An Evaluation of Procedures for the Determination of Vitamin B12 in Foods, Supplements and Premixes using HPLC and UPLC after selective extraction with immunoaffinity cartridges.

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An Evaluation of Procedures for the Determination of Vitamin B_{12} in Foods, Supplements and Premixes using HPLC and UPLC after selective extraction with immunoaffinity cartridges.

A Government Chemist Programme Report

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1. Executive Summary

Procedures for the determination of Vitamin B_{12} in foods, supplements and vitamin premixes using HPLC and UPLC were evaluated in this study. The study was undertaken to underpin the capability of the Government Chemist to perform referee analysis of vitamins by developing appropriate analytical methods.

The report includes an evaluation of a commercial, immunoaffinity cartridge used for the selective isolation of vitamin $B_{12.}$ The evaluation does not imply any endorsement of the product by LGC or the Government Chemist and alternative products may be available.

2. Background

Vitamin B_{12} is the collective name for cobalt-containing corrinoids that have biological activity in humans. The main cobalamins in humans and animals are hydroxocobalamin, adenosylcobalamin and methylcobalamin, the last two being the active coenzyme forms. Cyanocobalamin (CN-Cbl) is a form of vitamin B_{12} that is widely used clinically and for food supplementation due to its availability and stability.

Vitamin B_{12} in the diet is largely derived from microbiological synthesis. Small amounts can sometimes be found in plant products due to the presence of bacteria or due to microbial contamination but in the main it is found naturally, only in animal products, as a product of gut microflora synthesis. The richest food sources are meats (particularly offal), seafoods, eggs and dairy products. In addition, vitamin B_{12} can be added to foods such as breakfast cereals, infant formulae and dietary supplements.

Vitamin B_{12} naturally present in foods is bound to proteins and glycoproteins and must be released by acid or enzymatic digestion before analysis. It can be measured by chemical, spectrophotometric, microbiological, chromatographic, radioassay or immunoassay methods. Microbiogical assays have traditionally been used for the determination of Vitamin B_{12} in foods and much of the available data on this vitamin has been obtained using this technique. These assays are sensitive but are non-specific, require careful handling and can be prone to contamination. Incubation times of 24 hours or more are required. Assay of vitamin B_{12} in biological matrices by chromatographic methods is difficult due to the low concentrations usually present and the relatively high detection limits obtainable with typical chromatographic detectors. HPLC can be used for the analysis of fortified foods and supplements although the determination of vitamin B_{12} at natural levels remains a challenge.

In many samples, there is a need to enhance the vitamin B_{12} concentration prior to HPLC analysis and to reduce the amount of other sample components which may interfere with the chromatographic analysis. This has been made possible in recent years by the manufacture of commercial immunoaffinity cartridges which use immobilised antibodies to selectively extract cobalamins from food extracts. Such columns are available from a number of manufacturer's as below:

Easi-Extract® Vitamin B_{12} affinity columns - R-Biopharm Rhone Ltd (Europe)
Venture™ B_{12} Immunoaffinity columns - Grace Vydac (US)
Immunolab B_{12} Immunoaffinity columns – Immunolab GmbH (GE)

The Easi-Extract cartridges were used for this evaluation however this does not imply any endorsement of this product by LGC or the Government Chemist.

3. Objective

Procedures for the determination of vitamin B_{12} in foods and vitamin premixes using aqueous extraction, immunoaffinity clean-up / concentration and HPLC were evaluated and assessed in this study. The combination of HPLC or UPLC and sample preparation using immunoaffinity media offers a rapid means of determining vitamin B_{12} in foods and should improve the analytical uncertainty of the measurement compared to the microbiological assay procedures currently used.

4. Sample extraction

Cyanocobalamin (CN-Cbl) is water-soluble; therefore the free form can be extracted into aqueous solution. It is most stable at pH 4.0 – 4.5 therefore most extraction procedures are carried out at this pH. Vitamin B_{12} is stable to heat between pH 4 and 7 but should be protected from extremes of pH, strong light and oxidising agents.

In foods, vitamin B_{12} is bound to proteins and these must be denatured using acid or by digestion with proteases during extraction. For starchy samples, amylases are often used to break down starch and assist filtration. When determining natural vitamin B_{12} forms, excess cyanide is usually added to convert the cobalamins to the cyano form.

