



Characterisation of vitamin B12 immunoaffinity columns and method development for determination of vitamin B12 in a range of foods, juices and pharmaceutical products using immunoaffinity clean-up and high performance liquid chromatography with UV detection.

Elaine Catherine Marley, Eric Mackay, Gemma Young

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Characterisation of vitamin B12 immunoaffinity columns and method development for determination of vitamin B12 in a range of foods, juices and pharmaceutical products using immunoaffinity clean-up and high performance liquid chromatography with UV detection.

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2 1 **Characterisation of vitamin B12 immunoaffinity columns and method development**
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4 2 **for determination of vitamin B12 in a range of foods, juices and pharmaceutical**
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6 3 **products using immunoaffinity clean-up and high performance liquid**
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8 4 **chromatography with UV detection.**

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12 7 E.C. Marley, E. Mackay, G. Young
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16 9 Research Department, R-Biopharm Rhone (RBR) Limited, 3.06 Kelvin Campus, WSSP Maryhill
17 10 Road, Glasgow G20 0SP (UK)
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19
20 12 **Abstract**
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24 14 New rapid and simpler procedures using immunoaffinity columns, have been developed for the
25 15 determination of vitamin B12 in a range of samples including 3 different US National Institute of
26 16 Standard and Technology (NIST) Reference Materials, infant formula, powdered energy drinks
27 17 and bars, wheat breakfast cereal, carbonated soft drinks, fruit juices and vitamin B12 tablets. The
28 18 procedures involved extraction of vitamin B12 using water or sodium acetate buffer and enzyme
29 19 digestion (using pepsin or α -amylase or both) if necessary. The extract was clarified and passed
30 20 through 'EASI-EXTRACT[®] Vitamin B12', an immunoaffinity column containing monoclonal antibody
31 21 with high affinity and specificity to vitamin B12. Subsequently the vitamin B12 immunoaffinity
32 22 column was washed with 10 ml water and the vitamin B12 was released from the column with 3 ml
33 23 methanol. Following evaporation the samples were reconstituted in mobile phase and analysed by
34 24 HPLC-UV at 361 nm on an ACE 3AQ analytical column using a gradient elution consisting of
35 25 0.025% trifluoroacetic acid in water and acetonitrile. Analysis of 3 types of NIST Standard
36 26 Reference Materials in triplicate demonstrated the results of the immunoaffinity column method
37 27 were comparable to the microbiological assay results. Method repeatability was determined for all
38 28 samples analysed and ranged between 0.8-10% demonstrating the method was repeatable with
39 29 complex matrices (NIST 2383) containing low levels of vitamin B12 (0.44 μ g/100 g) as well as
40 30 simpler matrices such as vitamin tablets containing high levels (2000 μ g/0.849 g) of vitamin B12.
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53 32 *Keywords:* Vitamin B12, cyanocobalamin, immunoaffinity column, EASI-EXTRACT[®] Vitamin B12,
54 33 HPLC-UV.
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Introduction

Vitamin B12 is an important water-soluble vitamin; its primary functions are the formation of red blood cells and the maintenance of a healthy nervous system. In addition it is needed to process folic acid, another essential vitamin (Ball 1997). Deficiency can lead to pernicious anaemia due to failure of red blood cell formation (Food Standards Agency 2003). Prolonged deficiency can also lead to nerve degeneration and irreversible neurological damage due to failure to repair the myelin sheath protecting nerve cells (Scott 1992). Vitamin B12 originates from bacteria, fungi and algae (Tom 1994) and is found in virtually all types of meat, fish and dairy products. However, daily intake is required because the amount absorbed is limited (approximately 0.002 mg/meal) by the capacity of the (intrinsic factor-wall receptor) uptake system; consequently a range of foods are fortified to reduce the incidence of deficiency related diseases. Groups at risk of deficiency diseases include infants, the elderly and vegans. Hence all infant formulae are fortified with vitamin B12 and foods that are produced for vegans, such as soya products are often fortified voluntarily, as foods from plant sources do not contain vitamin B12.

Although the biologically active forms of vitamin B12 are methylcobalamin and adenosylcobalamin, the synthetic form used in vitamin supplements, pharmaceuticals and in the fortification of food is cyanocobalamin. Cyanocobalamin is converted to the biologically active methylcobalamin following uptake by ileal enterocytes and prior to binding transcobalamin II (TCII); the majority of cobalamin available for uptake from the circulatory system is associated with TCII (Food Standards Agency 2003).

