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The efficiency of ultrasonic-assisted extraction of cyanocobalamin is greater than heat extraction

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ABSTRACT

Cyanocobalamin, like other water-soluble vitamins, is susceptible to degradation due to exposure to heat, UV, oxygen and pH. Built on our previous finding, this study aimed to assess the extraction efficiency of cyanocobalamin from dietary supplements. Particularly, cyanocobalamin extraction in a 100 °C water bath was compared with ultrasonic-assisted extraction, with and without the addition of 1 mg/L sorbitol, xylitol and erythritol. Ground defatted samples of supplement tablets were initially treated for 15 min, centrifuged and filtered before quantitative HPLC analysis. Addition of sorbitol and xylitol significantly minimised the thermal degradation during extraction in a 100 °C water bath, as shown in measured cyanocobalamin (~145 μg/tablet) that was higher than the control (100 μg/tablet, $p < 0.05$). Despite the addition of sugar alcohols, mean cyanocobalamin in ultrasonic extracted samples (~170 μg/tablet) was not significantly different from those without ($p > 0.05$). Overall, mean cyanocobalamin in sonicated samples was higher than heat-extracted counterparts, suggesting that extraction in a 100 °C water bath was likely to cause thermal degradation. It was possible that ultrasonic-assisted extraction had no effect on cyanocobalamin stability and would lead to a higher extraction efficiency. Therefore, 15 min extraction in an ultrasonic bath can be suggested to be adequate to release cyanocobalamin before its quantitative determination.

1. Introduction

The water-soluble vitamin B_{12}, also known as cobalamin, is naturally present in a plethora of vitamers subject to varying ligands. The β-axial ligand is linked to a cobalt-centred corrin ring that is also attached to α-glucoside and nucleotide moieties. Cyanocobalamin, methylcobalamin and hydroxocobalamin are some of the vitamers that have a distinctively β-axial ligand of –CN, $–CH_3$ and OH, respectively. Cobalamin is susceptible to UV exposure [1], acidic pH conditions [2], heat and the presence of other B vitamins, such as thiamin, niacin and ascorbic acid [3]. Any such exposure affects the stability of the chemical structure causing cobalamin to degrade. Cyanocobalamin is known to be the most stable vitamer in contrast to hydroxocobalamin and methylcobalamin [3]. Consequently, cyanocobalamin has been extensively used in dietary supplements and fortified foods.

Vitamin B_{12} functions as a co-factor or a coenzyme in many biochemical reactions. It is pivotal in the methylation reactions, isomerisation, reductive dehalogenation and radical S-adenosylmethionine processes [4]. Inadequate intake of vitamin B_{12} from diet and/or decreased absorption would impair the nutritional status and increase the risk of cobalamin deficiency. One approach used to increase the dietary intake of cobalamin is through supplementation that improves the nutritional status and mild cobalamin deficiency [5]. Single vitamin supplements containing either cyanocobalamin or methylcobalamin are commercially available in Australia at a dose from 100 μg to 1000 μg per tablet. Also, multivitamins containing cyanocobalamin can generally be purchased over the counter from pharmacies and chemists. It is worth mentioning that in Australia, the recommended dietary intake (RDI) for individuals aged 19 years and above is 2.4 μg per day [6]. The levels of cobalamin in the supplements are 42 times or higher compared to RDI.

Quantitative determination of cyanocobalamin necessitates the vitamin to be released from the complex matrices. One of the most used methods is solvent extraction such as infusion and maceration. Infusion is a process that takes advantage of boiling temperature to release cyanocobalamin into the extraction solvent(s), while maceration allows soaking at certain conditions to release the compound of interest [7]. Different techniques have been documented in the literature to extract cyanocobalamin from ground dietary supplements, such as sample preparation in a 65 °C water bath for 10 min [8], using a rotary shaker [9] and in an ultrasonic water bath [10]. Ultrasound-assisted extraction (UAE) has been commonly practised in analytical chemistry for sample preparation using either an ultrasonic bath or a probe-based system. UAE

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is recognised for its advantages due to its simplicity, cost-effectiveness, high extraction yield, as well as a fast mass transfer and usage of green eco-friendly solvents [11]. Not only does UAE reduce the extraction time, but it also has high reproducibility and the solvents usage is much less compared with soxhlet extraction and maceration [12].

