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Original article

A rapid HPLC method for the extraction and quantification of vitamin B_{12} in dairy products and cultures of *Propionibacterium freudenreichii*

Jessy Van Wyk[1*], Trevor J. Britz[2]

[1] Department of Food Technology, Cape Peninsula University of Technology, Bellville 7535, South Africa
[2] Department of Food Science, University of Stellenbosch, Stellenbosch 7600, South Africa

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Abstract – To overcome nutritional vitamin B_{12} deficiencies in certain populations in Southern Africa, fortified functional foods have been developed. However, current microbiological methods used to accurately determine vitamin B_{12} levels in foodstuffs, important for quality control and regulatory purposes, are time consuming. This study describes an extraction and detection method for vitamin B_{12} in dairy products and growth media cultured with *Propionibacterium freudenreichii*. Samples were extracted by mixing in KCN buffer (pH 4.5), autoclaving at 121 °C for 25 min, cooling and centrifugation. The resultant supernatant was syringe-filtered prior to reversed-phase HPLC analysis using a methanol-water gradient that was effective in resolving the B_{12} peak. The method offered excellent linearity with a regression coefficient $R > 0.998$. The limit of quantification was 0.005 $\mu g \cdot mL^{-1}$ sample. For samples with vitamin B_{12} concentrations well within the linear range of the assay, good repeatability was demonstrated for the same sample with mean concentrations of 2.62 ± 0.02 and 2.61 ± 0.02 $\mu g \cdot mL^{-1}$ detected on day 1 and day 2, respectively. Recovery values ranged from 98.6% to 103.2%, indicating that the extraction method ensured complete dissolution of vitamin B_{12} from the matrices under study. Sensitivity was enhanced by sample concentration and purification using a series of solid phase extraction steps which resulted in improved peak resolution and removal of interfering peaks. The method was validated by comparison of the HPLC results of the same sample with those obtained using traditional microbiological methods. The method is a rapid alternative to the more time-consuming microbiological assay.

***Propionibacterium freudenreichii* / microbiological vitamin B_{12} assay / HPLC / vitamin B_{12}**

摘要 – HPLC 法快速提取和定量测定乳制品和费氏丙酸杆菌发酵产品中维生素 B_{12}。 为了治疗南非部分人群中出现的维生素 B_{12} 缺乏症，在一些功能性食品中强化了维生素 B_{12}。然而目前用于食品中维生素 B_{12} 检测的方法是微生物发酵法，该法特点是耗时。 本文研究了乳制品和费氏丙酸杆菌 (*Propionibacterium freudenreichii*) 发酵产品中维生素 B_{12} 的提取和测定方法。 乳样品与 KCN (pH 4.5) 缓冲溶液混合，在 121 °C 下高压灭菌 25 min，冷却、离心。上清液用于 RP-HPLC 分析，甲醇–水为流动相，进行梯度洗脱。方法的线性回归系数为 $R > 0.998$。定量检测限位为 0.005 $\mu g \cdot mL^{-1}$。样品中维生素 B_{12} 的浓度在测定方法的线性范围之内，方法的重现性较好，同一样品在第一天和第二天的测定值分别为 2.62 ± 0.02 $\mu g \cdot mL^{-1}$ 和 2.61 ± 0.02 $\mu g \cdot mL^{-1}$。 方法的回收率在 98.6% ～ 103.2%，表明所采用的提取方法能够完全溶

*Corresponding author (通讯作者): vanwykj@cput.ac.za

解所研究基质中的维生素 B_{12}。采用一系列固相萃取技术将样品浓缩和纯化后，消除了干扰峰，维生素 B_{12} 的色谱峰形好，使分析方法的灵敏度大大提高。将本研究方法的实验结果与传统微生物法测定的结果进行了对比，证明该法是快速、准确、可行的维生素 B_{12} 分析方法。

