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Original Article

Determination of Vitamin D_3 in Fortified Iranian Flat Bread (*Taftoon*) Using High-Performance Liquid Chromatography

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ABSTRACT

Background and Objectives: : Vitamin D_3 measurement in food and fortified food matrixes is usually interrupted by several variables. This study aimed to elaborate a specific vitamin D_3 quantification method for fortified *Taftoon* bread considering different influential variables in each step of measurement.

Materials and Methods: Dough was fortified with (4µg/100g) and the standard protocol No.13579 was followed with some modification in the extraction and determination steps.

Results: Addition of ethanol before adding potassium hydroxide and heating up the samples in tight containers in saponification step ceased clumping the extracts and helps it's homogeneity. In extraction, distilled water addition following extraction with diethyl ether and Petroleum ether, adding ethanol at each step of washing and using SPE column result in an appropriate experimental extract. In the chromatography step, mobile phase substitution from methanol to 5:95 water, methanol mixture provided the method with more differentiation power. Using the above condition, commercial vitamin D_3 was recovered from fortified *Taftoon* dough with 82-100% recovery and recovery of spiked vitamin D_2 from dough and bread were ranged as 94 and 86.5%, respectively, Limit Of Detection(LOD) and Limit Of Quantification(LOQ) of the method were 0.04 and 0.1mg L^{-1} respectively, which equaled 5µg/Kg and 10µg/Kg. True retention for the added commercial vitamin D_3 in *Taftoon* bread was obtained as 83.4% which increased to 92% after six months storage in freezer.

Conclusions: Results of this study can be used to specify a protocol for vitamin D_3 measurement in fortified bread samples. Modified experiment which is projected in this study would specify the general protocol of vitamin D_3 measurement that was previously proposed in the national standard of Iran for developing a new and practical national standard document.

Keywords: Vitamin D_3, Fortified bread, Extraction, High-performance liquid chromatography

Introduction

Vitamin D_3, (3β, 5Z, 7E)-9, 10-secocholesta- 5, 7, 10(19)-trien-3-ol) is a fat-soluble vitamin, which plays an important role in human health. Vitamin D_3 deficiency is known as a major hidden health problem worldwide. In addition to the typical and sever complications of vitamin D_3 deficiency, like rickets in children, osteomalacia and osteoporosis in adults, inadequate serum concentrations of this vitamin may induce long lasting negative effects.(1) Food fortification is a commonly accepted approach for preventing low vitamin D_3 status. Although animal originated foods are commonly considered as a suitable vehicle for vitamin D_3 fortification, bread has also been considered for this purpose in the recent years(1-3) . Being a staple food item and available to all social classes, vitamin D_3 fortified bread maybe more efficient for delivering this vitamin in to the low income individuals diet, (3) specifically in developing countries.

Although a few studies investigated the fortification of bread with vitamin D_3 and showed good stability of this vitamin in bulky bread

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manufacturing process (1, 3), relevant information regarding different types of beads is lacking in the literature. In particular, the methods which specifically describe the measurement of vitamin D_3 in bread are discussed by few studies so far (4).

Analytical determination of vitamin D_3 is highly affected by the variety of parameters e.g. physical condition, sample to solvent proportion, extraction process as well as the source of sample. It was shown that isomerization/oxidation to close derivatives during non-optimized measurement of vitamin D_3 negatively affects the precision and accuracy of the results (5).

In Iran all accredited analytical laboratories are highly recommended to follow the protocols provided by the Institute of Standards and Industrial Research of Iran. With regard to vitamin D_3 measurement, this organization drafted the document No. 6151 entitles as “diets foods – determination of vitamin D content” in 2003, introducing a general analytical method for measurement of vitamin D_3. Although the type of analyte was not indicated in the given standard protocol. The updated standard was issued by the same organization afterwards as document No.13579 entitled as "Foodstuffs - determination of vitamin D by high performance liquid chromatography-Measurement of cholecalciferol (D_3) or ergocalciferol (D_2)".