When carrying out analysis of vitamin B_{12} in foods by microbiological assay at LGC, samples are autoclaved at 121 °C for 10 minutes in pH 4.5 sodium acetate buffer. Potassium cyanide is added to ensure conversion of the active forms to CN-Cbl. (Note that potassium cyanide is a Schedule 1 poison and specific safety requirements must be implemented for its use). For starchy samples, a further incubation with amylase is carried out to aid filtration. Samples are then filtered or centrifuged before dilution to the required assay concentration.

In the Easi-Extract instructions, there are a range of extraction conditions given. For analysis of added cyanocobalamin in tablets, drinks and juices, extraction with water is all that is required. For infant formulae, and other foods that contain protein, an extraction with pepsin is used if total vitamin B_{12} is required in order to release the protein bound forms. An amylase digestion is suggested for starchy foods and potassium cyanide is added to ensure conversion to CN-Cbl. The extraction conditions vary but all except the tablets and drinks require a digestion with enzymes (pepsin and/or amylase), a heating step and control of the pH. Cyanide is used for determination of natural cobalamins forms. Vitamin B_{12} is most stable at ~ pH 4 – 5 therefore extraction is usually carried out in this range. Extraction conditions are given for tablets, drinks and juices, protein drinks and premixes, infant formula and a range of other foods. In general, similar extraction principles can be applied to all foods but the precise conditions required should be confirmed by the user in the matrix to be analysed.

5. Use of the Immunoaffinity cartidges (IAC)

The protocol given in the cartridge instructions was followed.

It is important to ensure that the extract pH is between 4.5 and 7.0 as the analyte may not bind sufficiently to the IAC cartridge outside of this range. Sample extracts should be filtered or centrifuged before the IAC procedure to avoid blocking of the cartridge and to ensure reproducible elution characteristics.

The columns were used with gravity elution although positive or negative pressure may be applied if required. Use of the IAC columns in these modes was not evaluated. A 10 mL plastic reservoir was attached to each cartridge before use. Sample loading should be between 2-3 mL/min to ensure adequate binding but in practice, the drip rate obtained by simple gravity elution was normally sufficient. Using this technique, there was some variation in elution times between cartidges but column performance was not adversely affected if the columns were allowed to “go dry”.

Following elution in methanol, the extracts were evaporated to dryness under nitrogen using a Turbovap evaporator and were redissolved in mobile phase.

An important consideration is that the cartidges have limited capacity to bind vitamin B_{12}. The maximum capacity is quoted as 1 µg of cyanocobalamin however low recovery of vitamin B_{12} was sometimes noted when the amount of vitamin B_{12} loaded, approached this value. Although this has not been fully investigated, normal practice at LGC is to limit the maximum amount of vitamin B_{12} to ~ 0.5 µg.

To standardise the elution characteristics, the "loading volume" was standardised to 5 mL. If the Vitamin B_{12} content in 5 mL of extract exceeded 0.5 µg, the extract was either diluted or the volume of extract used was reduced and an appropriate amount of buffer was added to the reservoir to bring the volume to 5 mL (e.g. 1 mL extract + 4 mL buffer). For samples containing lower concentrations of vitamin B_{12}, larger volumes of sample extract were used up to a maximum of 30 mL. (Note that higher volumes can be used i.e. up to 100 mL is suggested for carbonated drinks, but this was not tested.)

The binding capacity of cartridges is a limitation as a maximum of 0.5 (or 1) µg of vitamin B_{12} is obtained in the cartridge eluent. Since the minimum volume for redissolution is ~ 300 uL and preferably 0.5 – 1 mL for normal HPLC vials, the vitamin B_{12} concentration in the final extract is still quite low. A sensitive HPLC system and reasonably large injection volumes are required for subsequent analysis. In addition, for samples where the expected vitamin B_{12} concentration is unknown, care is required when interpreting the analytical results, to ensure that the IAC capacity has not been exceeded as the results for such samples will be low.

5.1 IAC recovery

The IAC instructions recommend that the IAC recovery should be tested using 10 mL of a 0.025 µg/mL solution of vitamin B_{12} in extraction buffer. When this was done, the recovery of vitamin B_{12} was around 97 %. However, lower and more variable recoveries were obtained when the amount of vitamin B_{12} on the column was between 0.5 µg and 1 µg. In addition, variable recoveries (between 65 and 130 %) have been noted when recovery was assessed using sample extracts to which vitamin B_{12} spikes had been added, even though the volumes were adjusted to keep the total expected vitamin B_{12} concentration within the theoretical limit for the cartridges. The latter recoveries would of course, include losses from other parts of the analytical procedure and not just the IAC cartridge. Although the general IAC column performance can be checked using a standard as described, acceptable recovery ranges should ideally be established using spiked sample extracts and an analytical procedure (loading volumes etc) as close as possible to that normally used.