***Propionibacterium freudenreichii* / 微生物测定法 / HPLC / 维生素 B_{12}**

Résumé – Une méthode HPLC rapide pour extraire et quantifier la vitamine B_{12} dans les produits laitiers et les cultures de *Propionibacterium freudenreichii*. Pour lutter contre les carences en vitamine B_{12} parmi certaines populations d'Afrique du Sud, des aliments fonctionnels enrichis ont été développés. Cependant, les méthodes actuelles utilisées pour analyser précisément le niveau de vitamine B_{12} dans les aliments, qui sont importantes à des fins de contrôle-qualité et de législation, sont coûteuses en temps. Cet article décrit une méthode d'extraction et de détection de la vitamine B_{12} dans les produits laitiers et les cultures de *Propionibacterium freudenreichii*. Les échantillons étaient extraits par mélange à du tampon KCN, pH 4,5 et autoclavage à 121 °C pendant 25 min, refroidissement et centrifugation. Le surnageant résultant était filtré puis analysé par HPLC en phase inverse en utilisant un gradient méthanol-eau, qui permettait une bonne résolution du pic de vitamine B_{12}. La méthode montrait une bonne linéarité, avec un coefficient de régression $R > 0{,}998$. La limite de quantification était de 0,005 $\mu g \cdot mL^{-1}$ d'échantillon. Pour des échantillons contenant des concentrations dans la moyenne de la gamme de linéarité, la répétabilité a été établie, avec des valeurs obtenues pour le même échantillon à deux jours différents de 2,62 ± 0,02 $\mu g \cdot mL^{-1}$ et 2,61 ± 0,02 $\mu g \cdot mL^{-1}$. Les rendements d'extraction variaient entre 98,6–103,2 %, ce qui indique que la méthode d'extraction permettait une dissolution complète de la vitamine B_{12} des matrices étudiées. La méthode HPLC était validée en comparant les résultats obtenus avec ceux de la méthode microbiologique de référence. La sensibilité de la méthode était améliorée par une série d'étapes d'extraction en phase solide pour concentrer et purifier les échantillons, ce qui améliorait la résolution des pics et éliminait les pics contaminants. La méthode décrite est une alternative rapide à la méthode microbiologique de référence.

***Propionibacterium freudenreichii* / analyse microbiologique / HPLC / vitamine B_{12}**

1. INTRODUCTION

Vitamin B_{12} plays many important roles in normal metabolism [6]. Subnormal vitamin B_{12} levels have been linked to reversible dementia [30], while vitamin B_{12} deficiency results in pernicious anaemia and is also manifested by neurological changes which could culminate in brain damage [6, 16]. Deficiency had also been associated with cerebellar ataxia [26], triggering seizures in epileptics [29] and reversible involuntary movements in adults [8]. More recently, low folate and vitamin B_{12} levels have been linked to psychiatric diseases, such as bipolar disorder [28]. Furthermore, not only were low vitamin B_{12} levels recorded in HIV-infected patients [14], but cobalamins demonstrated anti-retroviral activity against HIV-1 in vitro [23, 32], and the possibility that adequate vitamin B_{12} may slow the onset of AIDS in HIV-positive individuals was also reported [2].

Since chemical synthesis is extremely complex, only biosynthetic processes are used for the commercial production of vitamin B_{12} [27]. The microorganisms most widely used in these fermentations include *Propionibacterium* spp. [13, 27], *Pseudomonas denitrificans* [21], methanogens (*Methanosarcina barkeri*, *Methanobacterium* spp.) and *Bacillus* spp. [24, 34]. However, since some *Propionibacterium* species were granted GRAS (generally recognised as safe) status by the United States Food and Drug Administration [27], as well as European QPS (qualified presumption of safety) status [4], they are the preferred organisms for vitamin B_{12} production [27]. Although *Propionibacterium*

freudenreichii retains most of the synthesised vitamin intracellularly [35], the vitamin is readily released in acidic solutions (pH < 5.0), including the human stomach, thus ensuring bioavailability when ingested [31].

Since vitamin B_{12} deficiencies are prevalent among the poor in Southern Africa [10, 18], there is a need to produce fortified functional foods to combat these deficiencies. However, since activity may be lost during processing and storage, quantification of vitamin B_{12} is needed to ensure quality control, to satisfy government regulatory requirements, including nutrition labelling [3, 12].

Vitamin B_{12} assay techniques include microbiological assays, radioisotope dilution assay, electrothermal atomic absorption spectrometry, capillary electrophoresis, chemiluminescence techniques, biomolecular interaction analysis (BIA) and a combination of fluorescence resonance energy transfer (FRET) and flow-injection analysis (FIA) [1, 6, 12, 22, 33]. The microbiological assay is still the official method, but is very laborious [1]. High-performance liquid chromatography (HPLC) has been employed by a number of workers to assay vitamin B_{12} in multivitamin and mineral tablets. These matrices are less complex than foodstuffs and, therefore, extraction and resolution of the vitamin is simple in comparison [20, 22, 25]. More recently, reversed-phase HPLC methods with immunoaffinity extraction and UV detection [15], and coulometric electrochemical detection [19] were used to assay vitamin B_{12} in petfood and various infant formulae [15] and fortified fruit juice and various seafoods [19]. However, these food matrices did not include complex dairy products. Furthermore, none of the above-mentioned studies included extraction and analysis of vitamin B_{12} from propionibacteria (PAB). Hence, the aim of this study was to develop and optimise both an extraction protocol and an HPLC method that will afford a more rapid vitamin B_{12} analysis of milk and fermented dairy products, as well as of synthetic media cultured with *P. freudenreichii* strains.

