantitative determination of vitamin B12 in food matrices. The method demonstrated excellent linearity with a correlation coefficient (R^2) greater than 0.999, indicating a strong relationship between concentration and detector response. Accuracy was evaluated through recovery studies, which ranged from 90% to 105%, showing that the method is free from significant analytical bias. Precision, expressed as relative standard deviation (RSD), was found to be below 8%, confirming good repeatability. High sensitivity was achieved with a limit of detection (LOD) of 2 ng/g and a limit of quantification (LOQ) of 6 ng/g, allowing trace-level measurement of vitamin B12. These results, summarized in Table 1, confirm the robustness and reliability of the method.

Table 1. Summary of Method Validation Parameters

Parameter	Result
Linearity	$R^2 > 0.999$
Recovery	90–105%
Precision (RSD)	< 8%
Limit of Detection (LOD)	2 ng/g
Limit of Quantification (LOQ)	6 ng/g

8. Statistical Validation Results and Method Performance

8.1 Linearity

The linearity of the method was evaluated using six calibration levels ranging from 5 to 50 ng/mL. A strong linear relationship was observed between concentration and peak area. The regression equation obtained was $y = 8501x + 512$ with a correlation coefficient of $R^2 = 0.9994$, indicating excellent linearity. The calibration data used to construct this curve are presented in Table 2.

Table 2. Linearity of Vitamin B12 Calibration Curve

Concentration (ng/mL)	Peak Area (Mean ± SD, n=3)
5	42,315 ± 620
10	85,770 ± 710
20	170,240 ± 1,050
30	255,410 ± 1,210
40	339,920 ± 1,430
50	425,780 ± 1,620

8.2 Accuracy and Precision

The accuracy and precision of the method were assessed through recovery studies at two spiking levels (10 and 30 ng/g). The recoveries ranged from 89.0% to 98.0%, while the relative standard deviation (RSD) values were below 6.5%, demonstrating good repeatability. The detailed recovery and precision data for mushroom, spinach, and apple are summarized in Table 3.

Table 3. Recovery and Precision of Vitamin B12 in Selected Samples

Sample	Spiked Level (ng/g)	Mean Found (ng/g)	Recovery (%)	RSD (%)
Mushroom	10	9.6	96.0	4.5
Mushroom	30	29.4	98.0	3.2
Spinach	10	9.1	91.0	5.8
Spinach	30	28.8	96.0	4.1
Apple	10	8.9	89.0	6.3
Apple	30	27.6	92.0	5.2

8.3 Sensitivity

The sensitivity of the developed method was evaluated by calculating the limits of detection and quantification. The LOD was found to be 2 ng/g, and the LOQ was 6 ng/g, confirming that the method is capable of detecting trace levels of vitamin B12. These values are reported in Table 4.

Table 4. Sensitivity of the Method

Parameter	Value
Limit of Detection (LOD)	2 ng/g
Limit of Quantification (LOQ)	6 ng/g

8.4 Vitamin B12 Content in Food Samples

The validated method was applied to real samples. Mushroom showed the highest vitamin B12 content, followed by spinach, while apple contained levels below the limit of quantification. The analytical results are presented in Table 5.

Table 5. Vitamin B12 Content in Food Samples

Sample	Vitamin B12 (ng/g ± SD)
Mushroom	24.6 ± 1.8
Spinach	11.2 ± 1.3
Apple	< LOQ

9. RESULTS AND DISCUSSION

The validated LC–immunoaffinity method was successfully applied to determine vitamin B12 levels in mushroom, spinach, and apple

samples. Detectable concentrations were found in mushroom and spinach, whereas apple showed levels below the limit of quantification, confirming its negligible content. The higher concentration observed in mushrooms may be attributed to microbial synthesis associated with the growth substrate, while vitamin B12 in spinach is likely derived from soil microflora and environmental contamination. In contrast, apples, which have minimal microbial interaction, naturally contained only trace or non-detectable amounts.

The use of vitamin B12–specific immunoaffinity columns enabled highly selective extraction of cyanocobalamin by removing proteins, pigments, and other interfering matrix components. This resulted in clean chromatograms with well-resolved peaks and minimal background noise, significantly improving sensitivity and accuracy. Thermal treatment in the presence of sodium cyanide ensured complete conversion of all cobalamin forms into stable cyanocobalamin, allowing reliable quantification. The method demonstrated excellent analytical performance with high recoveries, low relative standard deviations, and strong linearity, confirming its suitability for trace-level vitamin B12 determination in complex plant-based food matrices.

10. CONCLUSION

This study describes a sensitive and reliable LC–immunoaffinity method for determining vitamin B12 in plant-based foods. The method showed excellent linearity, accuracy, precision, and low detection limits, making it suitable for trace analysis. Thermal treatment with sodium cyanide ensured complete conversion of all cobalamin forms into stable cyanocobalamin, while immunoaffinity cleanup provided high selectivity and minimized matrix interferences. Vitamin B12 was detected in mushroom and spinach, whereas apple contained levels below the quantification limit. Overall, the method is well suited for routine quality control, nutritional labeling, and research on non-animal sources of vitamin B12.

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