6. Analysis of Foods

High performance liquid chromatography (HPLC) and Ultra performance liquid chromatography (UPLC) systems were evaluated. The choice between these techniques was based only on availability of equipment but analysis using UPLC is much faster, where available. HPLC was used for the determination of the foods and supplements whilst UPLC was used for determination of vitamin premixes.

6.1 HPLC Conditions

For analysis of infant formulae and other food products, HPLC conditions similar to those given in the IAC instructions were used although these were modified to improve the speed of analysis. The conditions were as follows:

Column:	Waters, Cosmosil C18-AR-II 100 mm x 4.6 mm i.d.
Mobile Phase:	A: 0.025% TFA in water, pH 2.6 B: Acetonitrile
Gradient:	**Minutes** / **%A** / **%B** 0 / 100 / 0 0.21 / 100 / 0 2.80 / 85 / 15 5.20 / 75 / 25 5.44 / 90 / 10 6.80 / 100 / 0 11.0 / 100 / 0
Flowrate:	1.0 mL/min
Column Oven Temp:	30 °C
Detection:	UV(DAD) at 361 nm
Injection volume:	100 µL

Fig A: HPLC conditions for foods

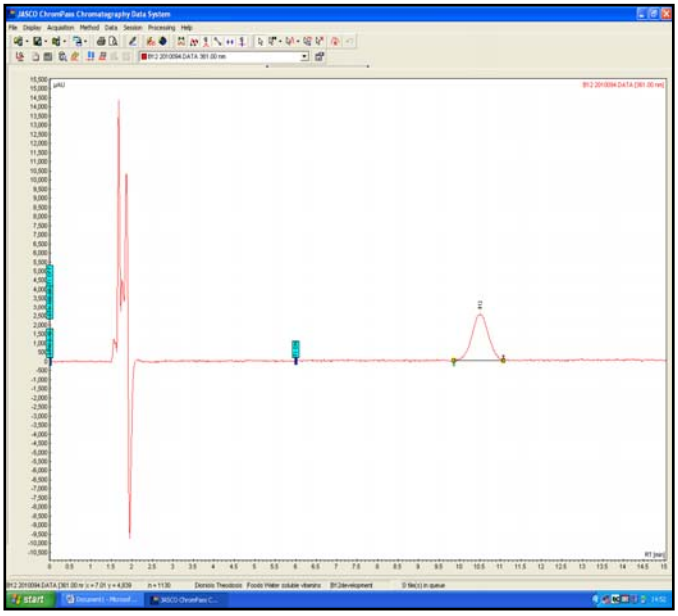

Fig B: Chromatogram of standard

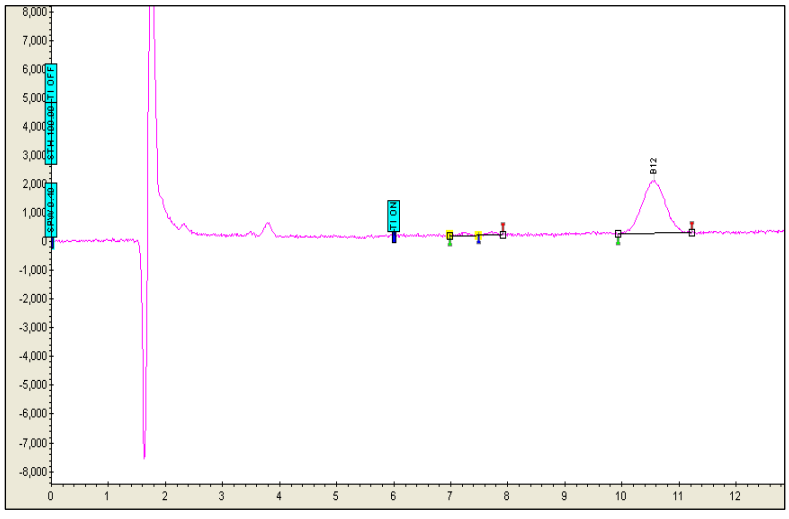

Fig C: Chromatogram of an Infant formula

6.2 Linearity, Range and injection repeatability

The HPLC system was assessed for linearity over a range from 0.5 µg/mL to 10 µg/mL. The calibration curve is shown below.