Vitamin supplements are widely used and manufacturers are required to test premixes and finished product to confirm vitamin B12 levels. Also food and drink manufacturers are obliged by law to determine the vitamin B12 content of a range of fortified commodities for process control purposes and to enable accurate labelling.

Currently the determination of vitamin B12 in food products is routinely carried out by means of the microbiological assay (MBA), which uses *Lactobacillus leishmanii* as the test organism (Anonymous 2000). Although the assay is extremely sensitive, the MBA lacks a high degree of specificity as food commodities can contain inactive cobalamins that interfere with the growth of the microorganism. Heudi et al. (2006) reviewed alternative methods such as an intrinsic factor based assay using radioisotopic dilution, a protein binding assay with surface plasma spectroscopy detection and solid phase extraction (SPE) techniques with subsequent concentration procedures prior to analysis by HPLC-UV for the determination of vitamin B12 in foods and biological samples. The majority of the methods previously identified lack sensitivity or

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2 1 specificity. HPLC-UV alone is not sensitive enough to detect vitamin B12 in non-fortified food
3 2 products and determination by fluorescence requires a lengthy derivatisation of vitamin B12 to
4 3 yield a fluorescent compound, as vitamin B12 does not naturally fluoresce.
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8 7 Due to the need for accurate determination of vitamin B12 and the lack of sensitivity and specificity
9 8 with existing methods an immunoaffinity clean-up column was developed. The column consisted of
10 9 a monoclonal antibody with high affinity and specificity to vitamin B12 (cyanocobalamin) linked to
11 10 cyanogen bromide activated agarose beads. Immunoaffinity column clean-up with HPLC-UV could
12 11 be used for the routine analysis of vitamin B12 in food because it enables concentration of the
13 12 analyte and removal of interfering compounds; thus, allowing accurate quantification of vitamin
14 13 B12 at low levels. In addition, this work involved method development for the determination of
15 14 vitamin B12 in a range of food products and pharmaceuticals (NIST standard reference material,
16 15 infant formula, high protein powdered energy drinks and bars, breakfast cereal, carbonated soft
17 16 drinks, fruit juice drinks and vitamin B12 tablets) and consequently has expanded upon previous
18 17 work by Heudi et al. (2006).
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28 17 **Experimental**
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31 19 **Reagents**
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33 20 Vitamin B12 (cyanocobalamin) (catalogue No. V2876), hydroxycobalamin (catalogue No. H7126),
34 21 sodium acetate (S8750), pepsin (EC 3.4.23.1, catalogue No. P7125), α -amylase (EC
35 22 3.2.1.1, catalogue No. A2771) and trifluoroacetic acid (TFA) were all purchased from Sigma-Aldrich
36 23 (Dorset, UK). Potassium cyanide (catalogue No. 424500250) was obtained from Acros
37 24 (Leicestershire, UK). HPLC grade acetonitrile and water were purchased from Lab-Scan Analytical
38 25 Sciences (Dublin, Ireland) and were used for mobile phase preparation and immunoaffinity column
39 26 elution solution. De-ionised water was obtained from a MilliQ water purification system (Millipore
40 27 UK Ltd) and was used for preparation of buffers and standards, sample dilution and immunoaffinity
41 28 column wash solution. NIST Standard Reference Materials were obtained from National Institute of
42 29 Standard and Technology (Gaithersburg, MD, USA). The immunoaffinity columns, EASI-
43 30 EXTRACT[®] Vitamin B12, (catalogue No. P80) are available from R-Biopharm Rhône Ltd
44 31 (Glasgow, UK).
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54 33 **Cautions**
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56 34 Vitamins B12 is subject to light deterioration. Protect analytical work from bright light and use
57 35 actinic glassware for sample extracts and standard solutions.
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Safety Considerations

Potassium cyanide is a highly toxic colourless crystalline compound. Consequently, solutions and extracts should be handled with extreme care. Gloves and other protective clothing were worn as a safety precaution during the handling of this compound. Potassium cyanide residues can be decontaminated using 15% sodium hypochlorite.

