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Simultaneously HPLC Analysis of B1, B9 and B12 Vitamins at Trace Levels via Cloud Point Extraction

Halil İbrahim Ulusoy [1,a], İpek Nur Yiğit [1,b], Ümmügülsüm Polat [1,2,c,*], Esra Durgun [3,d], Aslıhan Gürbüzer [4,e], Songül Ulusoy [5,f]

[1] *Department of Analytical Chemistry, Faculty of Pharmacy, Sivas Cumhuriyet University, Sivas, Türkiye.*
[2] *Department of Analytical Chemistry, Institute of Health Sciences, Ankara University, Ankara, Türkiye.*
[3] *Department of Analytical Chemistry, Institute of Health Sciences, Erciyes University, Kayseri, Türkiye.*
[4] *Department of Plant and Animal Production, Vocational School of Technical Sciences, Sivas Cumhuriyet University, Sivas, Türkiye.*
[5] *Department of Pharmacy, Vocational School of Technical Services, Sivas Cumhuriyet University, Sivas, Türkiye.*
**Corresponding author*

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ABSTRACT

An enrichment and determination method based on liquid chromatographically analysis and cloud point extraction (CPE) has been developed for trace levels of B vitamins (B1, B9 and B12) in the proposed study. Vitamin molecules were drawn into the non-ionic surfactant phase of Polyethylene Glycol (PEG-6000) in the presence of pH 9.0 medium. The surfactant-rich phase separated by centrifugation and then dissolved with 700 µL of ethanol. The obtained ethanol phase was filtered by 0.45-micron filter prior to the HPLC analysis. All parameters affecting the CPE method such as pH, buffer volume, incubation time, surfactant and electrolyte concentration, solvent for the surfactant-rich phase and its amount have been individually studied and optimized step by step. After the optimization of all parameters of the CPE process, the detection limits of the developed method for B1, B9 and B12 vitamins were calculated as 1.42 ng mL^{-1}, 7.14 ng mL^{-1} and 14.28 ng mL^{-1}, respectively. The linear working ranges for three vitamin molecules was obtained in the range of 5.0-500.0 ng mL^{-1}. After CPE procedure, determination of vitamin molecules was carried out by using HPLC system with diode array detector(DAD) at 244 nm for vitamin B1, 285 nm for vitamin B9, and 361 nm for vitamin B12, respectively.

Keywords: Cloud point extraction, HPLC, Vitamin B1, Vitamin B9, Vitamin B12.

[a] *hiulusoy@cumhuriyet.edu.tr* *https://orcid.org/0000-0002-8742-5145*
[b] *ipeknuryigit@gmail.com* *https://orcid.org/0009-0009-0593-5394*
[c] *ugulsum@cumhuriyet.edu.tr* *https://orcid.org/0000-0001-8393-2396*
[d] *esradurgun2017@gmail.com* *https://orcid.org/0009-0009-2613-4019*
[e] *aslihan@cumhuriyet.edu.tr* *https://orcid.org/0000-0002-6753-2808*
songululusoy@yahoo.com *https://orcid.org/0000-0003-3640-5416*

Introduction

Vitamins are organic substances that are necessary for the normal occurrence of metabolic events in the body and to maintain a healthy state which cannot be synthesized in the body or are synthesized insufficiently and which taken in small amounts from the environment in foods. Vitamins are an indispensable part of healthy life originate from the Latin word "vita" which means life. It is produced little or not at all in our body and therefore must be taken from outside with food. Vitamins are found in foods in active form or in the form of pro-vitamins that will become active in the body. In fact, each vitamin has its own name but they are referred to with the letters of the alphabet for easy understanding [1].

