

A Dispersive Liquid–Liquid Micro–Extraction Technique for the Pre–concentration and Quantification of Vitamin D₃ in Milk and Yogurt Samples Using a Non-Aqueous HPLC Method

Maryam Ghalebi^{a, b}, Elnaz Tamizi^a, Shirin Ahmadi^{a, c}, Ahad Sheikhloo^a and Mahboob Nemati^{a, b*}

^aDepartment of Pharmaceutical and Food Control, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran. ^bFood and Drug Safety Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. ^cPharmaceutical Analysis Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

Abstract

In present study, a DLLME-HPLC-UV method was developed and validated for the extraction, pre–concentration, and subsequently quantification of vitamin D₃ (Vit D₃) in milk and yogurt samples. In order to be able to extract Vit D₃ from studied samples efficiently, the DLLME procedure was optimized with respect to the parameters affecting the extraction efficacy, where acetonitrile (2 mL as disperser solvent) resulting from the protein precipitation procedure was mixed with 80 µL carbon tetrachloride (as an extraction solvent) respectively. The extracted samples were quantitatively analyzed with a HPLC technique using a C₈ column (250 mm × 4.6 mm, 5 µm) at room temperature (25 °C), mobile phase of acetonitrile/methanol (90:10% v/v) in isocratic elution mode at a flow rate of 1.2 mL/min and UV detection at 265 nm. The method validation results revealed that the method was linear in the concentration range of 2 to 60 ng/mL ($r = 0.9997$) with a LOD of 0.9 ng/mL and LLOQ of 2 ng/mL; the method was accurate ($-2.1\% \leq RE\% \leq +0.6\%$) and precise ($1.2\% \leq RSD\% \leq 11.3\%$) and its recovery was in the range of 86.6 to 113.3%. The obtained results indicated that the method could be utilized as an easy to use technique for the monitoring Vit D₃ in dairy products, especially milk and yogurt samples.

Keywords: DLLME; HPLC-UV; Vitamin D₃; Milk; Yogurt.

Introduction

Cholecalciferol, vitamin D₃ (Vit D₃), is one of the isomers of vitamin D that is converted to its active form via hydroxylation in liver and kidney. It is of great importance in keeping normal calcium metabolism (1). In the other words, the main biological function of Vit D₃ is to improve intestinal absorption of calcium and phosphate (2).

The required Vit D₃ for the human body can be provided through either exposure of its precursor (7-dehydrocholesterol), existing in the epidermal layer of skin, to ultraviolet (UV) radiation or food intake (3). However, it should be mentioned that the amount of Vit D₃ in food resources is little and only fishery products, particularly fatty fish such as salmon, mackerel, and also, herring and fish liver oils, are the good sources of Vit D₃ (4). In the case of dairy products, bovine milk is considered to be one of the Vit D₃'s dietary origins, nevertheless the amount of Vit D₃ in milk is too low and in

* Corresponding author:

E-mail: nematim@tbzmed.ac.ir

the range of 4–40 IU/L (0.1–1.0 µg/L) (3). It has been demonstrated that Vit D₃ deficiency is a common nutritional problem in all age groups in the world (5, 6). This deficiency could lead to the prevalence of rickets in children and osteomalacia in adults (7, 8). Owing to the important role of vitamin D₃ in human health, the development of analytical methods applicable to determine the accurate amounts of this micronutrient in food stuffs is necessary (9).

Since vitamins are present in low quantities and in combination with other compounds in foodstuffs, different techniques such as saponification, liquid–liquid extraction (LLE), and solid-phase extraction (SPE) have been utilized for the cleanup and extraction of Vit D₃ from food samples (10, 11). However, some drawbacks such as use of large volumes of toxic solvents and being time-consuming make these techniques as costly procedures which are incompatible with the environment.

In recent years, significant efforts have been devoted to achieve simple alternative methods for sample preparation. Dispersive liquid–liquid microextraction (DLLME) is a relatively new technique introduced by Rezaee *et al.* in 2006 (12). It is an easy to operate, rapid and environmentally friendly technique (13). Low amounts of required organic solvents and fast sample preparation procedure are the main advantages of DLLME (14).