Preparation of Vitamin B12 Standard Curve

A 10 µg/ml vitamin B12 stock solution was prepared by weighing 1 mg vitamin B12 into a 100 ml amber volumetric flask. The vitamin B12 was then diluted to mark with water and placed in an ultrasonic bath for 15 min to dissolve. The stock solution was stable for 3 months at 4 °C.

Serial dilutions of Vitamin B12 Stock solution were then used to prepare a five-point standard curve; 2 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml and 0.05 µg/ml. A correlation coefficient of >0.995 was obtained routinely. The limit of detection (LOD) was 1.5 ng of vitamin B12 injected and the limit of quantification (LOQ) was 5 ng of vitamin B12 injected. The same protocol was used for the preparation of hydroxycobalamin stock and diluted solutions. The stability of the hydroxycobalamin stock solution was 1 month at 4 °C.

Samples

A range of samples were selected to demonstrate the clean-up capacity of the immunoaffinity columns, these included; NIST Standard Reference Materials, whey-based Infant Formula, high protein powdered drinks and bars, wheat breakfast cereal, carbonated soft drinks, fruit juices and vitamin B12 tablets. Three test portions of each sample were analysed and the mean result reported.

Analysis of Vitamin B12 content in NIST Standard Reference Materials, Infant Formula, breakfast cereal and high protein powdered drinks and bars

Sample preparation

Prior to analysis it was critical to consider the saturation of the immunoaffinity column and the LOQ of the HPLC-UV system; hence the optimum amount of vitamin B12 loaded onto the immunoaffinity column should be within the range of 0.1-1.5µg. A 5-30g test portion of sample (depending on label claim) was mixed with 100 ml of 50 mM sodium acetate buffer (pH 4.0), 1 ml potassium cyanide (1%), 0.5 g α-amylase and 2 g pepsin for 10 minutes. The sample was transferred to a shaking water bath set at 37 °C for 30 min followed by a further 30 min incubation in a 100 °C shaking water bath. After cooling, the sample was filtered through a Schleicher & Schuell filter (597½, Whatman).

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4 2 *Purification of Vitamin B12*
5 3 A 5-15 ml aliquot (corresponding to 0.1–1.5 µg vitamin B12) of the sample extract was passed
6 4 through the column followed by 10 ml of water to wash the column. The vitamin B12 was eluted
7 5 with 3 ml methanol and subsequently evaporated to dryness by heating (50-60 °C) under a stream
8 6 of air and reconstituted in 300 µl of 0.025% TFA in water. When analysing infant formula the
9 7 sample volume applied to the column was restricted to ≤5ml as method development
10 8 demonstrated a decrease in vitamin B12 recoveries when applying volumes greater than 5ml. A
11 9 mean recovery of 99% (RSD_r =0.71%, n=6) was obtained when 5ml of infant formula extract was
12 10 applied to the column, the recovery decreased to 88% (RSD_r =3.22%, n=6) when 10ml was
13 11 applied and 78% (RSD_r =3.57%, n=6) when 15ml was applied.
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15 13 **Analysis of vitamin B12 content in carbonated soft drinks and fruit juices**
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17 15 *Sample preparation*
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19 17 Carbonated soft drinks were degassed by placing the sample in an ultrasonic bath for 10 min and
20 18 upon removal adjusting the pH to 7.0 with 1M sodium hydroxide solution. For fruit juices, the pH
21 19 was adjusted to 7.0 with 1M sodium hydroxide solution and centrifuged for 10 min at 4000 rpm.
22 20 Subsequently the supernatant was filtered through glass microfibre filter paper (GMF2, Satorius).
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24 22 *Purification of vitamin B12 from carbonated soft drink and fruit juice extracts*
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26 24 A 20 ml-100 ml aliquot of sample (corresponding to 0.1–1.5 µg vitamin B12) was passed through
27 25 the immunoaffinity column. The column was washed with 10 ml of water and the vitamin B12
28 26 eluted with 3 ml methanol. The column eluate was evaporated to dryness by heating (50-60 °C)
29 27 under a stream of air and reconstituted in 500 µl of 0.025% TFA in water.
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31 29 **Analysis of vitamin B12 content in vitamin tablets**
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33 31 *Sample preparation*
34 32