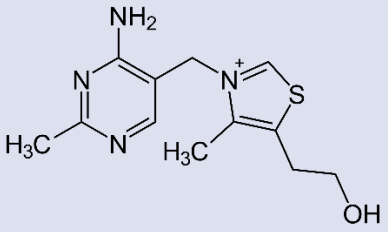
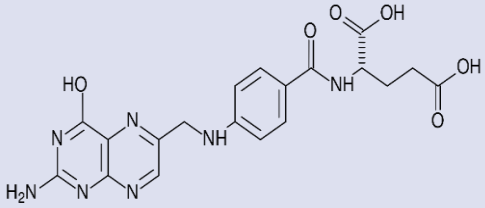
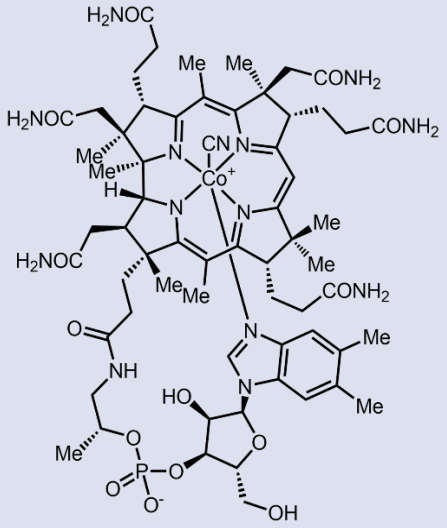
Vitamins are important compounds for the normal functions of the body which take part as coenzymes or enzymes in many vital processes. According to the general view that emerged because of many studies each vitamin has a separate task in the body and the health problem or diseases caused by a vitamin deficiency can't be eliminated with another vitamin. If one or more of the vitamins we take with our daily foods are missing or not in the required density, growth retardation, low productivity, decreased reproductive performance and some similar disorders appear. Essential vitamins are taken with diets and vitamin supplements[2].

Vitamin B1 is very important for body and mental health which is in the group of powerful vitamins called B vitamin complex. Vitamin B1, also known as thiamine, was obtained purely for the first time in 1926 as an anti-beriberi factor. In the following years, it was determined that it took part as a cofactor in important enzyme reactions [3]. Folic acid, also called folacin or folate, is used to describe a family of compounds related to pteroic acid. Pteroic acid consists of a pteridine ring joined by a para-aminobenzoic acid (PABA) structure, while a variable number of glutamyl structures are linked by peptide bonds[4]. It is slightly soluble in water and completely soluble in alcohol[5,6]. Vitamin B12 is the most complex of the water-soluble vitamin. Its most common form, Cobalamin, which is in the form of red crystals, dissolves in water and alcohol at high temperatures. However, it is insoluble in acetone, ether and chloroform [7,8]. Chemical structures of the studied vitamins were shown in Table 1.

Despite the development of high-precision analytical devices for the analysis of biological, environmental, food and pharmaceutical products, the analytical systems often fail to determine in the complex matrix medium. Therefore, pre-treatment is usually required for the

extraction (separation) and concentration (enrichment) of the analytes from the matrix medium.

Table 1. Molecular structure of the studied vitamins

Chemical Name	Vitamin Name	Molecular Structure
Thiamine	Vitamin B1	
Folic Acid	Vitamin B9	
Cobalamin	Vitamin B12	

Separation and enrichment processes are generally carried out by processes such as distillation, adsorption into a solid surface, and extraction[9]. Extraction processes are mostly applied as liquid-liquid, solid-liquid and solid phase extraction [10]. The cloud point extraction method was first developed by Hiroto Watanabe et al. in 1976. Especially after the 1990s, it has been frequently used by analytical chemists for the enrichment and analysis of organic molecules in the presence of surfactant for separation and enrichment [11].

Sample preparation is one of the most important steps in chemical analysis. A pre-treatment procedure is often required to separate interfering species and to concentrate trace analytes prior to detection[12]. Although new sample preparation methods such as solid phase microextraction (SPME), supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE) are used more frequently; conventional solid phase extraction (SPE), classical liquid-liquid extraction (LLE) and cloud point extraction (CPE) are still widely used [13] thanks to easy operation[14,15]. Cloud point extraction (CPE) as a pre-concentration method attracts great interest as a green analytical approach by limiting the use of toxic organic solvents[14]. In the CPE experiments, mostly non-ionic surfactants are used and the samples is heated till a certain temperature called as cloud point (CP) temperature. In this point, the solution spontaneously splits into two separate phases. The first phase is the surfactant-rich phase including target molecules and aquatic phases including the other components [16].

Briefly, a new analytical methodology was developed for vitamin B1, B9, and B12 based on CPE combined HPLC-DAD system. Experimental variables of the proposed method were studied and optimized step by step and analytical parameters of method were calculated and presented by means of model solutions.