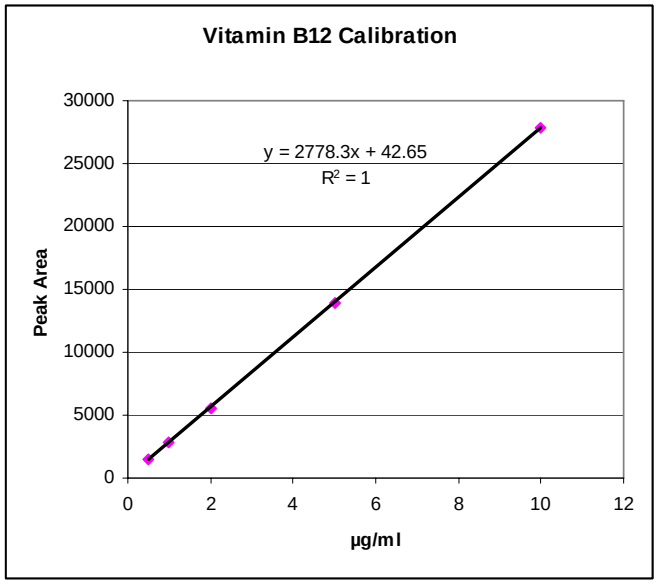

Fig D: Calibration curve for Vitamin B_{12} by HPLC

The calibration was linear over the range tested. The injection repeatability (RSD %) at 0.1 µg/mL and 0.8 µg/mL was 3.6 % and 1.4 % respectively.

6.3 Limit of detection

The limit of detection of the HPLC system as described was approximately 0.02 µg/mL in the injected solution. When using a sample weight of 30 g, an extract volume of 100ml, an IAC load-volume of 10 mL and an IAC elution volume of 0.5 mL, this is equivalent to a Vitamin B_{12} concentration of 0.003 µg/g.

6.4 Sample Extraction

Sample (1 g to 30 g depending upon vitamin B_{12} content) was dispersed in sodium acetate buffer at pH 4 and was then digested with pepsin and amylase in the presence of excess cyanide. The filtered extract was then passed through an IAC cartridge, which was washed with water before elution with methanol. The methanol was removed by evaporation and the vitamin B_{12} re-dissolved in 0.5 – 1 mL of extraction buffer for HPLC.

6.5 Method Performance

The accuracy and precision of the procedure was evaluated by replicate analysis of a certified reference material (Pig liver; BCR 487). The CRM was analysed six times by the same analyst under the same conditions in order to asses the repeatability of the procedure. The results are shown in Table 1 below:

Table 1: Results of replicate extractions of CRM 487

Replicate	B_{12} Concentration (µg/g)
1	1.181
2	1.035
3	1.044
4	1.136
5	1.057
6	1.124
mean	**1.10**
s	**0.054**
%RSD	**4.94**

The certified value for CRM 487 is 1.12 ± 0.09 mg/kg, therefore the analytical mean result was very close to the expected value. The expected, within–laboratory repeatability (%RSD) calculated from the Horwitz equation at 1 µg/g is 10.72, therefore the experimental %RSD of 4.94 was acceptable.

6.6 Method specificity

The immunoaffinity cartridge uses a monoclonal antibody which is highly specific to vitamin B_{12}.

6.7 Method applicability

The IAC kit gives extraction conditions for tablets, powdered energy drinks and premixes, carbonated drinks and fruit juices, infant formula and food (cereal, dairy, cocoa powder, meat homogenate and baby food composite). The method was tested successfully on vitamin tablets, infant formula, and yeast extract.

Table 2: Results for Vitamin B_{12} in foods and supplements

Sample	Measured Concentration (µg/g)	Label Declaration (µg/g)
Infant milk formula	0.09	0.1
Multivitamin tablet	1.12	1
Multivitamin chewable tablet	0.97	1
Marmite	0.22	0.15

A cereal bar with fruit and chocolate however, gave problems due to poor filtration indicating that a modified extraction was necessary.

The IAC procedure provides a rapid and selective means of isolating vitamin B_{12} from foods and supplements however; the analyst must establish the optimum extraction procedure for the sample to be examined and the sample weights, extraction volumes and IAC volumes to be used. This can be problematic for unknown samples and may require some investigation before an analysis can be carried out although the manufacturer's instructions give guidance for some sample types.