Barr et al underpinned the stabilising effect of sorbitol in vitamin B_{12} aqueous solutions, while sucrose and dextrose cause the degradation of the vitamin [13]. Also, sorbitol improves the stability of cobalamin in mixed preparations of vitamin B_{12} and ascorbic acid due to its water--binding capacity causing water to be less available for chemical interaction of the vitamins [14]. There is a paucity of evidence in the field until our recent study that found that sorbitol improved the stability of cyanocobalamin in solution by minimising the degradation loss during 30 min of 100 °C heat treatment [3]. To further this knowledge, vitamin B_{12} dietary supplement used as a solid matrix was subjected to heat extraction to assess the stability of cyanocobalamin. Furthermore, the heat extraction of cyanocobalamin was then compared with UAE and the impact of different types of added sugar alcohol on the cyanocobalamin stability. Ultimately, this study aimed to optimise the extraction efficiency indicative of the release of cyanocobalamin from dietary supplement samples prior to HPLC analysis.

2. Methods

2.1. Chemicals

Cyanocobalamin, D-sorbitol (99%, CAS-50-70-4), xylitol (≥99%, CAS-87-99-0), erythritol, n-hexane (HPLC grade, CAS-110-54-3) and methanol (HPLC grade, CAS-67-56-1) were purchased from Sigma-Aldrich (Sydney, NSW, Australia). Ammonium acetate (97%, CAS-631-61-8) was acquired from Ajax Finechem (Sydney, NSW, Australia).

2.2. Sample preparation

A dietary cyanocobalamin supplement was obtained from a local pharmacy in Sydney (NSW, Australia) in October 2018. According to the nutrition information panel each tablet contained 100 μg cyanocobalamin. Supplement tablets (15 g) were ground to a fine powder (IKA, Sydney, NSW, Australia) and samples were used to evaluate the extraction efficiency.

The need for de-fatting the ground samples before cyanocobalamin extraction was assessed at the beginning of the study and was compared with the non-defatted counterparts. To remove the fatty component, 30 mL of hexane was added into centrifuge tubes (in triplicate) containing 4 g ground samples (Figure 1). Samples were placed on the ten-roller mixer (Thermo Scientific, Sydney, NSW, Australia) and were mixed overnight (approximately 16 h). Afterwards, the samples were centrifuged (Thermo Fisher, Sydney, NSW, Australia) for 15 min at 10,000 g at 4 °C, and the supernatants containing fat-soluble components were discarded. The pellets were dried under a gentle flow of nitrogen gas for approximately 4 h until completely dried. Defatted samples were stored at -20 °C freezer before cyanocobalamin extraction. The tubes were covered with aluminium foil to minimise degradation due to UV exposure and the experiments were carried out in a UV-free laboratory. Cyanocobalamin was extracted from defatted and non-defatted samples in an ultrasonic water bath for 15 min, as described in more detail below.

2.3. Sugar alcohol preparation

Besides sorbitol, this study evaluated the effect of added xylitol and erythritol on the stability of cyanocobalamin during extraction. Sugar alcohol solutions were prepared by dissolving each sugar alcohol in Milli-Q water on the day of analysis. The concentrations of sorbitol, xylitol and erythritol tested in the present study were 1 mg/mL (1000 ppm) according to our previous finding [3].

2.4. Heat extraction

To evaluate the stability of cyanocobalamin during heat extraction, each sugar alcohol solution (10 mL) was dispensed into centrifuge tubes containing defatted samples (0.5 g, n = 3), vortexed and placed in a 100 °C shaking water bath for 15 min and then cooled down (Figure 1). Thereafter, the samples were centrifuged for 15 min at 10,000 g at 4 °C. The supernatants (3 mL) were filtered using Millex-GP 0.22 μm syringe filter (Millipore, Sydney, NSW, Australia) into HPLC vials then analysed