Literature review revealed that different analytical methods mainly based on high performance liquid chromatography (HPLC) have been reported for determination of Vit D₃ in foodstuffs (3, 7, 10 and 15-20). Analytical characteristics such as shorter analysis time, higher specificity and selectivity, acceptable precision and reasonable accuracy make HPLC as a favorable method for the food analysis compared to the other methods (21). An overview of the reported methodologies for the determination of Vit D₃ in food samples was shown in Table 1. As it can be seen in this table, although DLLME technique was utilized for the extraction of Vit D₃ from infant cereals, it has not been utilized for the extraction of Vit D₃ in milk and dairy products so far (10). Therefore, since dairy products are accessible and affordable foods for different social classes that could

be suitable carriers for Vit D₃ fortification, the aim of the present study was optimization of a simple and sensitive DLLME technique coupled to HPLC with UV detection applicable to the quantification of vitamin D₃ in some dairy products including milk and yogurt samples.

Experimental

Apparatus

Analytical balance (A&D Weighing, San Jose, CA), vortex mixer (Scientific Industries Inc., Bohemia, NY), and high-speed centrifuge (Eppendorf, Hamburg, Germany) were used for sample preparation.

Chemicals and Reagents

Reagents

Chloroform, carbon tetrachloride, isopropyl alcohol, and ethanol were purchased from Merck (Darmstadt, Germany). Methanol and acetonitrile were obtained from Duksan Pure Chemicals Co. (Gyeong gi-do, Korea). All solvents were of analytical or HPLC grade.

Standard Solutions

Cholecalciferol (Vit D₃) was prepared from Sigma-Aldrich. Stock solution of Vit D₃ with a concentration of 1000 µg/mL was freshly prepared via dissolving accurately weighed amount of cholecalciferol standard powder in chloroform. Working standard solutions of Vit D₃, in a concentration range of 1 to 60 ng/mL, were prepared through diluting fresh stock solution using mobile phase (acetonitrile/methanol 90:10% v/v). All solutions were stored at 4 °C and covered with aluminum foil to be protected from light, prior to the analysis.

HPLC-UV Instrumentation

The chromatographic analyses were performed using a HPLC system (Knauer, Berlin, Germany). The detection was carried out using a UV detector (Knauer, Berlin, Germany) at 265 nm. The separation was achieved using a C₈ column (250 mm × 4.6 mm, 5 µm) (Knauer, Berlin, Germany) at room temperature (25 °C). The mobile phase composed of a mixture of acetonitrile and methanol (90:10% v/v) used in isocratic elution mode at a flow rate of 1.2 mL/min.

Table 1. The previously reported methods for the extraction and quantification of Vit D₃ in foodstuffs.

Sample	Extraction procedure	Analytical method	Sample volume/weight	Extraction solvent's type and volume	LOQ (ng/mL)	Retention time (min)	Linear range (ng/mL)	Recovery	Reference
Fresh bovine milk Commercial milk	SPE	LC-MS/MS	10 mL	Hexane 50 mL	0.1	17.7	-	30-40%	3
Skimmed Milk Whole milk	Saponification	HPLC	15 mL	Diethyl ether in hexane 30 mL	2.6	16.0	20 - 1000	94-110%	7
Infant cereals	DLLME	LC-DAD	0.2-2 g	Carbon tetrachloride 150 µL	1-100	12.6	-	95-103%	10
Non-enriched milk Enriched milk	LLE	HPLC	100 mL	Hexane 100 mL	-	19.3	1 - 7.5	-	15
Fortified infant formula Milk Milk powder	LLE	LC/MS	4 mL	Isooctane 10 mL	0.14	3.60	-	103.2%	16
Whole milk	SPE	HPLC	100 g	Diethyl ether: petroleum ether	-	20.7	-	95.4%	17
Milk	Saponification	LC-MS/MS	4 mL	Hexane: dichloromethane 12 mL	-	17.7	-	98.9%	18
Fortified milk	Saponification - Extraction with hexane	UPLC-PDA	20 mL	Hexane 8 mL	-	11.9	2.5 - 60	93%	19
Milk-based infant formulas	Non-heating saponification	LC-MS	0.5-2 g in 10 mL water	Isopropyl alcohol 10 mL	-	10.7	1 - 100	93-110%	20
Milk and yogurt	DLLME	HPLC-UV	1 mL	Carbon tetrachloride 80 µL	2	5	2-60	86.7-113.3%	Present work