Experimental

Instruments and Reagents

Shimadzu (Prominence) HPLC (Kyoto, Japan) device was used for all chromatographic measurements. The HPLC device used; It is equipped with LC 20 AD quaternary pump, SPD-M20 A PDA detector, DGU-20A vacuum degasser and CTO-10 AS VP column furnace. All separations and determinations were performed on a reverse phase C18 column (Inertsil ODS-3, 250 mm×4.6 mm, 5 µm). Evaluation of chromatograms was done using LC Solution 2.0 software. A pH meter (pH-2005, JP Selecta, Barcelona, Spain) was used to adjust pH of solutions.

All reagents used during the experiments were of analytical grade and were purchased from Sigma or Merck companies. All solutions used were prepared with ultrapure water with 18.2 mΩ/cm resistance obtained from ELGA Pure Lab Flex III instrument.

pH 1.0-10.0 Britton-Robinson(BR) Buffer Solution: This buffer solution was prepared by dissolving of 2.4732 g H_3BO_3, 2.67 mL of H_3PO_4 and 2.32 mL of acetic acid in 1.0 L of ultrapure water. The desired pH valued of buffer solution was adjusted to appropriate pH ranges according to their acidity constants, and pH was checked with the help of a pH meter, and then protected from light until use.

- *Vitamin B1, B9 and B12 Stock Solution, 500 mg L^{-1} :* 50 mg of pure vitamins B1, B9 and B12 (Sigma Aldrich) were weighed and taken into a flask, dissolved with methyl alcohol and made up to 100.00 mL, transferred to a dark glass bottle and stored at +4 °C.
- *20 % Polyethyleneglycol (PEG) 6000 Stock Solution:* 20.000 g of analytical grade polyethyleneglycol was weighed and dissolved in water and made up to 100 mL, transferred to a dark glass bottle and stored at +4 °C.
- *20 % Na_2SO_4 Stock Solution:* 20 g of analytical grade sodium sulphate and dissolved by heating with the help of some water in the beaker and completed to 100.00 mL.

HPLC Analysis Conditions for Determination of Vitamins B1, B9 and B12 Molecules

Before proceeding to the CPE experiments, directly determination parameters by HPLC were optimized for vitamin B1, B9 and B12. For this purpose, based on the literature, a C-18 column was chosen as the stationary phase. Many experiments were performed to determine the suitable mobile phases, including methanol, ethanol, acetonitrile, and buffers with different pHs, using isocratic and gradient elution modes. Experiments were continued until ideal peaks were obtained for vitamins B1, B9, and B12. As a result of the experiments, it was determined that the most ideal mobile phase conditions were pH 6.5 phosphate buffer and acetonitrile. The ideal HPLC operating conditions obtained after optimization were given in Table 2.

Table 1. HPLC operating conditions

Parameter	Value
HPLC Mode	Isocratic
Mobil Phases	ACN : pH 6.5 Phosphate Buffer (0.02 M)
Flow Rate	1.0 mL/min
Wavelength in DAD	244 nm for vitamin B1, 285 nm for vitamin B9, 361 nm for vitamin B12
Column	C18- Inertsil ODS-3 (250 mm×4.6, 5.0 µm)
Column Temperature	40°C
Injection Volume	10 µL

Calibration parameters were given in Table 3 under the optimized HPLC conditions. - Chromatogram peaks obtained from standard solutions of vitamin species were given in Figure 4.

Table 2. Direct determination results by HPLC before CPE

Parameter	Vitamin B1	Vitamin B9	Vitamin B12
Retention Time, min	9.70	5.10	11.50
Wavelength in DAD Detector	244 nm	285 nm	361 nm
Calibration Range	1.0-50.0 µg mL $^{-1}$	1.0-50.0 µg mL $^{-1}$	1.0-50.0 µg mL $^{-1}$
Limit of Detections, (LOD)	0.23 µg mL $^{-1}$	0.23 µg mL $^{-1}$	0.23 µg mL $^{-1}$
R^2	0.9986	0.9995	0.9975
The number of repetitions, (N)	3	3	3