Although more expanded protocols have been given in the latter documents, the method presented is as unspecific as the old one. On the other hand, a variety of chemicals and experiments have been included as the possible choices to use that is confusing rather than being a help. In addition, the exact quantity of the analyte and chemicals, also suitable containers to use are missing in this document, making it difficult to be applicable for determination of vitamin D_3 in the fortified *Taftoon* bread (Iranian flat bread).

To our knowledge, there are few studies on the fortification and determination of vitamin D_3 in flat bread, while there is a great tendency to launch such a product in some developing countries as flat bread in some areas like Iran, Afghanistan, Pakistan and Turkey is the most common bakery product in the diet. Previous studies carried out on bread fortification with vitamin D_3, mostly addressed the vitamin assays which had been used for vitamin D_3 measurement in food articles other than bread(1, 3, 6).

A recent study speculated the effect of diffident conditions in extracting vitamin D_3 added to flour and bread powder samples and showed that the solvent volume, dispenser volume, pH and ionic strength needs to be optimized while using dispersive liquid liquid microexraction as an efficient extraction protocol (4).

The aim of this study is to determine the optimized method for vitamin D_3 measurement in *Taftoon* breads as a popular Iranian bread based on principal steps of saponification, extraction and quantification. Specifications of this standard for measurement of vitamin D_3 in flat bread is therefore would be the main result of this study. Moreover, true retention of the added vitamin in the fortified bread would be of our interest.

Materials and Methods

Equipment: High pressure reverse phase liquid chromatography (Jasco, Japan), Intelligent HPLC pump, PU-2080 plus (Jasco, Japan), Solvent mixer, Model MX-2080-32, Dynamic Mixer (Jasco, Japan), Degasser, Model 2080-53, 3-Line Degasser(Jasco, Japan), Automatic injector, Model AS-2055 plus, Intelligent sampler(Jasco, Japan), Intelligent column thermostat, Model CO-2060 plus(Jasco, Japan), Intelligent UV detector, Model UV-2075 plus(. Jasco, Japan) C18 column 4.6 × 150m MN (Macherey-Nagel, Germany) Evaporator (Heidolph, Germany), Micro Silica-Column(SPE)(RS Tec, Korea),Oven (Behdad , Tehran)

Standards and Solutions Vitamin D_3 powder(DSM Company, Switzerland), Vitamin D_2 analytical standard (Sigma-Alderich, Germany),Methanol HPLC grade (Merck, Germany),Water HPLC grade (Merck, Germany) Potassium hydroxide (Merck, Germany) ,Ethanol(Hamoun Teb, Iran),Ascorbic Acid (Merck, Germany), Diethyl Ether (Merck, Germany), Petroleum Ether (TatChem, Iran).

Preparation of Vitamin D_3 Fortified Bread**:** Taftoon bread flour with 88% extraction rate was provided from a traditional bakery in Isfahan. Tap water, salt and yeast were added at the 55-85%, 1-2% and 1% of the flour weight, respectively to formulate the *Taftoon* bread dough. Commercial water soluble vitamin D_3 powder (5010950004, DSM Company Switzerland) containing 100000IU of vitamin D_3 per gram was used in this study. In order to enrich the dough, four ml of 1 mg/L of the vitamin D_3 solution was added to the 100gr mixture and mixed manually, covered with

a plastic sheet and kept at room temperature (30°C) for one hour. Then the fermented dough flattened and oven cooked (Behdad Company, 50 liter, Iran, Tehran) at 220°C temperature for an average of 20 minutes.

Saponification: Each of the bread samples (dough) were fluxed with 100-150 mL ethanol, 2-2.5 grams ascorbic acid and then 50ml of 28-50% potassium hydroxide solution. Processed samples were kept in water bath (80-100°C) for 25-30 minutes. In Table 1(a) varied parameters in the saponification step are shown. The order of ingredient addition and the type of container used in water bath were considered as changing variables in this step.