2. MATERIALS AND METHODS

2.1. Chemicals, reagents and other materials

Vitamin B_{12} (cyanocobalamin) (Fluka) standard was used. Methanol (BDS) and acetonitrile (BDS) were of HPLC grade. All other chemicals were of Analar grade. Reverse osmosis Milli-Q water (18 MΩ), purified with a Milli-Q water purification system (Millipore, Microsep, Bellville, South Africa), was used for all solutions and dilutions.

The KCN-acetate buffer consisted of ($g \cdot L^{-1}$) 20 g KCN (Saarchem) and 544.32 g sodium acetate (CH_3COONa) (Saarchem). Prior to dilution to the required concentration, the pH was adjusted to 4.5 using glacial acetic acid (BDH Chemicals) [19]. The medium (B_{12} medium) used to enhance vitamin B_{12} synthesis consisted of ($g \cdot L^{-1}$): polypeptone (BBL) 12.5; casamino acids (Difco) 11.0; yeast extract (Difco) 2.5; $NaH_2PO_4 \cdot 2H_2O$ (Merck) 2.25; $MgCl_2 \cdot 6H_2O$ (Merck) 0.4; K_3P0_4 (Merck) 1.76; $CoCl_2 \cdot 6H_2O$ (Merck) 0.018; $FeSO_4 \cdot 7H_2O$ (Merck) 0.01; D-calcium-pantothenate (Fluka) 0.004; (+)-Biotin (Fluka) 0.0003; Tween 80 (Saarchem) 1.0 mL; glucose (BDH Chemicals) 40; and 5,6-dimethylbenzimidazole (DBI) (Merck) 0.07. The pH of the medium was adjusted to 7.0 with 40 $g \cdot L^{-1}$ NaOH prior to autoclaving at 121 °C for 15 min [35]. Yeast extract sodium lactate (YEL) broth contained in $g \cdot L^{-1}$: yeast extract (Oxoid) 5.0; sodium lactate syrup (500 $g \cdot L^{-1}$) (Saarchem) 20.0; peptone (Biolab) 2.0; KH_2PO_4 (Merck) 10.0; and Tween 80 (Saarchem) 1.0 mL [7].

Eight strains of *P. freudenreichii* were used. Strains J8, J9, J10, J15 and J16 were obtained from the culture collection of the Department of Food Science, University of Stellenbosch, strain J17 (ATCC 13673) from

the American Type Culture Collection and strains J18 and J19 from the All Russian Collection of Microorganisms, Pushino IBFM, Russian Academy of Sciences. The inoculum volume was 200 μL·100 mL^{-1} broth, with a cell count of 1×10^7 cfu·mL^{-1} (variation of 5% at final count). The test organism used in the microbiological assay, *Lactobacillus delbrueckii* ssp. *lactis* (ATCC 7830), was obtained from the South African Bureau of Standards (SABS).

UHT skim milk, 10 g·L^{-1} fat content, UHT full cream milk, 33 g·L^{-1} fat content and "Amasi", 50 g·L^{-1} fat content, a fermented milk product generally consumed in Southern Africa, were purchased from a local supermarket. Kefir grains were obtained from the Department of Food Science, University of Stellenbosch. Reactivation of the Kefir grains was achieved by adding 30 mL milk to 10 g grains and incubating at 25 °C for 12 h, with agitation. The milk phase was drained using a sterilised metal strainer. The grains were then inoculated into 100 mL UHT full cream milk and incubated at 25 °C for 12 h, after which the grains were separated from the Kefir beverage. Reactivated grains were also co-inoculated with strain J15 to obtain Kefir beverage samples fortified with vitamin B_{12}.

2.2. Standard solutions

The stock solution was prepared bimonthly by weighing out 4.0 mg cyanocobalamin and diluting it to 200 mL with KCN-acetate buffer in amber glassware and covering the container with aluminium foil. This solution, stored at 4 °C, was used to make dilutions as required for both the HPLC and microbiological assays and was completely stable for a minimum of five months. Since cobalamin powders are very hygroscopic [6] and sensitive to light [17], prior to preparing a fresh stock solution, the powder was dried to constant mass in a desiccator containing fresh silica gel in the dark.