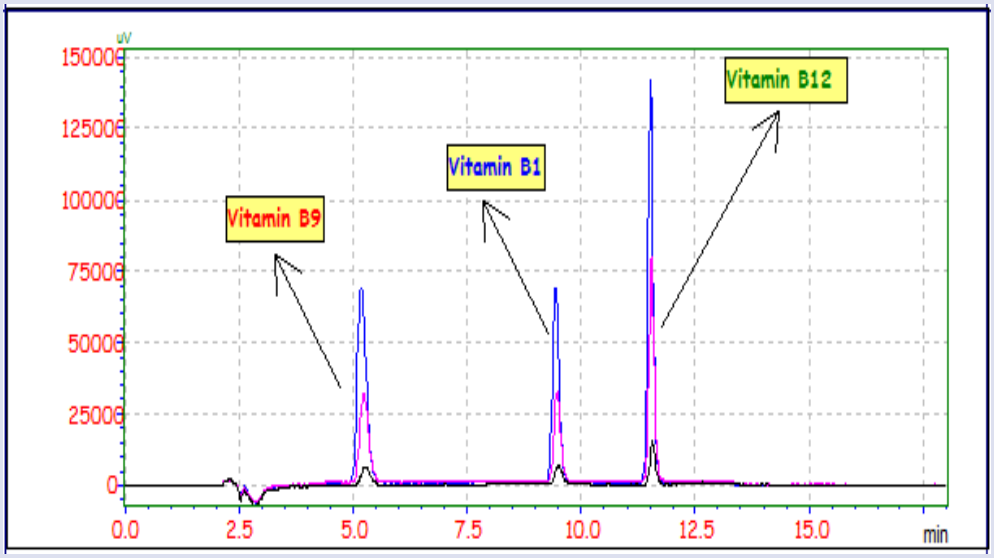


Figure 4. Chromatogram obtained under HPLC Conditions before CPE for B1, B9 and B12 vitamins

The Proposed Method

CPE experiments based on cloud point extraction, parameters such as pH, analyte concentrations, surfactant amount, incubation temperature and time, vortex time were studied and optimized step by step. In the proposed method, 2 mL of sample solution was transferred into tubes and 2.0 mL pH 9.00 BR buffer, 1.0 mL 20% (w/v) Polyethylene glycol (PEG-6000), 10.0 mL 20 % (w/v) Na_2SO_4 were added on this solution. After that, the solution was made up to 15 mL by adding with water and incubated in a water bath at 50°C for 30 minutes in order to increase temperature to cloud point temperature of surfactant. Then the solutions were centrifuged at 4000 rpm for 5 minutes in order to separate the surfactant-rich phase and the aqueous phase. Sample tubes were kept in the refrigerator for 20 minutes to facilitate the separation of the surfactant-rich phase and the aqueous phase. At the end of this period the surfactant phase with a high density was collected in the upper part of the tube and the aqueous phase in the lower part was separated with the help of an injector. The surfactant-rich phase was diluted with 700 µL of ethanol and completely homogenized with the help of a vortex. Then the samples filtered with a 0.45 µm injector-tipped filter were transferred to HPLC bottles. Vitamin contents of enriched samples were determined by HPLC device.

Experimental Studies and Discussion

Basic Approach of Experimental Studies

The developed method based on cloud point extraction and HPLC-DAD detection has been optimized for the determination of target group B vitamins. At the beginning of the experimental studies, preliminary trials were carried out on all parameters that would enable the quantitative transition of the related vitamin molecules to the surfactant-rich phase. B vitamins may be existed as charged or uncharged form depending on the pH of the medium. Therefore, all parameters CPE method such as pH, buffer volume, incubation time, surfactant and electrolyte concentration, solvent for the surfactant-rich phase and its amount, were individually studied and optimized.

Optimization of the Developed Method

pH effect

Ambient pH is very important as it affects both the reactions between the analytes and other species and the enrichment in the next steps. As the pH of the medium moves to the acidic region, the amount of positively charged ions in the medium increases which reduces the activity of the hydrophilic head of the surfactant in the solution medium.

For this purpose, buffers with pH values between 2-10 were used in the presence of PEG-6000, which is used as a non-ionic surfactant in the sample solution. The obtained results were shown in Figure 5, it is seen that the most suitable pH value for the CPE processes is pH 9.00. Therefore, pH 9.00 BR buffer system was used in later studies.