7. Analysis of vitamin premixes

7.1 UPLC conditions

Column:	Zorbax RRHD Eclipse Plus C18 (Agilent) 2.1 x 100 mm, 1.8 µm.
Mobile Phase:	**A**: 1-pentane sulphonate (0.1 %w/v) in 0.015M phosphoric acid. **B**: A + Acetonitrile (2:1)
Gradient:	**Minutes** / **%A** / **%B** 0 / 70 / 30 0.5 / 40 / 60 2.0 / 40 / 60
Flowrate:	0.8 mL/min
Column Oven Temp:	30 °C
Detection:	UV(DAD) at 361 nm
Injection volume:	20 µL

Fig E: UPLC conditions for vitamin premixes

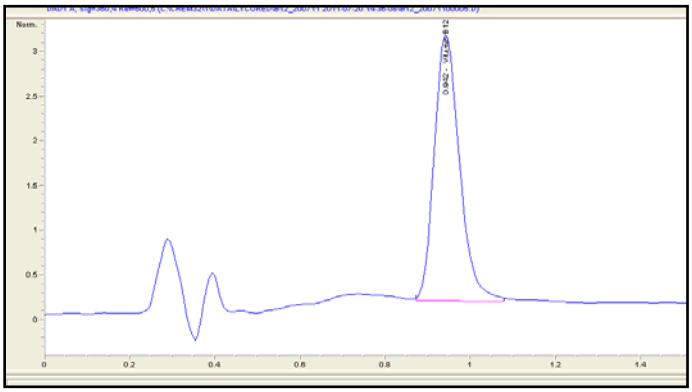

Fig F: UPLC chromatogram of Vitamin B12 standard @ 0.5µg/ml

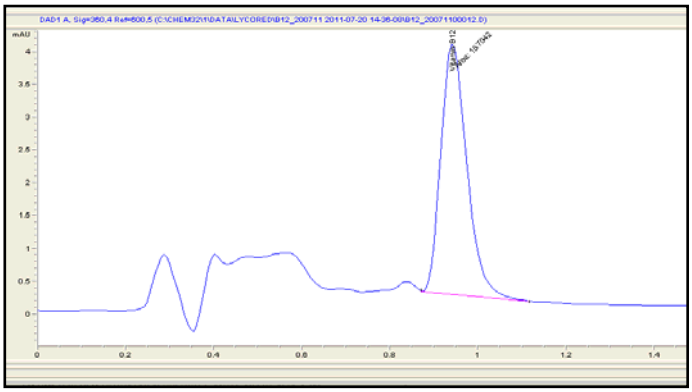

Fig G: Chromatogram of vitamin premix sample

7.2 Linearity, Range and injection repeatability

The UPLC system was assessed for linearity over a range from 0.015 µg/mL to 1 µg/mL. The calibration curve is shown below.

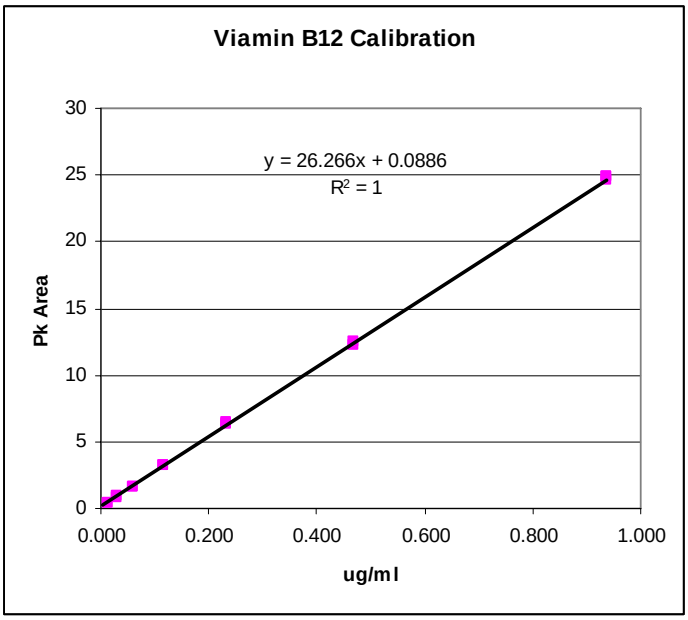

Fig H: Calibration curve for UPLC

The calibration was linear over the range tested. The injection repeatability (RSD %) at ~0.5 µg/mL was 1.3 %.