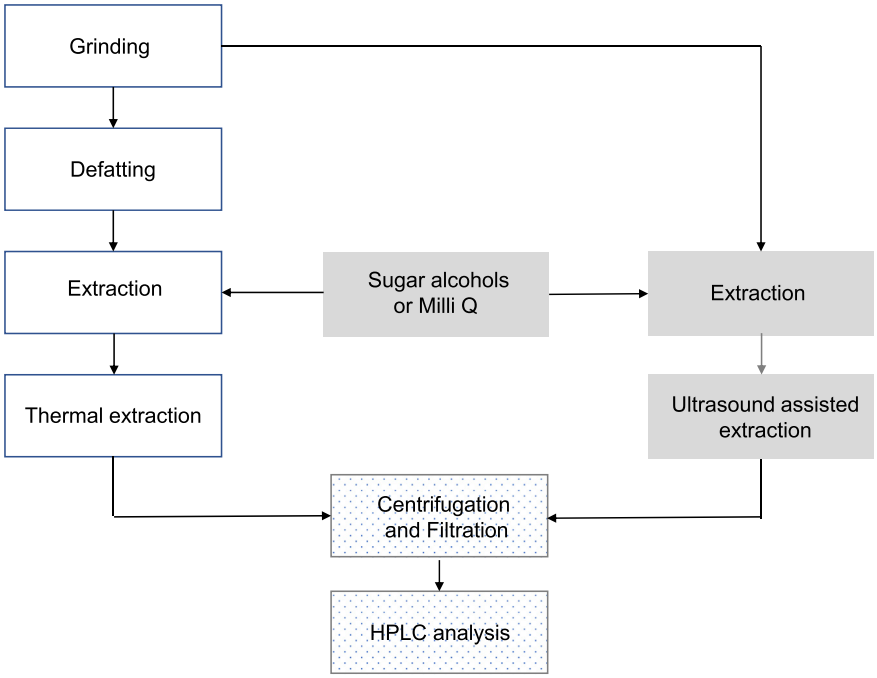

Figure 1. The experimental set up to assess the extraction efficiency of cyanocobalamin.

using HPLC. Controls samples were prepared alongside without added sugar alcohol.

2.5. Ultrasonic-assisted extraction (UAE)

The stability of cyanocobalamin during heat extraction was compared with UAE to assess the extraction efficiency. In this experiment, defatted samples (0.5 g, in triplicate) were initially dissolved in 10 mL of sugar alcohol solutions, vortexed and placed in an ultrasonic bath (Thermo, Sydney, Australia) for 15 min (Figure 1). The power and the frequency of the ultrasonic bath were 700 Watt and 40 kHz, respectively. The temperature of the water bath (volumetric power of 33.8 Watt/L) was set at 20 °C.

Thereafter, the samples were centrifuged, and the supernatants were filtered using Millex-GP 0.22 μm syringe filter. Control samples with no added sugar alcohols were also processed together with treated samples.

2.6. Cyanocobalamin standard solutions

Stock standard solutions (100 μg/mL) were prepared by adding 10 mL of Milli Q water to 1 mg of cyanocobalamin. Working standard solutions used for the calibration curves were prepared fresh on the day of analysis.

2.7. Cyanocobalamin in selected supplements

Four different brands of vitamin B_{12} supplements (1000 μg/tablet) and one brand of multivitamins containing cyanocobalamin were purchased in September 2019. Samples were defatted (in triplicate) prior to UAE and processed before HPLC analysis as previously described. Additionally, the sample that was purchased in 2018 and stored in the amber bottles at -20 °C was also de-fatted, extracted and measured alongside the recently purchased samples.

2.8. Cyanocobalamin in the fortified wheat flour

Ultrasound-assisted extraction was carried out to determine cyanocobalamin in fortified wheat flour. Commercial organic white flour was obtained from a supermarket in Sydney and the vitamin premix containing 30.5 μg/g cyanocobalamin was a gift from DSM (Auckland, New Zealand). To 100 g flour ($n = 3$) 0.1 g of the vitamin premix was added and mixed thoroughly using Thermomix (Vorwerk, Wuppertal, Germany). The extraction solution used for cyanocobalamin extraction was 40% (v/v) methanol in 100 mM hydrochloric acid. The pH of this solution was adjusted to 4.5 using 2.5 M sodium acetate. Extraction solution (5 mL) was aliquoted to 1 g fortified flour ($n = 3$), sonicated for 15 min and centrifuged as previously described. The immunoaffinity columns (r-Biopharm, Sydney, Australia) were then employed to clean-up the supernatants to remove the matrix interference [15]. Following this, the eluents were evaporated to dryness using a CentriVap (Labconco, Kansas City, MO, USA). Samples were resuspended in 1 mL of the mobile phase prior to HPLC analysis.

2.9. HPLC analysis

An HPLC system (Shimadzu Prominence UFLC, Kyoto, Japan) interfaced with a photodiode array detector was employed in this study, as previously reported [3]. Chromatography was carried out on a reversed-phase column (4.6 mm × 150 mm, 5 μm, Kinetex Biphenyl, Phenomenex, Sydney, Australia). The mobile phase system consisted of 50 mM ammonium acetate (pH 5.0, solvent A) and 50% (v/v) methanol in Milli Q water (solvent B). The initial condition was set at 90% solvent A and 10% solvent B. The composition of solvent B was then raised to 100% in 4 min and remained at this condition for 10 min before going back to the initial condition (10% B) at 15 min. Each run was 25 min in duration, the flow rate and the injection volume were set at 0.8 mL/min and 50 μL, respectively.