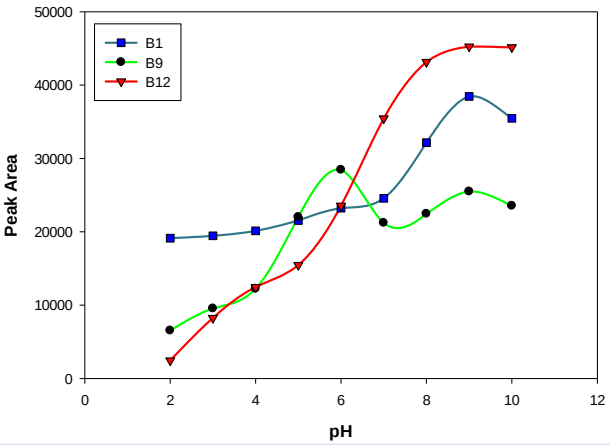


Figure 5. pH effect on the proposed method

After choosing the optimal pH value for the next experimental studies, optimization study was carried out with volume of buffer solution volume. For this purpose, volume of the optimum pH 9.00 BR was scanned between 0-5.00 mL. As can be seen in Figure 6, the highest signals were obtained with 2.0 mL of buffer.

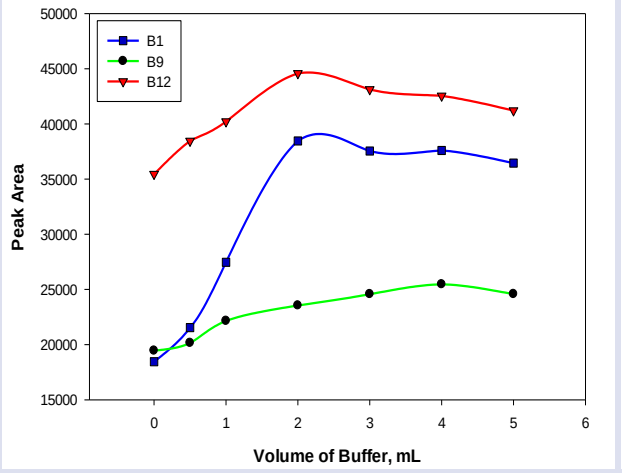


Figure 6. Effect of volume of buffer solution on the proposed method

Effect of non-ionic surfactant concentration

The amount of surfactant in the samples is one of the most important parameters affecting the effectiveness of cloud point extraction. If the surfactant concentration is low, micelle formation is limited and the extraction efficiency decreases. The non-ionic surfactant used for this experiment is Polyethylene glycol 6000 (PEG-6000) and a concentration of 20 % (w/v) surfactant was prepared before the experiments. Concentration of PEG-6000 was studied in the range of 0.4- 2.0 % (w/v). As a result of these procedures, it was seen that the best signal was obtained with 1.1 % (w/v) PEG-6000. Next studies were continued with this value.

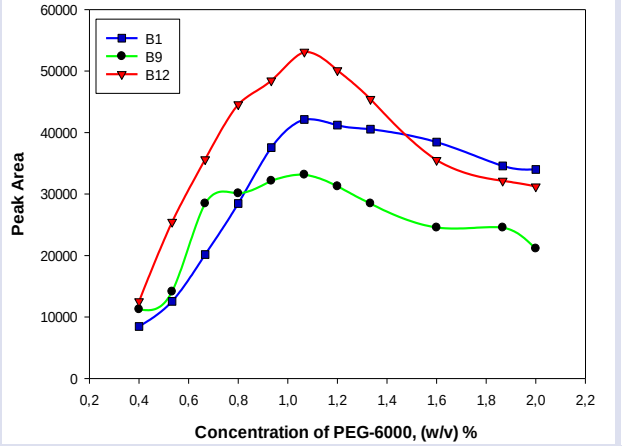


Figure 1. Effect of concentration of non-ionic surfactant

Electrolyte effect on CPE

Surfactants are macromolecules with high molecular mass that can form a micelle structure when a certain concentration and temperature is exceeded. As being proteins, the solubility of surfactants is reduced due to the salt effect. This effect, known as the salting effect, facilitates the separation of surfactant molecules from the

aqueous phase. When the literature is examined, it is seen that strong electrolytes such as NaCl, KCl, KNO_3, Na_2SO_4 or $NaNO_3$ are used for this aim. In our study, sodium sulphate was preferred to allow the phases to be separated from each other more easily. Concentration of Na_2SO_4 was studied in the range of 5.0-15.0 % (w/v). As can be seen in Figure 8, the best signals were obtained with 11.0 % (w/v).