2.3. Linearity, repeatability, recovery and validity

2.3.1. Linearity

The inherent sensitivity of the HPLC method was determined by analysing a series of vitamin B_{12} standard solutions, increasing linearly in concentration from 0.005 to 15 μg·mL^{-1} sample. The range of concentrations was chosen based on vitamin B_{12} levels (in μg·mL^{-1} sample) reported for dairy products (0.003–0.012 [11]) and synthesised by PAB (0.02 [5]; 0.14 [9]; and 12.50 [35]).

Seven samples per concentration were analysed ($n = 7$) and the resultant computed vitamin B_{12} concentration was plotted against the actual (i.e. expected) vitamin B_{12} concentration. A linear regression line, calculated by the method of least squares, was indicated. In addition, the correlation coefficient (R^2) and the regression coefficient (R) were calculated.

2.3.2. Repeatability

An homogeneous sample was divided into 14 aliquots, and the vitamin B_{12} concentration was determined in two series of seven aliquots on two successive days.

2.3.3. Recovery

A sample or placebo, i.e. a sample that initially contained no cyanocobalamin, was spiked with a known quantity of cyanocobalamin. After the normal extraction procedure, the added amount of cyanocobalamin was measured to determine the percentage recovery.

2.3.4. Validity

Using a selection of samples, the validity of the HPLC results was established by performing the microbiological assay

on aliquots of the same extract. All assays were performed in triplicate.

2.4. Sample extraction

The ratio of KCN-acetate extraction buffer (pH 4.5) to broth sample was 10:4 (v/v). The samples were protected from light as described. Autoclaving at 121 °C for 25 min was followed by rapid cooling and centrifugation at 15 000× *g* for 10 min at 4 °C. This was followed by (1) filtration through a 0.22-μm syringe filter (Cameo, Osmonics, Sigma Aldrich) into amber sample vials with solid caps (HPLC method) and (2) suitable dilutions, followed by inoculation (microbiological assay). HPLC samples that were not analysed immediately were stored at 4 °C in the dark.

The presence of cyanide in the extraction buffer is critically important to ensure quantitative conversion of all forms of vitamin B_{12} to cyanocobalamin, the most stable form of the vitamin, while the heat treatment liberates all protein-bound cobalamin [6]. Hence, this extraction method was used for both the HPLC and microbiological assays.

2.5. Sample purification and concentration

Since the vitamin B_{12} content of some dairy products is as low as 0.003 $\mu g \cdot mL^{-1}$ [11], sample purification and concentration was investigated to improve the sensitivity of the analysis. Solid phase extraction (SPE) columns were used to both purify and concentrate the sample. Purification involved application of 9–12 mL sample extract to a preconditioned SPE column (Chromabond SB/3 mL/500 mg, Macherey-Nagel, Düren, Germany) and collecting the eluate, i.e. the purified sample extract, for HPLC analysis. When sample concentration followed purification, immediately after collecting the purified sample extract, any vitamin B_{12} remaining on the SPE column was eluted with one column volume 400 $mL \cdot L^{-1}$ methanol (HPLC grade, BDS). This second eluate was collected separately. A preconditioned Chromabond C18ec/6 mL/1000 mg column (Macherey-Nagel) was then used to concentrate the sample. To achieve this, the first eluate (i.e. the purified sample extract) was applied to the column. The vitamin B_{12}, which remains as a visible pink to red band on the column, was then eluted using the second eluate (collected separately during the purification step), followed by 1 mL 900 $mL \cdot L^{-1}$ methanol. After flash evaporation of the methanol in a rotary evaporator at 40 °C, the concentrate was diluted with Milli-Q water to 3 mL in a volumetric flask. Syringe filtration (0.22-μm Cameo, Osmonics, Sigma Aldrich) into amber glass vials preceded the HPLC analyses.

2.6. HPLC

The HPLC separations were performed using a Hewlett-Packard 1100 HPLC system, equipped with a vacuum degassing unit, a quaternary pump and a Rheodyne 7125 injection valve fitted with a 20-μL sample loop. A 50-μL syringe was employed to ensure improved accuracy with respect to delivery of the desired 20-μL sample volume. A variable wavelength detector set at 361 nm was used. The HP Chemstation software (Hewlett-Packard, Waldbron, Germany) integrated, recorded and calculated the peak areas and was used to store data.