2.10. Statistical analysis

The data set were analysed using IBM SPSS Statistics 22 (Sydney, NSW, Australia). The Kolmogorov–Smirnov test was carried out to assess the normal distribution. Normally distributed continuous variables were presented as mean values and standard deviations. Tukey's Studentized Range (HSD) and Bonferroni tests, as well as one-way analysis of variance (ANOVA), were applied to compare the mean difference. The level of significance was reported by a p-value 0.05.

3. Results and discussion

3.1. The need for de-fatting prior to extraction

The experimental data showed that measured cyanocobalamin in defatted samples was 20% higher than in non-defatted ones, and the difference was significant ($p < 0.05$). This suggested that de-fatting of samples was necessary to accurately determine the cyanocobalamin concentrations. De-fatting also produced clearer extracts due to fewer matrix effects, thus facilitating a greater release of cyanocobalamin during the sonication. Similar to pharmaceutical formulations, dietary supplements often contain excipients, which should maintain the dosage, stability and bioavailability of the active ingredients [16]. Excipients, such as binders, emulsifiers, humectants [17] incorporated into the B_{12} supplements might have been removed during the de-fatting process as indicated in our experiment, thus assisting in the quantitative determination of the vitamin.

3.2. Heat extraction of cyanocobalamin

Samples without added sugar alcohols that were treated at 100 °C showed 43% less cyanocobalamin compared to the ultrasonic treated counterparts ($p < 0.05$). This suggested that degradation of cyanocobalamin might have occurred in the water bath when samples were exposed to 100 °C temperature. The thermal sensitivity of cyanocobalamin observed here was consistent with the literature [18]. It has been reported that increasing the storage temperature from 4 °C to 30 °C causes a greater loss of cyanocobalamin in beverages [19].

When sorbitol and xylitol were added into heat-treated samples, mean cyanocobalamin concentrations per tablet were 152 μg and 136 μg, respectively. These were significantly higher than those without added sugar alcohols (100 μg/tablet; $p < 0.05$). Thus, the addition of either sorbitol or xylitol to heat-treated samples seemed to effectively minimise the degradation loss of cyanocobalamin (Table 1). Mean cyanocobalamin in samples with added sorbitol was 52% higher than those without. When xylitol and erythritol were added to samples, measured cyanocobalamin levels were 37% and 8% higher than those without added sugar alcohols. This data supported our previous findings [3] that decomposition of cyanocobalamin is reduced because sugar alcohols bind to available water and slow down the hydrolysis rate [13]. It was found that mean cyanocobalamin in samples with added erythritol was 109 μg/tablet and was not significantly different to samples without added erythritol ($p > 0.05$). The result revealed that addition of erythritol might

Table 1. Mean cyanocobalamin and degradation losses in heat-treated samples compared with sonicated samples (± corresponds to standard deviation).

Samples	Heat-treated (μg/tablet)	Sonicated (μg/tablet)	Difference (%)
Control (no sugar alcohols)	100 ± 17	177 ± 13	
Sorbitol	152 ± 11	173 ± 11	12
Xylitol	136 ± 10	159 ± 24	14
Erythritol	109 ± 4	168 ± 18	35

exert a less protective effect against cyanocobalamin degradation during 15 min of heat treatment at 100 °C.

3.3. Extraction in an ultrasonic bath

Overall, mean cyanocobalamin in ultrasonic treated samples (with and without added sugar alcohols) was higher than the heat-treated ones with added sugar alcohols (Table 1). Sonicated samples with added sugar alcohols contained approximately 170 μg/tablet cyanocobalamin and the mean difference between samples with added sorbitol, xylitol and erythritol was not significant ($p > 0.05$). Also, mean cyanocobalamin in sonicated samples with added sugar alcohols was not significantly different than those without added sugar alcohol ($p > 0.05$). The data suggested that independent of the types of sugar alcohol added, sonication would be enough to release and isolate cyanocobalamin from dietary supplements as opposed to heat extraction. Furthermore, it can be implied that no degradation loss might have occurred during extraction in an ultrasonic bath. This could possibly be due to the low-intensity of sonication (<1 W/cm^2), a non-destructive technique that allows determination of the composition, structure and physical state of matter [11].