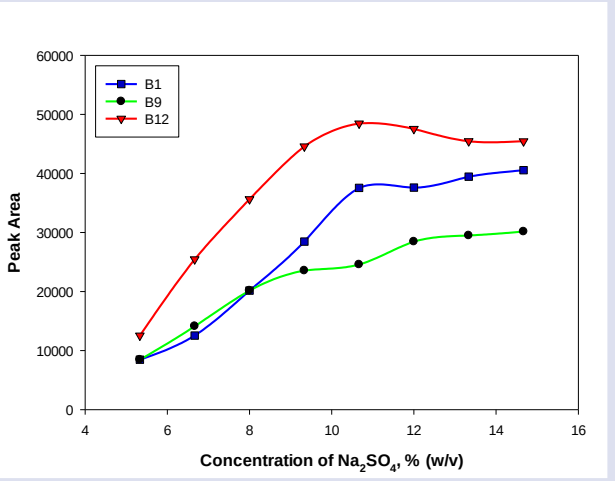


Figure 8. Electrolyte effect on the proposed method

Effect of incubation time

The another studied parameter is the effect of incubation time. Temperature is an important parameter in cloud point extraction and it is important in the formation of micelle structure. In order that optimize the effect of incubation time on cloud point extraction, the samples were place to water bath at 50 °C. The optimization of time was carried out in the rage of 5-90 minutes. As can be seen in Figure 9, 30 min is enough for extraction efficiency. Therefore, in future studies, 30 minutes was used as the incubation time.

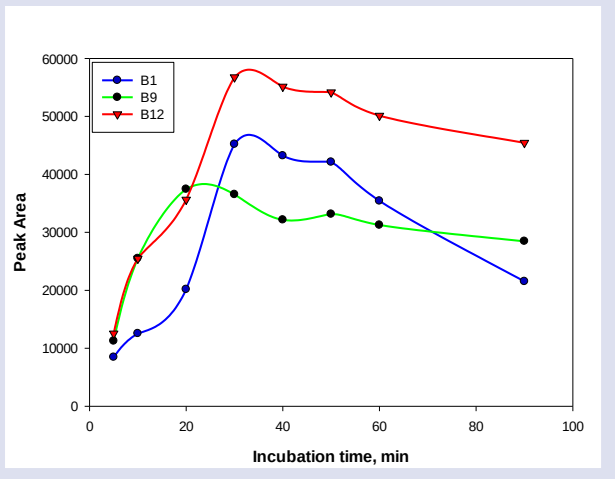


Figure 9. Effect of incubation time on the proposed method

Preparation of surfactant-rich phase for analysis

After centrifugation, the surfactant-rich phase was collected at the top of the tube. In order to facilitate the surfactant-rich phase (SRP) to be easily separated from the aqueous phase, the sample solutions were kept in the refrigerator for a while thus increasing the viscosity of the surfactant-rich phase. At the end of this period, the aqueous phase was easily separated from the surfactant-rich phase with the help of an injector. After the aqueous phase has been separated, the surfactant-rich phase must be diluted with a suitable solvent before being introduced into the HPLC device. Because the phase with high viscosity is not suitable for injection as such and is not sufficient to receive a signal in the device. For this purpose, various solvents were tried to dissolve the surfactant-rich phase before the analysis. The solvent chosen should both completely dissolve the surfactant-rich phase and not damage the device to be determined.