35 33 The sample was ground to a fine powder with pestle and mortar. An amount of tablet containing
36 34 the equivalent of 2.5 µg vitamin B12 was dissolved in 100 ml water and placed in an ultrasonic
37 35 bath for 15 min. A 1 ml aliquot of the extract was made up to 1 litre with water in a volumetric flask.
38 36 The pH of the diluted extract was then confirmed to be between 4.5 and 7. If the pH was greater
39 37 than 7, it was adjusted to within range using citric acid. If the pH was less than 4.5 the sample was
40 38 discarded and re-extracted using phosphate buffered saline (PBS).
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Purification of vitamin B12 from tablet extract

Passed 20 ml of the sample extract through the immunoaffinity column. The column was washed with 10 ml water and the vitamin B12 eluted with 3 ml methanol. The column eluate was evaporated to dryness by heating (50-60 °C) under a stream of air and reconstituted in 1 ml of 0.025% TFA in water.

Analysis by HPLC-UV

Samples were analysed using a Dionex HPLC-UV system equipped with Chromeleon software, a UVD 170U/340U detector, a P680 gradient pump, a TCC-100 thermostatted column compartment and sample injections of 100 µl were made from an ASI-100 autosampler (Dionex, CA, USA). The chromatographic separations were performed via an ACE 3 AQ, 150 mm x 3.0 mm column (Hichrom, Reading, UK), at a flow rate of 0.250 ml/min. The mobile-phase consisted of 0.025% TFA in HPLC grade water (solution A) and acetonitrile (solution B) and followed a series of gradients (ratio of solution A: solution B): range 0-3.5 min 100:0, range 3.5-11 min 85:15, range 11-19 min 75:25, range 19-20 min 90:10 and range 20-35 min 100:0 (v/v). The column effluent was monitored by UV at 361 nm.

Results and discussion

Initially dilutions of the stock vitamin B12 (cyanocobalamin) and hydroxycobalamin solutions were prepared in water to challenge the immunoaffinity column. When 200 ng vitamin B12 in water was applied to the immunoaffinity column >90% mean recovery ($RSD_r = 2.1\%$, $n=10$) was obtained and when 200 ng hydroxycobalamin in water was applied to the column a mean recovery of 75% ($RSD_r = 1.0\%$, $n=6$) was achieved. Pure solutions of adenosylcobalamin and methylcobalamin could not be obtained as hydroxycobalamin was always found to be present; adenosylcobalamin and methylcobalamin are reduced cobalamin derivatives that are unstable and degrade to the hydroxo compound (Hoffbrand 1979). The column was challenged with a mixture of 100 ng vitamin B12 and 200 ng hydroxycobalamin in water; 91% ($RSD_r = 1.7\%$, $n=6$) mean recovery of vitamin B12 and 45% ($RSD_r = 1.1\%$, $n=6$) mean recovery of hydroxycobalamin was obtained. Hence, both cyanocobalamin and hydroxycobalamin were shown to interact with the antibody on the immunoaffinity column. However, the results demonstrated the antibody had greatest affinity to vitamin B12. This finding is particularly relevant because the synthetic form of cobalamin used in vitamin B12 supplements, pharmaceuticals and in the fortification of food is cyanocobalamin.

At the preliminary stages of method development, the effectiveness of methanol and acetonitrile as elution solvents for releasing bound vitamin B12 from the immunoaffinity column were