Sampling

A total of 10 dairy products including 7 brands of milk and 3 brands of yogurt among available and well-known dairy brands in Tabriz, Iran were purchased from local supermarkets in a time period of May to July 2016. Among purchased products, four brands of milk samples (A, B, C, and E) and one brand of yogurt samples (D) were fortified with Vit D₃. It should be mentioned that three samples of each brand with different batch numbers were prepared and analyzed in triplicate.

Sample Preparation/Clean up Procedure

One mL of milk samples and 1 mg of yogurt samples were shaken for 1 min. Then, 2 mL of acetonitrile was added into the samples and vortex mixed for 2 min. After that, the samples were centrifuged for 10 min at 6000 rpm and finally the upper aqueous layer was transferred to another centrifuge tube for DLLME procedure.

DLLME Procedure

The applied DLLME technique was based on a previously reported work (10). However, some modifications were performed in reported technique to optimize the efficacy of the pre-concentration procedure for the intended purpose. In order to conduct DLLME, 80 μ L of carbon tetrachloride as an extracting solvent was added to the obtained fraction from the previous step that was used as a dispersive solvent as well. After that the mixture was quickly injected into 5 mL of double distilled water using a syringe that resulted in the formation of a cloudy solution. Then, the mixture was gently shaken for 1 min followed by centrifugation at 11000 rpm for 2 min that led to the sedimentation of the extracting solvent at the bottom of the conical tube. Finally, the sedimented phase was totally transferred to a microtube and evaporated to dryness at room temperature. The residue was reconstituted with 50 μ L of acetonitrile and 20 μ L of the obtained solution was injected into the HPLC.

Validation of Optimized DLLME-HPLC Method

The optimized DLLME-HPLC technique was validated for the quantitative purposes based on

the ICH guidelines on validation of analytical procedures (22). In order to investigate linearity of the method and lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) amounts, the peak areas of calibration samples with concentrations in the range of 2 to 60 ng/mL calculated from the equation of "the obtained peak area from calibration sample – peak area of blank sample" were plotted against Vit D₃ concentrations (n = 3 at each concentration). For preparation of calibration samples, 100 μ L of working standard solutions with concentrations in a range of 10–600 ng/mL were spiked into 1 mL of samples. To illustrate, 100 μ L of a working standard solution with a concentration of 10 ng/mL was spiked into 1 mL of a milk sample to achieve a calibration sample with a concentration of 1 ng/mL.

LOD (limit of detection), of the method has been calculated using the equation of "LOD = $3.3 \times \sigma/s$ ", where σ is the standard deviation of peak areas from the analysis of six individual blank samples prepared based on the optimized conditions and s is the slope of the calibration equation.

The precision, including repeatability and intermediate precision, and accuracy of the method were investigated at 3 concentrations at the lower, the middle, and the upper levels of the calibration curve (at 2, 20 and 60 ng/mL Vit D₃). Repeatability and intermediate precision were derived from the calculated amounts for each concentration using the calibration equation and peak areas obtained from the repeated analyses (n = 3) in one day and 3 days in a row, respectively. For accuracy determination, the samples with known concentrations of 2, 20, and 60 ng/mL Vit D₃ were analyzed in triplicate, then the experimentally derived concentrations were calculated from the peak areas and calibration equation.

The accuracy of the method was presented as a relative error (RE%) calculated using the equation of "((calculated concentration – nominal concentration)/nominal concentration) \times 100". The recovery of the method was evaluated at three concentration levels including 2, 10, and 40 ng/mL and reported as a percentage of the experimentally derived concentration to the nominal concentration.