A range of chromatographic conditions was employed in an attempt to optimise peak resolution and response (peak area). These included the use of different analytical columns, adjusting the flow rate and increasing and decreasing the slope of the solvent gradient. Optimum conditions were found when the analytical column used was a 5-μm BDS-C_{18} column (250 × 2 mm internal diameter, i.d.) produced by Hewlett-Packard (Waldbron, Germany). A guard

cartridge (ThermoQuest, Needham, Massachusetts) (4 × 4 mm i.d.) packed with 5 μm Hypersil BDS-C_{18} packing material was installed ahead of the analytical column. The guard cartridge was consistently replaced after 150–200 injections. The mobile phase was a 15:85 to 50:50 (v/v) methanol-water linear gradient over 25 min with a flow rate of 0.35 mL·min^{-1}. The retention time of cyanocobalamin was 12.46 ± 0.48 min under these conditions.

Utilising the calibration function of the HP Chemstation software to construe the calibration curve, quantification of the vitamin B_{12} levels of samples was performed by the external standard method. Hence, a standard solution diluted to a known concentration on the day was analysed in duplicate and the software programmed to use the resultant peak area to calculate the concentration of vitamin B_{12} in the samples. Regeneration of the column between injections entailed post-run flushing with 100% acetonitrile (5 min), followed by 100% water (5 min), and then recycled to initial conditions (5 min).

2.7. Microbiological assay

The microbiological assay method of Angyal [1], with modifications, was employed. The test organism, *L. delbrueckii* ssp. *lactis* ATCC 7830, was routinely cultured in MRS broth or MRS agar (Biolab). One day prior to the assay, the culture was inoculated into Bacto Lactobacilli broth AOAC (Difco) liquid culture medium and incubated at 37 °C. On the day of the assay, the cultures were centrifuged (10 000× *g*, 10 min, 4 °C), the cells were washed and resuspended in a 10 mL sterile physiological saline solution (p.s.s.) under aseptic conditions. To obtain the inoculum, this cell suspension was diluted with sterile p.s.s. to 60 %T (read against sterile saline set at 100 %T).

The requisite standard solutions (with cyanocobalamin concentrations of 0.01, 0.014 and 0.02 ng·mL^{-1}) were prepared by diluting the stock solution with the KCN-acetate buffer and not with 25% alcohol as suggested by Angyal [1].

As mentioned earlier, since the KCN buffer ensures superior vitamin B_{12} stability in the sample extract [6], it was preferred to the metabisulphite buffer described by Angyal [1]. Furthermore, since replicate samples were assayed using the HPLC, the extraction procedure was as described earlier.

After preparation and sterilisation of the standard and assay tubes, a Distriman repeater pipette equipped with a gamma-sterilised tip (Gilson, Villiers-le-Bel, France) was used to deposit one drop of inoculum directly onto the surface of the tube contents. Check tubes (replicates of the primary standard tubes) were prepared as prescribed.

The tubes were then incubated at 37 °C for up to 72 h. Two check tubes were removed on a 24 h basis and titrated against 4 g·L^{-1} NaOH. After a maximum of 72 h, or when the check tube with the highest concentration of vitamin B_{12} primary standard required 8–12 mL NaOH, the tubes were refrigerated at 4 °C to retard further acid production. The relative growth in all the standard and assay tubes was then determined by titrating against 4 g·L^{-1} NaOH to a set end-point of pH 6.8, using a Methrohm 702 SM Titrino automatic titrator (Swiss Lab Technologies, Standton, South Africa).

Averages of the replicate values were used to plot the standard curves and these, in turn, were employed to reference the amount of vitamin B_{12} in the assay tubes. The vitamin B_{12} content of the original sample was calculated taking all dilution factors into account.

2.8. Data analyses

All statistical analyses of data were performed using SPSS 16.0 for Windows®. The relative standard deviation (RSD) was calculated for the recovery data as a measure of inter-assay variability.

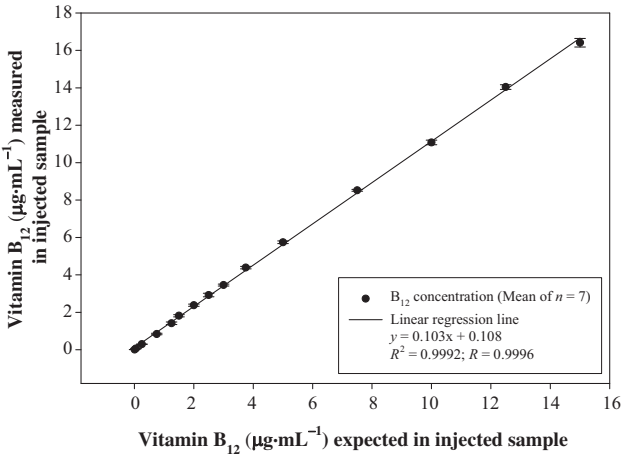

Figure 1. Linearity of vitamin B_{12} analyses. Error bars indicate the SD.