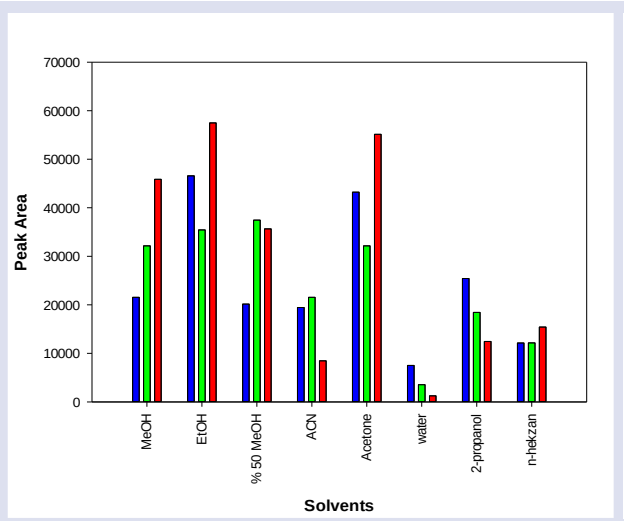


Figure 10. Effect of solvents for SRP

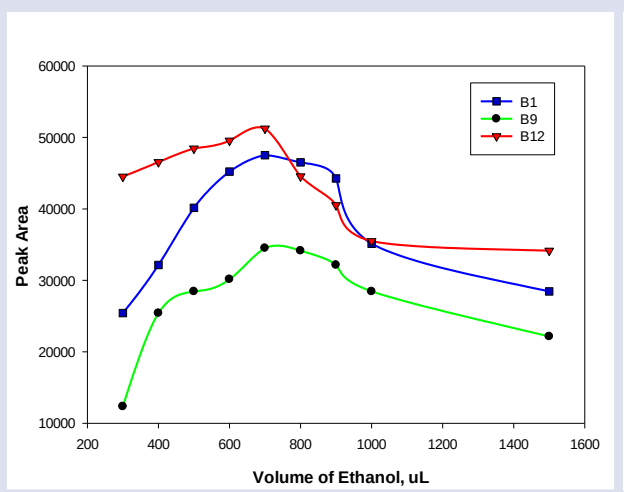


Figure 11. Volume of ethanol used for SRP

While selecting the solvents to be used for this purpose, it is desirable that the HPLC system be suitable for the operating stage and be strong enough to dissolve the SRP quantitatively. As can be seen in Figure 10, the best signal was obtained with ethanol. The surfactant-rich phase was dissolved with this solvent. Since the amount of solvent used to dissolve the surfactant-rich phase will directly affect the enrichment factor, it is important how much solvent volume is taken. To obtain a high enrichment coefficient, the solvent volume must be at the smallest value. The volume of ethanol was optimized in the range of 300-1500 µL of solvent as can be seen in

Figure 11. As a result of this study, the maximum signals were obtained with 700 µL of ethanol and this value was used in further studies.

Analytical Performance of the Developed Method

After each experimental variable of CPE was optimized, analytical merits of the developed method were studied and calculated. The CPE experiments were applied to vitamin B solutions at different concentrations in order to determine the linear working range. As a results of this study, the signals increase in proportion to the concentration. All analytical parameters of the developed method were presented in Table 4.

Table 4 . Analytical parameters of the developed method

Parameter	Before CPE	After CPE		
	B1, B9, B12	B1	B9	B12
Linearity	1.0-50.0 µg mL $^{-1}$	5-500.0 ng mL $^{-1}$	25.0-500.0 ng mL $^{-1}$	50.0-500.0 ng mL $^{-1}$
Limit of Detection	0.23 µg mL $^{-1}$	1.42 ng mL $^{-1}$	7.14 ng mL $^{-1}$	14.28 ng mL $^{-1}$
Limit of Quantification	0.94 µg mL $^{-1}$	4.71 ng mL $^{-1}$	23.57 ng mL $^{-1}$	47.14 ng mL $^{-1}$
Slope of Calibration	1,528	40.3	45.5	58.8
Correlation Coefficient (R^2)	0.9986	0.9945	0.9912	0.9875
Enrichment Factor[a]	-	21,4	21,4	21,4
Pre-concentration Factor[b]	-	26.4	29.8	38.5

[a] It was calculated by taking the ratio of the volume initial and final (15 mL and 0.7 mL)
[b] It was calculated by taking the ratios of the calibration slopes before and after CPE.

Conclusion

Cloud point extraction (CPE) is a widely used and increasingly common method for the separation and enrichment of both organic and inorganic species, especially in the last two decades. Hundreds of studies were published every year in this area. The CPE covers wide application areas for many different species and sample types. The main factors in finding so many application areas of the method are; It can be listed as simplicity, environmental friendly, low cost and easy applicability in almost every laboratory. All parameters that may have an effect on the method developed described throughout the experimental section of this paper have been optimized one by one. For the CPE process, the most effective CPE conditions were determined for method variables such as ambient pH, type and concentration of nonionic surfactants, electrolyte effect, incubation temperature, selection of suitable solvent for the surfactant-rich phase. After the optimization of a newly developed CPE method was completed and its analytical parameters were determined, it was applied to several model solutions in order to calculate analytical merits. This method can be easily applied for determination of B1, B9 and B12 vitamins simultaneously.

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Conflicts of interest

The authors state that did not have conflict of interests.

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