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2 1 compared. The columns were challenged with 1500 ng vitamin B12 in water, washed with 10 ml
3 2 water and the vitamin B12 eluted with methanol or acetonitrile, the recoveries were >90% (RSD_r
4 3 =6.2%,) and <5% (RSD_r =8.3%) respectively. Hence methanol was used as elution solvent for all
5 4 further work.
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10 6 Three NIST Standard Reference Materials (NIST 1846 Infant Formula, NIST 1546 Meat
11 7 Homogenate and NIST 2383 Baby Food Composite) were analysed using immunoaffinity clean
12 8 up. In all cases the value of vitamin B12 given by NIST was established using the MBA and a
13 9 specified uncertainty was stated (Table 1). It should be noted that NIST 2383 and NIST 1546 were
14 10 complex matrices containing low levels of vitamin B12 and in all cases the results obtained using
15 11 immunoaffinity clean-up in conjunction with HPLC-UV were within the uncertainty limit obtained
16 12 using the MBA (Table 1). In particular, NIST 2383 Baby Food Composite was a very challenging
17 13 matrix to extract vitamin B12 from as the sample contained (in order of decreasing mass) orange
18 14 juice, infant formula, corn, rice flour, creamed spinach, carrots, papaya juice, tomato paste, beef,
19 15 macaroni, wheat flour, non-fat milk, Romano cheese, soya protein, onion powder, green pepper,
20 16 celery oil, oregano in conjunction with a low-level of vitamin B12 (0.44 µg/100 g). The sample
21 17 preparation and purification procedure clarified the sample filtrate (Figure 1) and concentrated the
22 18 vitamin B12 sufficiently, to allow quantification by HPLC-UV analysis. In addition the sensitivity of
23 19 the method was further improved as immunoaffinity clean up removed interfering peaks from
24 20 HPLC chromatograms allowing accurate determination of vitamin B12 at low levels using λ (max.)
25 21 of 361 nm. In support of this Choi et al. (2003) showed without prior clean up monitoring at 550 nm
26 22 was necessary to avoid interfering peaks; monitoring vitamin B12 at 550nm resulted in a decrease
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46 28 In addition to NIST 1846, three other commercial brands of infant formula were purchased at a
47 29 local supermarket and the vitamin B12 content assessed and compared to the label claim. With
48 30 the exception of Brand 1 Plus Infant Formula all recoveries were greater than 100% of the
49 31 declared value with % RSD_r < 7% (Table 1). EU legislative levels for vitamin B12 allow ± 15% of
50 32 the declared value (European Commission Directive 2002). For Brand 3 Infant Formula and NIST
51 33 1846 Infant Formula the determined vitamin B12 levels were within the legislative range. For
52 34 Brand 1 and Brand 2 Infant Formula the determined values were 3% (RSD_r = 1.6%) below the
53 35 lower limit and 2% (RSD_r = 5.0%) above the upper limit respectively. Previous work by Casey et al.
54 36 (1982) demonstrated more efficient extraction of vitamin B12 when the sample was autoclaved for
55 37 10 min at 121 °C rather than heated on a boiling water bath for 10 min. However when autoclaved
56 38 Brand 1 Plus Infant Formula as described the recovery of vitamin B12 was found to decrease.

Naturally occurring forms of vitamin B12 (methylcobalamin and adenosylcobalamin) are often associated with proteins within food matrices. Pepsin was added to the extraction mixture prior to incubation at 37 °C to aid protein digestion and release protein bound methylcobalamin and adenosylcobalamin. Also α -amylase was added to promote digestion of the starch content of the sample and aid filtration. The methylcobalamin and adenosylcobalamin were converted to cyanocobalamin (vitamin B12) by the addition of potassium cyanide; the cyano group replaces the methyl or adenosyl group to yield vitamin B12 (Heudi et al. 2006).

In addition two types of high protein powdered drinks and three types of high protein bars were analysed. The protein powdered drinks contained high levels of vitamin B12; 103% and 94% of the label claim were obtained (Table 2). For high-level vitamin B12 samples in complex matrices it is critical the amount of vitamin B12 applied to the immunoaffinity column does not exceed 1.5 μ g as amounts greater than this are above the upper limit for optimum column performance. In contrast protein bars are fortified with relatively low levels of vitamin B12. However, due to the concentration effect of the immunoaffinity column the quantity of vitamin injected onto the HPLC system was above the LOQ and good chromatographic separation was achieved. Recoveries relative to declared value of greater than 100% were obtained (Table 2).

INSERT TABLE 2 HERE

For the wheat flake sample 107% of the declared level (1.7 μ g/100 g) was obtained with RSD_r of 4.4% ; this is within \pm 15% of the declared value and complies with EU legislation (European Commission Directive 2002). Cereals are a challenging matrix because they absorb much of the extraction buffer and generally contain low level vitamin B12. The use of immunoaffinity columns to purify the vitamin from the sample filtrate coupled with the evaporation of the column eluate, concentrates the vitamin B12 sufficiently to allow detection by HPLC-UV.