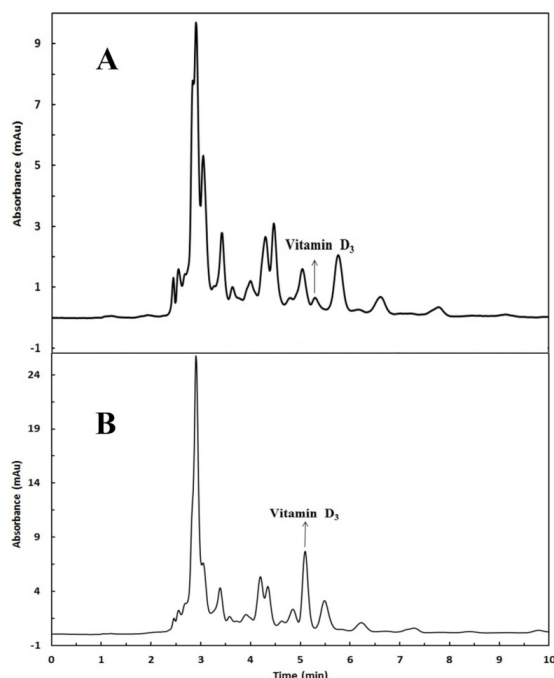


Figure 1. A dispersive liquid–liquid micro–extraction technique for the pre–concentration and quantification of vitamin D₃ in milk and yogurt samples using a non–aqueous HPLC method. The obtained chromatogram from the analysis of samples using a optimized HPLC method, separation conditions: C₈ column (250 mm × 4.6 mm, 5 μm), room temperature (25 °C), mobile phase of acetonitrile/methanol (90:10% v/v), flow rate of 1.2 mL/min, UV detection at 265 nm. (A) blank milk sample; (B) milk sample spiked with 50 ng/mL Vit D₃.

Results and Discussion

Optimization of the HPLC Condition

The chromatographic separation was optimized for the intended purpose. In order to select the stationary phase, performance of C₈ and C₁₈ (4.6 mm × 250 mm, 5 μm) columns for the separation of Vit D₃ were investigated. Due to the poor peak shapes obtained using C₁₈ column, C₈ column was selected as the suitable stationary phase. To select the optimized mobile phase, different mixtures of solvent consisting of 5% acetonitrile in methanol, 10% acetonitrile in methanol, and 10% acetonitrile in isopropyl alcohol, were evaluated.

The obtained results indicated that the best separation was achieved using the mixture of acetonitrile and methanol with a ratio of 90:10% v/v with flow rate of 1.2 mL/min and an isocratic elution mode at 25 °C. The obtained chromatograms from the HPLC-UV analysis can be seen in Figure 1.

Optimization of the DLLME Procedure

Selection of Extracting Solvent's Type and Volume

Selection of a suitable extracting solvent is a critical factor that affects the efficiency of the DLLME procedure. Low solubility in water, lower density than water, good chromatographic behavior and high affinity to the analyte of interest are important criteria to choose a suitable extracting solvent (23). In the present study, tetrachloroethane, carbon tetrachloride, and chloroform were utilized as extracting solvents. The extractions were carried out with injecting 80 μL of each extracting solvent in combination with 2 mL of acetonitrile extract (as a disperser solvent) into the 5 mL water. Considering the volume of sedimented phase and the required time for the evaporation of the applied solvent at the end of DLLME procedure, carbon tetrachloride was selected as the extracting solvent for the further analyses. In addition to the extracting

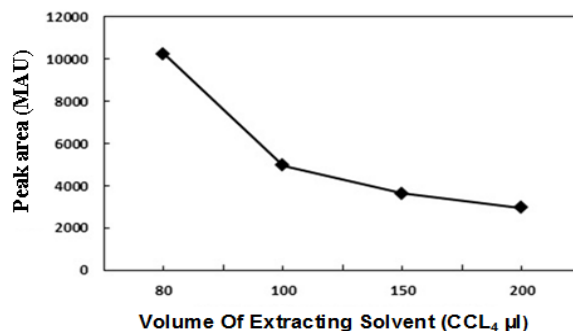


Figure 2. A dispersive liquid–liquid micro–extraction technique for the pre–concentration and quantification of vitamin D₃ in milk and yogurt samples using a non–aqueous HPLC method. Effect of extracting solvent's volume on the obtained responses from the analysis of vitamin D₃. Experimental conditions: type and volume of disperser solvent: acetonitrile, 2 mL; Vit D₃ concentration: 20 µg/mL; HPLC conditions: C₈ (4.6 mm × 250 mm, 5 µm) column, acetonitrile/methanol (90:10% v/v) with flow rate of 1.2 mL/min as a mobile phase, isocratic elution mode at 25 °C, UV detection at 265 nm.

solvent's type, the impact of its volume on the efficacy of DLLME procedure was evaluated, where different volumes of carbon tetrachloride (80, 100, 150 and 200 µL) were utilized to accomplish the best extraction output.