Extraction: Saponified samples were extracted with (2 × 50) mL diethyl ether and (2 × 50) mL petroleum ether in decantation. Some extracts were washed several times to attain neutral pH and then undergone evaporation by Heidolph evaporator (30°C). The out dried precipitants were dissolved in heptane and some samples were passed through the micro silica-column (INOPAK 500mg, 3ml volume, a product of RS Tec Korea). Applying distilled water fallowing the application of organic solvents, different washing solvents, using solid phase extraction (SPE) and different SPE rinsing and washing solvents[7] were considered as changing variables in this step (Table 2a).

Table 1. Variables (a) in saponification step and their outcomes (b)

a) Variable Conditions	b) Outcomes
The order of adding the ingredients	
Ascorbic acid, potassium hydroxide solution, ethanol	Clumping of the sample, improper homogenization.
Ascorbic acid, ethanol, potassium hydroxide solution	Uniform disintegration of sample, well homogenous mixture
The type of container used in water bath	
Glass container covered with aluminum foil	Sample dryness negatively altered the sample texture
Shot Bottle	Uniform homogenous mixture

Table2. Variables (a) in extraction step and their outcomes (b)

a) Variable Conditions	b) Outcomes
Extraction with organic solvents	
Diethyl ether+Petroleum ether	Inappropriate separation
Diethyl ether+Petroleum ether with increasing polarity by adding water	Ease in decantation of the saponified product
Washing solvents:	
Distilled water	Foam creation, inappropriate separation.
Distilled water and ethanol	Proper partitioning of the created foam.
Use of SPE column	
Nonuse of SPE column	Merged peaks in chromatogram
Use of SPE column	Individuals peaks identical to vitamin D_3 and its derivatives
Ratio, type and the amount of solvents used in SPE:	
10ml diethyl ether (6%) in heptane in Rinsing and washing with 20 ml diethyl ether (20%) in heptane.	Merged peaks in chromatogram
Rinsing with 8ml propanol (20%) in heptane and washed with8 ml propanol (6%) , 4 ml propanol (10%) and 8 ml propanol (20%) in heptane, respectively.	Individuals peaks identical to vitamin D_3 and its derivatives

Evaporation: Extracts were vacuum dried below 40°C using two different evaporators: 1) vacuum rotary evaporator (Heidolph Germany), 2) and handmade vacuum evaporator. Dried sediments were dissolved in 2mL of HPLC methanol.

Vitamin D_3 Measurement by

High-Performance Liquid Chromatography: High-performance liquid chromatography, equipped with a compact-sized HPLC pump (Model PU-2080 plus), solvent mixer (Model MX-2080-32, Dynamic Mixer), degasser (Model 2080-53, 3-Line Degasser), automatic injector (Model AS-2055 plus, Intelligent sampler), column thermostat (Model CO-2060 plus, Intelligent column thermostat), LC-Net Π / ADC, UV detector (Model UV-2075 plus, Intelligent UV/Vis Detector) all of which were purchased from Jasco, Japan. C18 column 4.6 × 150m manufactured by MN (Macherey-Nagel), Germany was used for vitamin D_3 measurement. The column oven temperature was 30°C. HPLC grade methanol/ distilled water were provided from Merck and used as mobile phase. Two different mobile phases including (95%/5%) methanol /water or pure methanol were used separately as two different conditions. The flow rate of the mobile phase was 1 mL/min applying the injection volume of 100 µL, quantitation was performed based on peak area. Vitamin D_3 peak was identified at 265nm (Figure 1).