Vitamin B12 sample preparation procedures have the dual purpose of liberating protein-bound cobalamins and converting the naturally occurring labile forms to cyanocobalamin. Consequently total vitamin B12 content can be determined by the quantification of cyanocobalamin. However to prevent the use of toxic cyanide solutions, required to form cyanocobalamin, it is possible to convert all the vitamin B12 to sulphitocobalamin by reaction with metabisulphite ($\text{Na}_2\text{S}_2\text{O}_5$) (Eitenmiller 1999). To establish if this route could be a practical solution and so avoid the use of cyanide solutions, optimisation of the HPLC-UV procedure would be required to assess the affinity of the antibody present on the immunoaffinity column to sulphitocobalamin. An alternative solution to this problem is to derive the α -ribozole, a fluorescent marker for the HPLC determination of

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2 1 vitamin B12 in foodstuffs (Pakin et al. 2005). The disadvantage associated with this method is the
3 2 overnight incubation required to form the α -ribozole.
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6 5 Commercially available carbonated energy activation and rejuvenation drinks containing 0.1
7 6 $\mu\text{g}/100\text{ ml}$ vitamin B12 according to the label claim were also tested. The drinks were found to
8 7 contain 0.11 $\mu\text{g}/100\text{ ml}$ and 0.95 $\mu\text{g}/100\text{ ml}$ respectively (Table 3). In order to obtain an indication
9 8 of column capacity, the activation and rejuvenation drinks were spiked with an additional 2 $\mu\text{g}/100$
10 9 ml and 15 $\mu\text{g}/100\text{ ml}$ or 20 $\mu\text{g}/100\text{ ml}$ vitamin B12. Recoveries of vitamin B12 from activation and
11 10 rejuvenation drinks spiked at 2 $\mu\text{g}/100\text{ ml}$ were 95% and 99% respectively with 0.4-0.6 μg vitamin
12 11 B12 being applied to the immunoaffinity column. In addition when the drinks were spiked at very
13 12 high levels (20 $\mu\text{g}/100\text{ ml}$ or 15 $\mu\text{g}/100\text{ ml}$) the recoveries were 85% and 84% with 3-4.5 μg
14 13 vitamin being applied to the column. Hence the capacity of the column (defined as that level where
15 14 recovery is equal to 70%) exceeds 4.5 μg . It should be noted the performance of the column could
16 15 be affected by more complex matrices especially at levels of vitamin B12 close to column capacity.
17 16 Therefore for assessment of routine samples the recommended maximum amount of vitamin B12
18 17 applied to the immunoaffinity column should not exceed 1.5 μg . In addition, the dilution of
19 18 activation and rejuvenation drinks with PBS prior to passage through the column resulted in a 3-
20 19 4% increase in recovery. This was not significant and therefore a change to protocol was not
21 20 recommended in this instance but dilution of sample with PBS may be used to greater effect when
22 21 analysing more complex matrices.
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24 23 To challenge the immunoaffinity column with samples containing low levels of vitamin B12 (<0.14
25 24 $\mu\text{g}/100\text{ ml}$) four types of fruit drinks (apple, orange and peach, blackcurrant and apple, and
26 25 strawberry juice) were analysed and the vitamin B12 content quantified (Table 3). The innate level
27 26 of vitamin B12 in the fruit drinks was not declared. Therefore, to determine a percentage recovery
28 27 of the vitamin, the fruit drinks were spiked with 1 $\mu\text{g}/100\text{ ml}$ vitamin B12 and recoveries in the
29 28 range of 78 - 86% were obtained (Table 3).
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31 30 INSERT TABLE 3 HERE
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33 32 The vitamin B12 content of 2 different brands of vitamin B12 supplement tablets were analysed to
34 33 compare the percentage of vitamin B12 recovered relative to the declared value. Brand 1 with a
35 34 declaration of 2000 $\mu\text{g}/0.849\text{ g}$, yielded 100% ($\text{RSD}_r = 3.1\%$) of the declared vitamin value and
36 35 Brand 2 with a declaration of 1000 $\mu\text{g}/0.580\text{ g}$, yielded 91% ($\text{RSD}_r = 3.7\%$) of the declared value.
37 36 Hence the described method can be successfully used to quantify the amount of vitamin B12 in
38 37 tablets.