3. RESULTS AND DISCUSSION

3.1. Linearity and sensitivity

The correlation coefficient (R^2) and the regression coefficient (R) were 0.9992 and 0.9996, respectively. Confidence of linearity, signified by $R > 0.998$, is closely related to the rate of recovery, i.e. the accuracy of the analysis [19]. The linear appearance of the plot depicted in Figure 1, combined with the fact that the correlation coefficients approached unity, indicated that the measured vitamin B_{12} concentration was linear over the actual vitamin B_{12} concentration range from 0.005 to 15 $\mu g \cdot mL^{-1}$ sample. The HPLC method could, therefore, be adjudged sufficiently sensitive to be used as an analytical tool.

The limit of quantification (LOQ), i.e. the lowest vitamin B_{12} concentration where reproducible results were obtained, was 0.005 $\mu g \cdot mL^{-1}$ sample. This compares favourably to the LOQ of 0.01 $\mu g \cdot mL^{-1}$ sample reported by Heudi et al. [15]. However, since it excludes the lowest vitamin B_{12} concentration reported for dairy products, viz. 0.003 $\mu g \cdot mL^{-1}$ [11], the sensitivity of the method was improved by employing sample concentration successfully (Fig. 3).

3.2. Repeatability, recovery and validity

When a standard sample with expected vitamin B_{12} concentration of 2.63 $\mu g \cdot mL^{-1}$ was divided into 14 aliquots and analysed repeatedly on different days, reproducible results were obtained. Mean vitamin B_{12} concentration, 2.62 ± 0.02 $\mu g \cdot mL^{-1}$ (day 1) and 2.61 ± 0.02 $\mu g \cdot mL^{-1}$ (day 2), did not differ significantly ($P = 0.73$) and was close to the expected value of 2.63 $\mu g \cdot mL^{-1}$. Good repeatability (intermediate precision) was, therefore, demonstrated.

The recovery of vitamin B_{12} from the samples and placebo (Tab. I) ranged from 98.6% to 103.2% (average values), with the RSD ranging from 2.68% to 6.79%. It was, therefore, reasonable to conclude that the extraction regime and the analytical procedure effected complete dissolution of vitamin B_{12} in the matrices analysed in this study.

Statistical analysis of the results of the microbiological assays vs. the HPLC analyses of the same samples indicated that no significant difference existed ($P > 0.05$)