As shown in Figure 2, 80 µL carbon tetrachloride was selected as an optimal extracting solvent, since in low volumes of extracting solvent (<80 µL) the two phase system was not formed.

Selection of Disperser Solvent's Type and Volume

Miscibility of disperser solvent with both the organic phase (extracting solvent) and the sample solution is an essential factor in the selection of a suitable disperser solvent. It helps to disperse the extracting solvent in aqueous phase and as a result improves the extraction performance (23). In this study, acetonitrile, ethanol, and methanol were utilized as disperser solvents. According to the obtained results, application of acetonitrile as a disperser solvent resulted in the acceptable extraction efficiency. In addition to the disperser solvent's type, its volume plays an important role in the performance of extraction procedure, because it could directly influence formation of cloudy solution between ternary component solvent systems. Therefore, the impact of dispersive solvent's volume on the extraction efficacy was evaluated, where distinctive volumes of acetonitrile (500, 1000,

2000 and 3000 µL) were utilized to conduct DLLME procedure. The observations revealed that at low volumes (>2000 µL), acetonitrile couldn't appropriately scatter extraction solvent resulting in negligible formation of the cloudy solution. Additionally, it is worth saying that application of low volumes of acetonitrile could not effectively precipitate the proteins of the samples in the cleanup step. Higher volumes of acetonitrile (<2000 µL) resulted in increased solubility of analytes in the aqueous phase and consequently less extraction productivity. Therefore, acetonitrile with a volume of 2000 µL was selected as the optimal dispersive solvent.

Selection of the Extraction Time

In DLLME, extraction time is characterized as a time period between the injection of the blend of a disperser solvent, extracting solvent and sample in the water and centrifugation (23).

In the present study, observations showed that alterations in the extraction time from 30 sec to 2 min did not affect the extraction efficiency, significantly suggesting the independency of the applied DLLME technique from extraction time. However, in order to keep the uniformity among the different analyses, 1 min was selected as an extraction time.

Method Validation Results

Method validation results indicated that the

Table 2. The obtained results from the accuracy and precision evaluations.

Concentration (ng/mL)	Accuracy (RE%)	Repeatability (RSD%)	Intermediate precision (RSD%)
2	-1.5	11.4	7.2
20	-2.1	7.9	4.4
60	+0.6	3.9	1.2

method was linear in the concentration range of 2 to 60 ng/mL with a calibration equation of $y = 452385x - 526/73$, where y was peak area and x was concentration of Vit D₃ in µg/mL, and correlation coefficient of 0.9997. The LLOQ and ULOQ of the method was 2 and 60 ng/mL, respectively, owing to the possibility of the quantifications with acceptable precision (RSD% ≤ 20%) and accuracy (|RE%| ≤ 20%) at mentioned concentrations.

Since the obtained standard deviation among the responses of 6 blank samples was 136.0, the LOD of the method was calculated as 0.9 ng/mL.

The accuracy and precision results are reported in Table 2. As it can be seen in this table, the method was accurate (-2.1% ≤ RE% ≤ +0.6%) and precise (RSD% of less than 11.4%) enough for the quantification of Vit D₃ in the linear range, and its recovery was in

the acceptable range of 86.7 ± 9.5% to 113.3 ± 5.6%. It should also be noted that the recovery of vitamin D₃ in yoghurt samples (D, E and G brands), by the proposed method was from 88.55%, 89.23, and 99.05%, respectively.