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5 3 **Conclusion**

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7 4 In conclusion the immunoaffinity column isolates and concentrates vitamin B12 from a wide range
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9 5 of commodities prior to quantitative HPLC-UV determination at 361nm resulting in improved
10 6 sensitivity. Robust and reliable ($RSD_r = 0.8-10\%$) procedures for the accurate determination of
11 7 vitamin B12 in complex matrices (NIST 2383) containing low level vitamin B12 ($0.44 \mu\text{g}/100 \text{ g}$) as
12 8 well as simpler matrices such as vitamin tablets containing high levels ($2000 \mu\text{g}/0.849 \text{ g}$) of vitamin
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14 9 B12 were developed. The analysis of NIST Standard Reference Materials demonstrates the
15 10 immunoaffinity column and the microbiological assay generate comparable results in complex
16 11 matrices. In addition the method is rapid, inexpensive, and easy to use compared to existing
17 12 methods for vitamin B12 analysis.
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4 2 Chromatograms obtained for the determination of vitamin B12 in (a) NIST 1864 Milk-based Infant
5 3 Feed (3.9 $\mu\text{g}/100\text{ g}$), (b) NIST 1546 Meat Homogenate (0.6 $\mu\text{g}/100\text{ g}$) and (c) NIST 2383
6 4 Composite baby food (0.44 $\mu\text{g}/100\text{ g}$)
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Table 1. Percentage recoveries of vitamin B12 from a range of NIST SRM and Infant Formula

Product	Claim ($\mu\text{g}/100$ g)	Acceptable range ($\mu\text{g}/100$ g)	Amount determined ($\mu\text{g}/100$ g) (n=3)	Recovery relative to claim (%)	RSD _r (%) ¹
NIST Meat Homogenate 1546	0.6	0.5-0.7 ^a	0.5	83	2.9
NIST Baby Food Composite 2383	0.44	0.25-0.63 ^a	0.55	125	2.1
NIST Infant Formula 1846	3.9	3.6-4.2 ^a (3.23-4.49) ^b	4.1	106	0.8
Brand 1 Plus infant Formula	1.37	1.16-1.58 ^b	1.13	82	1.6
Brand 2 infant Formula	1.25	1.06-1.44 ^b	1.47	118	5.0
Brand 3 Infant Formula	1.30	1.11-1.50 ^b	1.48	114	5.9

^a As defined by NIST^b As defined by EU Legislation ($\pm 15\%$ of declaration)RSD_r represents the repeatability when analysing 3 test portions of each sample.

Table 2. Percentage recoveries of vitamin B12 from protein drinks and bars

Product	Claim	Recovery relative to claim (%)	RSD _r (%) ¹
Protein powdered drink 1	5.26 µg/0.580 g	103	2.7
Protein powdered drink 2	5.26 µg/0.580 g	94	10.2
Protein bar A	3.5 µg/100 g	124	7.8
Protein bar B	0.90 µg/100 g	166	8.8
Protein bar C	0.94 µg/100 g	183	5.4

RSD_r represents the repeatability when analysing 3 test portions of each sample.

Table 3. Percentage recoveries of vitamin B12 fortified and spiked energy drinks and fruit drinks

Product	Initial concentration (µg/100 ml)	Added concentration (µg/100 ml)	Concentration found (µg/100 ml)	Recovery (%)	RSD _r (%) ¹
Activation energy drink	0.1	2	1.2	95	9.9
Activation energy drink	0.1	20	17.1	85	
Rejuvenation fruit drink	0.1	2	2.1	99	
Rejuvenation fruit drink	0.1	15	12.7	84	
					RSD _r (%) ²
Apple juice	Not declared	1	0.76	78	6.5
Orange and peach juice	Not declared	1	0.96	86	
Blackcurrant and apple juice	Not declared	1	0.94	82	
Strawberry juice	Not declared	1	0.90	82	

¹RSD_r represents the repeatability data in analysing 2 different energy drinks at 3 levels of vitamin B12 using 3 test portions of juice at each level.

²RSD_r represents the repeatability data in analysing 4 different types of fruit juice drinks spiked at 1 µg/100ml vitamin B12 using 3 test portions of juice.

% Recovery values were corrected for natural vitamin B12 content.

Figure 1
Chromatograms obtained for the determination of vitamin B12 in (a) NIST Milk-based Infant Feed 1846 (3.9 µg/100 g), (b) NIST Meat Homogenate 1546 (0.6 µg/100 g) and (c) NIST Composite baby food (0.44 µg/100 g)