3.4. Cyanocobalamin in dietary supplements

Our data showed that both de-fatting and UAE were adequate to release cyanocobalamin from dietary supplements (Figure 2). Mean cyanocobalamin in brand A that was determined in 2018 was 177.0 μg/tablet (Table 2) and was greater than the declared label value (100 μg/tablet). When analysed in 2019, mean value of this brand was reduced to 54.8 μg per tablet, suggesting that cyanocobalamin in the tablets has degraded after a year. Despite being stored at -20 °C in the amber glass container, the data showed that only 31% of cyanocobalamin was retained in the brand A supplement. It is likely that exposure of air to vitamin tablets when opening and closing of the container could affect the stability of cyanocobalamin. A previous study reported that oxygen from the air promotes oxidation of vitamin B_{12} in milk [20] and this might have a similar effect on vitamin tablets. How oxidative degradation affects the stability of cyanocobalamin in supplements warrants further investigation in the future. Notably, the shelf life of brand A supplement was still within the use-by date, and yet the amount of cyanocobalamin (54.8 μg/tablet) was lower than the declared value of 100 μg/tablet.

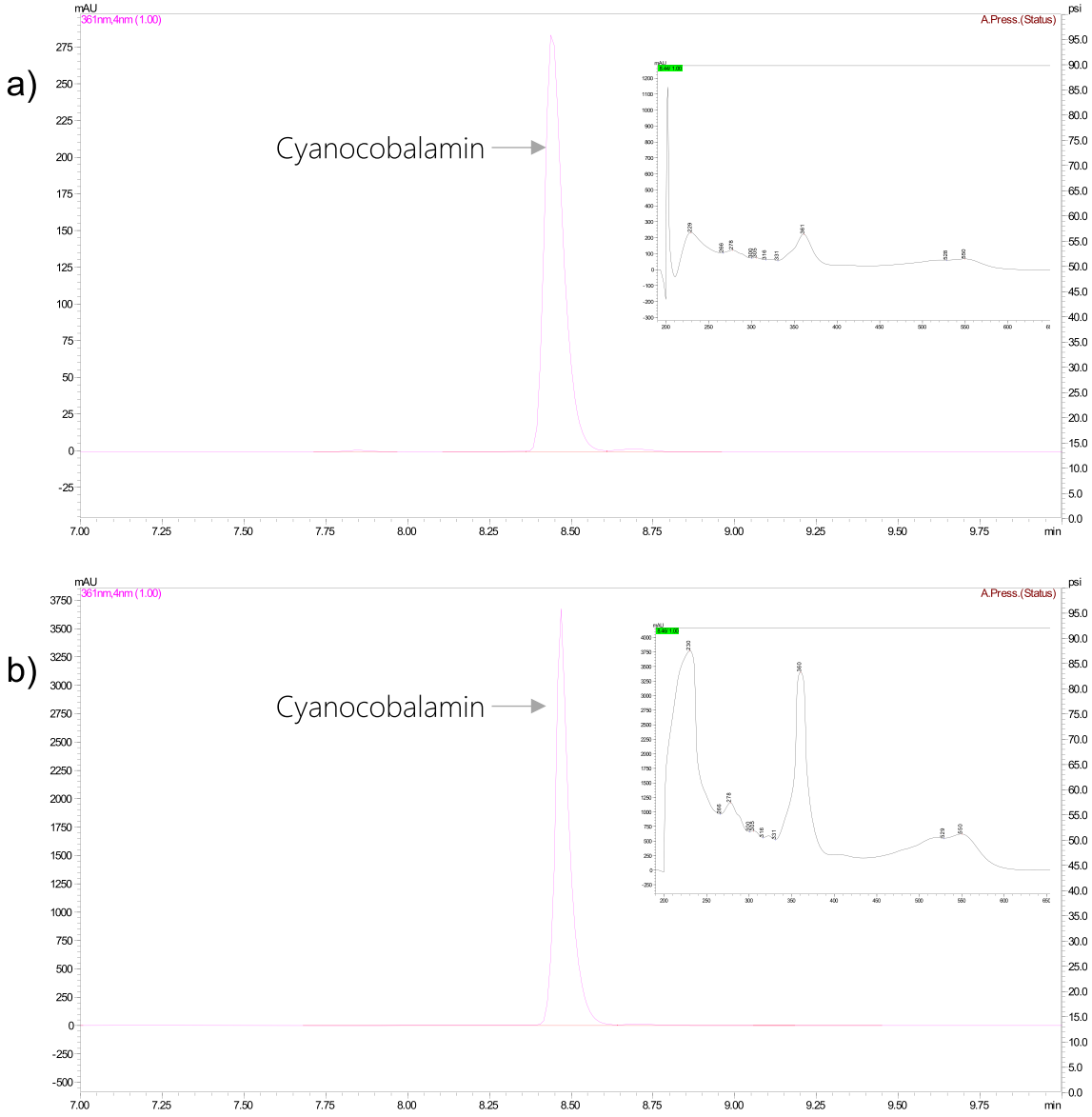

Figure 2. The chromatogram of cyanocobalamin with its specific absorption spectra. The peaks of the cyanocobalamin standard (a) and a supplement sample (b) both eluted at 8.4 min. The absorption spectra, maximum at 361 nm, was used for cyanocobalamin quantitation.

Table 2. Measured cyanocobalamin in selected dietary supplements (Most supplement brands contained only cyanocobalamin, only brand F was multivitamins; ± corresponds to standard deviation).

Different brands of the supplements	Mean ± sd (μg/tablet)	Declared value (μg/tablet)	Mean mass per tablet (g)
A (purchased in 2018)	177 ± 13	100	0.52 ± 0.002
A (purchased in 2018 reanalysed in 2019)	54.8 ± 6.0	100	0.52 ± 0.002
B	1125.2 ± 156.8	1000	0.35 ± 0.003
C	1587.0 ± 78.6	1000	0.26 ± 0.004
D	1205.8 ± 21.5	1000	0.41 ± 0.007
E	1116.2 ± 77.8	1000	0.40 ± 0.007
F (Multivitamins containing B_{12})	38.0 ± 1.5	50	0.52 ± 0.002

Measured cyanocobalamin in two brands was ~1100 μg/tablet and 12% greater than the declared label value (Table 2). The maximum amount of cyanocobalamin was found in one brand with 1578.0 μg/tablet and was 57% greater than the declared label value. The experimental data were consistent with previous findings that reported greater analysed values than declared label values [19, 21]. Overage is commonly practised in the manufacturing of dietary supplements to compensate for the inhomogeneity and losses of active ingredients during processing and storage [22]. Degradation losses undeniably occur during the storage of vitamins [21] as shown previously in our experimental data. Although, the shelf life of the vitamin tablets was still before the 'use-by date', 30% of cyanocobalamin was retained during the storage.