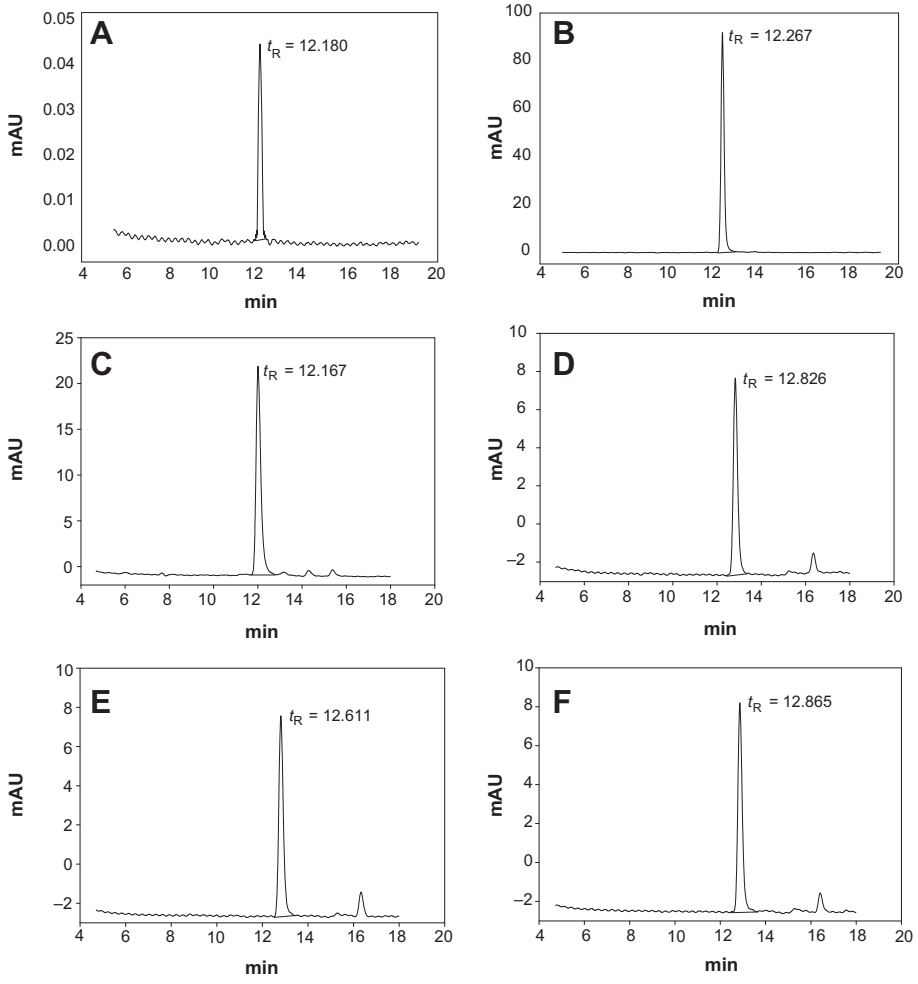

Figure 2. Sample chromatograms of the vitamin B_{12} standard (A, 0.005 μg·mL^{-1}) and (B, 15 μg·mL^{-1}), vitamin B_{12} extracted from B_{12} medium fermented by strain J15 (C, 3.61 μg·mL^{-1}), fortified skim milk (D, 1.28 μg·mL^{-1}), fortified Amasi (E, 1.28 μg·mL^{-1}) and fortified Kefir (F, 1.31 μg·mL^{-1}). Retention time (t_R) next to the peak marks the B_{12} peak.

(Tab. II). The HPLC method is, therefore, a valid alternative to the microbiological assay.

3.3. Sample extraction and HPLC assays

Sample chromatograms depict the results for various samples (Fig. 2). The excellent resolution of the B_{12} peak, together with the absence of baseline noise, illustrated the excellent selectivity of the HPLC method. The results also reinforced the fact that the extraction and analytical procedures produced acceptable results when applied to these food products and to synthetic media fermented by *P. freudenreichii* spp.

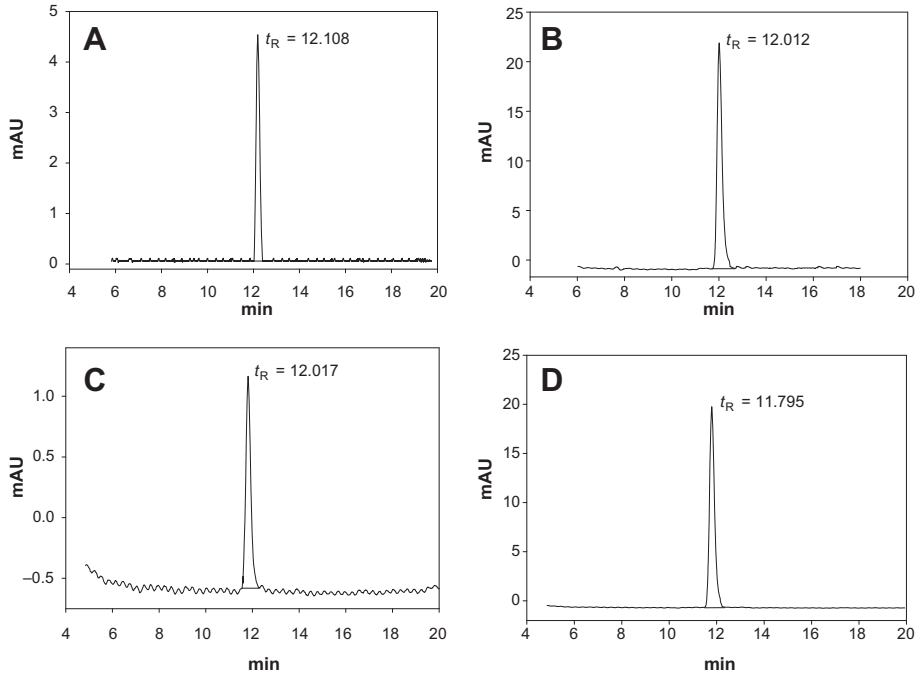

Figure 3. Chromatograms depicting sample purification (A and C), followed by sample concentration (B and D) using SPE techniques. Samples were extracted from B_{12} medium cultured with strain J15 (A and B) and fortified Kefir (C and D). Vitamin B_{12} concentration was measured (in $\mu g \cdot mL^{-1}$): 0.77 (A) and 0.34 (C) before and 3.77 (B) and 3.36 (D) after sample concentration. Retention time (t_R) marks each B_{12} peak.

Table I. Recovery results for HPLC determination of vitamin B_{12} in different samples, with vitamin B_{12} added in various known concentrations.