Real Sample Analysis

The samples purchased from local supermarkets as described in sampling section, were analyzed using the optimized DLLME-HPLC-UV method. As it can be followed in Table 3, the method successfully quantified the amount of Vit D₃ in different samples and the findings indicated that in only one brand of fortified milk samples the obtained amount of Vit D₃ was in accordance with the label claim; the finding highlights the need to consider stricter legislation for the monitoring of the claims on the labels of dairy products in Iran.

Table 3. The obtained results from the quantification of Vit D₃ in studied real samples using the developed DLLME-HPLC-UV method.

Real samples (milk and yoghurt brands)	Label claim (IU*/mL)	Vit D ₃ (ng/mL) ± SD	Vit D ₃ (IU*/mL) ± SD
A	0.2	10.5 ± 1.5	0.42 ± 0.1
B	—**	12.4 ± 1.7	0.49 ± 0.1
C	0.2	17.1 ± 1.2	0.68 ± 0.1
D	0.5	14.3 ± 3.3	0.57 ± 0.0
E	0.1	6.30 ± 0.9	0.25 ± 0.5
F	—	9.10 ± 2.7	0.36 ± 0.1
G	—	7.60 ± 1.3	0.30 ± 0.0
H	—	6.40 ± 1.8	0.25 ± 0.1
I	—	5.20 ± 0.8	0.21 ± 0.0
J	—	6.30 ± 0.2	0.25 ± 0.0

*Each IU is equal to 25 ng Vit D₃.

**Not mentioned.

Conclusion

A DLLME-HPLC-UV method has been developed and validated for the reliable extraction and determination of Vit D₃ in dairy products, particularly milk and yogurt. The inherent advantages of DLLME procedure including simplicity and time and cost effectiveness, low amounts of required organic solvents and smaller sample volume along with lower LOQ value and shorter retention time (5.0 ± 0.8 min) are the main superiorities of the developed method to the previously reported ones. Nevertheless, it is worth saying that application of more sensitive detectors such as mass spectroscopy would result in the lower LOD and LOQ values as it can be seen in Table 1. Finally, derived from the all above mentioned discussions, it can be concluded that the developed method could be considered as a promising technique in the monitoring of Vit D₃ in dairy products and evaluation of the label claims of the fortified samples in food quality control laboratories.

Acknowledgment

This work is a part of thesis by Maryam Ghalebi submitted for MSc degree (No. 4) and is supported by the Research Council, Tabriz University of Medical Sciences.