Brand F multivitamins tablets contained 38 μg of cyanocobalamin per tablet and the amount was lower than the declared label value of 50 μg/tablet (Table 2). The result suggested that the presence of other water-soluble vitamins, such as thiamin affects the stability of cyanocobalamin [3, 23]. An explanation for this is that thiamin degrades during storage producing a thiazole moiety [24]. It is the breakdown product of the thiazole moiety, particularly cysteine hydrochloride, that is responsible for the degradation of cyanocobalamin [24].

3.5. Extraction of cyanocobalamin fortified wheat flour

In order to test the efficiency of UAE in a different matrix, cyanocobalamin was extracted from fortified flour samples. Mean cyanocobalamin measured in the samples was 0.037 ± 0.004 μg/100 g and was consistent with the expected concentration of 0.035 μg/100 g. The data presented here suggest that there were no losses of cyanocobalamin during sample preparation and HPLC determination. It could be deduced that cyanocobalamin was efficiently released from the flour samples following UAE and immunoaffinity clean-up, consistent with the finding of Marley et al [10]. Authors reported that immunoaffinity columns successfully removed any matrix interferences, isolated and concentrated the analyte, thus allowing even low cyanocobalamin concentrations to be detected and quantified using HPLC [10].

4. Conclusion

Thermal degradation of cyanocobalamin occurred during heat treatment in a 100 °C water bath for 15 min to extract cyanocobalamin from the dietary supplements and this was minimised by the addition of sorbitol, xylitol and erythritol. In fact, our results suggest that cyanocobalamin extraction in an ultrasonic bath is adequate to release cyanocobalamin, even without the addition of sugar alcohols. The cavitation bubbles that are formed by ultrasound lead to mechanical and chemical effects. Consequently, this causes the supplement's matrix to break down and cyanocobalamin to be released, as shown in this study.

Declarations

Author contribution statement

Han Xu: Performed the experiments; Analyzed and interpreted the data.

Jayashree Arcot: Contributed reagents, materials, analysis tools.

Maria Veronica Lisa Chandra-Hioe: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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