Sample	Amount added (range) in $\mu g \cdot mL^{-1}$	Recovery (%)		RSD[b] (%)
		Range	Mean ± SD[a]	
YEL with added vitamin B_{12} (n = 18)	0.1–12.5	94–107	98.6 ± 4.17	4.23
J10 in YEL broth with added vitamin B_{12} (n = 10)	0.1–12.5	95–107	100.77 ± 4.19	4.16
J19 in vitamin B_{12} broth with added vitamin B_{12} (n = 10)	0.1–12.5	98–106	100.4 ± 2.95	2.94
Skim milk with added vitamin B_{12} (n = 12)	0.05–5.0	96–111	99.9 ± 6.79	6.79
Amasi with added vitamin B_{12} (n = 16)	0.05–5.0	98–115	103.2 ± 5.08	4.92
Kefir with added vitamin B_{12} (n = 10)	0.05–5.0	100–106	102.2 ± 2.74	2.68

[a] Mean ± SD.
[b] Relative standard deviation (%) = (SD/Mean) × 100.

Table II. Comparison of vitamin B_{12} results from HPLC and microbiological assays ($n = 3$). Values are reported as mean ± SD.

Sample (strain and growth medium)	Microbiological assay ($\mu g \cdot mL^{-1}$ broth)	HPLC results ($\mu g \cdot mL^{-1}$ broth)	P value[c]
J10 (YEL)[a]	0.25 ± 0.01	0.23 ± 0.01	0.40
J17 (YEL)[a]	0.22 ± 0.01	0.22 ± 0.01	0.82
J8 (B_{12} medium)[b]	2.23 ± 0.19	2.25 ± 0.03	0.84
J9 (B_{12} medium)[b]	0.29 ± 0.02	0.32 ± 0.04	0.31
J10 (B_{12} medium)[b]	2.04 ± 0.07	2.12 ± 0.05	0.38
J15 (B_{12} medium)[b]	1.69 ± 0.04	1.77 ± 0.01	0.06
J16 (B_{12} medium)[b]	0.66 ± 0.01	0.77 ± 0.06	0.09
J17 (B_{12} medium)[b]	0.25 ± 0.01	0.27 ± 0.01	0.29
J18 (B_{12} medium)[b]	0.26 ± 0.01	0.33 ± 0.01	0.92
J19 (B_{12} medium)[b]	0.14 ± 0.00	0.16 ± 0.02	0.20

[a,b] YEL and B_{12} media were sampled at 216 and 73 h, respectively.
[c] The Student's t test (independent samples) was performed to establish whether the results of the two methods differed significantly. $P \leq 0.05$ indicates significance.

3.4. Sample purification and concentration

When comparing the vitamin B_{12} levels before (Figs. 3A and 3C) and after (Figs. 3B and 3D) sample concentration using SPE, it is clear that this technique resulted in a substantial increase in the apparent concentration of vitamin B_{12} in a broth sample cultured with strain J15 (from 0.77 to 3.77 in $\mu g \cdot mL^{-1}$, i.e. an almost five-fold increase, Figs. 3A and 3B), as well as in a fortified Kefir sample (from 0.34 to 3.36 $\mu g \cdot mL^{-1}$, i.e. an almost 10-fold increase, Figs. 3C and 3D).

The significance of this enhanced response is that it increases the sensitivity of the analysis. In other words, while the LOQ was established at 0.005 $\mu g \cdot mL^{-1}$, with a five-fold increase in sensitivity, as demonstrated for the J15 sample, the LOQ will be decreased to 0.001 $\mu g \cdot mL^{-1}$. Hence, using SPE concentration makes this method suitable for the analysis of dairy products with vitamin B_{12} levels as low as 0.003 $\mu g \cdot mL^{-1}$.

Sample purification resulted in cleaner chromatograms. This is illustrated by the absence of any peaks other than the vitamin B_{12} peak (the KCN buffer and solvent peaks are not shown since they elute at $t_R < 6$ min) (Figs. 3A–3D). Furthermore, the chromatograms depicted in Figures 2C and 3A were both obtained when analysing sample extracts of strain J15 cultured in B_{12} medium. It is clear that the compounds eluting immediately after vitamin B_{12} (Fig. 2C) are absent after purification using SPE (Fig. 3A). Hence, this is further confirmation that effective purification was achieved.

4. CONCLUSIONS

The extraction protocol was shown to effect complete recovery of vitamin B_{12} from the sample matrices. The HPLC method was found to be selective, linear ($R > 0.998$), accurate (recovery from 98.6% to 103.2%) and sensitive (LOQ = 0.005 $\mu g \cdot mL^{-1}$). It was also shown that the sensitivity could be improved using sample concentration. The method, therefore, is a rapid alternative to the more time consuming microbiological assay.

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