7.3 Limit of detection

The limit of detection of the UPLC system as described was approximately 0.02 µg/mL in the injected solution. When using a sample weight of 1 g of vitamin premix, an extract volume of 100 mL, an IAC load-volume of 5 mL and an IAC elution volume of 1ml, this is equivalent to a Vitamin B_{12} concentration of 0.4 µg/g.

7.4 Sample Extraction

Sample (1-3 g, depending upon vitamin B_{12} content) was dispersed in a suitable volume (100 -250 mL) of potassium phosphate buffer at pH 5.5. After shaking to disperse the sample, the solution was placed in an ultrasonic bath for 5 minutes. The extract was filtered through a 0.45 µm syringe filter and diluted with buffer if necessary to contain approx. 0.5 µg/mL of vitamin B_{12}. 1 ml of extract was then passed through an IAC cartridge, which was washed with water before elution with methanol. The methanol was removed by evaporation and the vitamin B_{12} re-dissolved in 1 mL of extraction buffer for UPLC.

7.5 Method Performance

Method performance was assessed by replicate analysis (n=5) of vitamin premixes containing known amounts of vitamin B_{12} at approx. 1000 µg/g and 350 µg/g. Single analyses were also carried out of samples containing lower amounts of vitamin B_{12}. The results are as shown in the table below:

Table 3: Vitamin B12 results for vitamin premixes

Sample ID	1	2	3	4	5	6	7	8	9
Declaration	*948 - 1158*			*315 - 385*	*69 - 79*	*12.3 - 15.1*	*10.0 - 11.9*	*2.9 - 3.7*	*1.4 - 1.7*
Vitamin B_{12} µg/g	1130	1080	1026	367	76	14.1	10.8	3.2	1.6
	1106	1065	1038	348	-	-	11.1	3.2	1.5
	1061	1092	1055	360	-	-	-	-	-
	1153	1096	1018	367	-	-	-	-	-
	$1_{12}2$	1056	1017	355	-	-	-	-	-
Mean	1114	1078	1031	360	76	14.1	10.9	3.2	1.5
Std Dev.	34.3	17.2	15.9	8.3	-	-	-	-	-
%RSD	3.1	1.6	1.5	2.3	-	-	-	-	-

The results obtained were within the expected ranges for all concentrations and samples tested. The repeatability was acceptable for samples 1 – 4 (see table) and although this has not been fully assessed for the samples containing lower concentrations of Vitamin B_{12}, the duplicates for samples 7 -9 were in good agreement.

8. Conclusion

The analysis of vitamin B_{12} in foods using liquid chromatography has, until recently been hampered by the low concentrations found in natural foodstuffs. Although radioassays or protein binding assays are available for analysis of vitamin B_{12} in blood and sera, these are less suitable for use with foods. Much of the available data on foods has therefore been obtained using microbiological assay but these are difficult to perform and require long analysis times. HPLC has been less used as the concentration of vitamin B_{12} in foods at natural levels is too low to enable reliable detection using UV detection at 361 nm without analyte concentration.

The availability of the immunoaffinity extraction cartridges enables the selective extraction of vitamin B_{12} from foods. In addition to removing other food components which could interfere with subsequent analysis, the technology allows the vitamin B_{12} content of the sample extract to be concentrated before analysis. Analysis by HPLC using UV detection then becomes a realistic option.

The cartridges tested worked well with vitamin premixes, vitamin tablets and infant formulae and gave good results using a certified reference material prepared from pig liver. Its use with other foods has not been fully tested although the selective nature of the product should enable its use with many different food matrices. The instructions provided give a range of extraction options and users should ensure that the extraction used is suitable for the product being determined.

The cartridges have a limited binding capacity which is not normally a problem when analysing foods at natural levels but care is needed when analysing unknown products with higher concentrations of vitamin B_{12} to ensure that the binding capacity is not exceeded. Recovery of vitamin B_{12} from the matrix of interest should also be checked.

Chromatographic analysis using HPLC or UPLC can be used although the latter technique is faster. Acceptable performance was obtained using both techniques with the conditions described.

The cartridges are easy to use and provide a rapid method for sample clean-up and analyte concentration, thereby facilitating HPLC analysis. Combined with a suitable extraction protocol, the technology enables rapid and reliable analysis of vitamin B_{12} in foods to be undertaken.

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