References

- (1) Kwak BM, Jeong IS, Lee MS, Ahn JH and Park JS. Rapid determination of vitamin D₃ in milk-based infant formulas by liquid chromatography-tandem mass spectrometry. *Food Chem.* (2014) 165: 569-74.
- (2) Bilodeau L, Dufresne G, Deeks J, Clément G, Bertrand J, Turcotte S, Robichaud A, Beraldin F and Fouquet A. Determination of vitamin D₃ and 25-hydroxyvitamin D₃ in foodstuffs by HPLC UV-DAD and LC-MS/MS. *Food Comp.* (2011) 24: 441-8.
- (3) Trenerry VC, Plozza T, Caridi D and Murphy S. The determination of vitamin D₃ in bovine milk by liquid chromatography mass spectrometry. *Food Chem.* (2011) 125: 1314-9.
- (4) Phillips K M, Byrdwell WC, Exler J, Gebhardt SE, Harnly JM, Holden JM, Horst RL, Linda EL, Patterson KY and Wolf WR. Development and validation of control materials for the measurement of vitamin D₃ in selected US foods. *J. Food Comp. Anal.* (2011) 24: 299-306.
- (5) Black LJ, Seamans K M, Cashman KD and Kiely M. An updated systematic review and meta-analysis of the efficacy of vitamin D food fortification. *J. Nutr.* (2012) 142: 1102-8.
- (6) Omidvar N, Abtahi M, Neyestani T and Hajifaraji M. Sensory evaluation and assessment of compliance with vitamin D and Calcium fortified-milk among school-age children in Tehran. *Iran. J. Nutr. Sci. Food Technol.* (2012) 7: 61-7.
- (7) Chen Y, Reddy MR, Li W, Yettlla RR and Lopez S. Development and validation of a high performance liquid chromatographic method for simultaneous determination of Vitamins A and D₃ in fluid milk products. *J. AOAC Int.* (2015) 98: 390-6.
- (8) Kiely M and Black LJ. Dietary strategies to maintain adequacy of circulating 25-hydroxyvitamin D concentrations. *J. Scand. J. Clin. Lab. Inves.* (2012) 72: 14-23.
- (9) Kasalova E, Aufartova J, Kujovska L, Mova K, Solichova D and Solich P. Recent trends in the analysis of vitamin D and its metabolites in milk – A review. *Food Chem.* (2015) 171: 177-90.
- (10) Viñas P, Bravo-Bravo M, López-García I and Hernández-Córdoba M. Dispersive liquid-liquid microextraction for the determination of vitamins D and K in foods by liquid chromatography with diode-array and atmospheric pressure chemical ionization-mass spectrometry detection. *Talanta* (2013) 11: 806-13.
- (11) Rezaee M, Yamini Y and Faraji M. Evolution of dispersive liquid-liquid microextraction method. *J. Chromatogr. A* (2010) 1217: 2342-57.
- (12) Rezaee M, Assadi Y, Milani MR, Aghae E, Ahmadi F and Berijani S. Determination of organic compounds in water using dispersive liquid-liquid microextraction. *J. Chromatogr. A* (2006) 1116: 1-9.
- (13) Viñas P, Bravo-Bravo M, López-García I, Pastor-Belda M and Hernández-Córdoba M. Pressurized liquid extraction and dispersive liquid-liquid microextraction for determination of tocopherols and tocotrienols in plant foods by liquid chromatography with fluorescence and atmospheric pressure chemical ionization-mass spectrometry detection. *Talanta* (2014) 119: 98-104.
- (14) Viñas P, Bravo-Bravo M, López-García I and Hernández-Córdoba M. Quantification of β -carotene, retinol, retinyl acetate and retinyl palmitate in enriched fruit juices using dispersive liquid-liquid microextraction coupled to liquid chromatography with fluorescence detection and atmospheric pressure chemical ionization-mass spectrometry. *J. Chromatogr. A* (2013) 1275: 1-8.
- (15) Barba FJ, Esteve MJ and Frígola A. Determination of vitamins E (α -, γ - and δ -tocopherol) and D (cholecalciferol and ergocalciferol) by liquid chromatography in milk, fruit juice and vegetable beverage. *Eur. Food Res. Technol.* (2011) 232: 829-36.
- (16) Abernethy GA. A rapid analytical method for cholecalciferol (vitamin D₃) in fortified infant formula, milk and milk powder using Diels-Alder

- derivatisation and liquid chromatography–tandem mass spectrometric detection. *Anal. Bioanal. Chem.* (2012) 403: 1433-40.
- (17) Jakobsen J and Saxholt E. Vitamin D metabolites in bovine milk and butter. *J. Food Comp. Anal.* (2009) 22: 472-8.
- (18) Gomes FP, Shaw PN, Whitfield K and Hewavitharana AK. Simultaneous quantitative analysis of eight vitamin D analogues in milk using liquid chromatography–tandem mass spectrometry. *Anal. Chim. Acta* (2015) 891: 211-20.
- (19) Alvi SN, El Tabache C and Hammami MM. Determination of vitamin-D level in fortified milk by ultra-performance liquid chromatography. *World J. Pharm. Pharm. Sci.* (2015) 4: 170-81.
- (20) Kwak BM, Jeong IS, Lee MS, Ahn JH and Park JS. Rapid determination of vitamin D₃ in milk-based infant formulas by liquid chromatography-tandem mass spectrometry. *Food Chem.* (2014) 165: 569-74.
- (21) Nollet LML. *Food Analysis by HPLC*. 3rd ed. Taylor & Francis, New York (2013) 330.
- (22) ICH International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use, Q2 (r1): validation of analytical procedures: text and methodology (2005).
- (23) Shammugasamy B, Ramakrishnan Y, Ghazali HM and Muhammad K. Combination of saponification and dispersive liquid–liquid microextraction for the determination of tocopherols and tocotrienols in cereals by reversed-phase high-performance liquid chromatography. *J. Chromatogr. A* (2013) 1300: 31-7.

This article is available online at <http://www.ijpr.ir>