Validity parameters: Unfortified bread has almost no detectable vitamin D_3. Therefore, Limit Of Detection (LOD) was determined as the lowest vitamin D_3 concentration that was detected in this experiment as 0.04 mg/L measured up to 4 µg/Kg in bread which confirmed by signal to noise (S/N) as 3.75. Limit Of Quantification (LOQ) was obtained as higher concentration 0.1mg/L since in this concentration S/N was obtained as 12.71, this value allowed us to quantify concentrations as low as 10µg/Kg. A stock standard solution (1000 mg/L) of vitamin D_3 (Merck-USA) in methanol was made. From which, working standard solutions were prepared at 0.1, 0.5, 1 and 2 mg/L concentration levels. Plotting the peak areas against concentration levels calibration curve was constructed, and equation NO.1 was well fitted with R^2= 0.99.

Equation 1: Y=1980.5X+6.887.

Working standard solutions of vitamin D_2 at 0.05, 0.1, 0.5, 0.8, 1 and 2 mg/L concentration levels were made from a stock standard solution (1000mg/L) of vitamin D_2 in methanol and calibration curve (equation NO.2) was obtained with R^2= 0.98**.**

Equation 2: Y=890.51X+98.834.

Using three standard solutions including 0.1, 0.5 mg/L intra-assay CV of the measurement method were obtained as 9.79% and 11.47%. While, inter-assay CV of the method yields 12.41% and 21.57% respectively.

The amounts of vitamin D_2 in dough and bread samples were measured with two replicates. The dough and bread samples were spiked with 1 ppm (0.5mL) vitamin D_2 standard solution twice, in two independent experiment runs. Recovery of vitamin D_2 in dough samples were calculated as 94% and in bread samples varied between 75 and 98 percent. Recovery of commercial vitamin D_3 in dough samples varied between 76 and 92 percent.

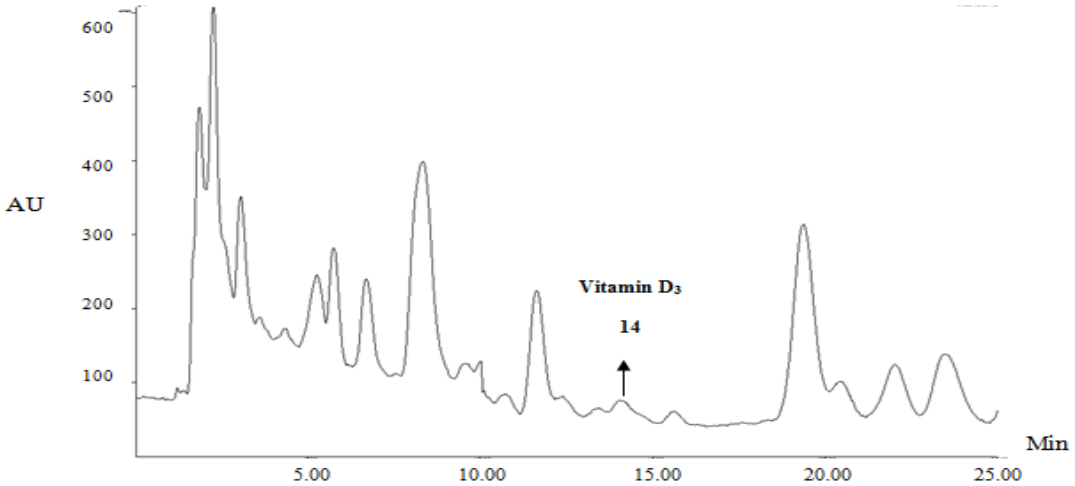


Fig1. Chromatogram of dough fortified sample at 10ppb commercial vitamin D_3, the peak with 14 min retention time is identical to vitamin D_3 from analytical standard.

Calculation of Vitamin D_3 purity in commercial vitamin D_3: The 0.05 ppm solution of commercial vitamin D_3 was prepared in water and vitamin D_3 was measured in 20 ml of this solution, which was quite similar in the dough and bread samples. Purity of commercial vitamin D_3 was obtained as 92%.

True Retention: Adapted from Jette Jakobsen(1), true retention in *Taftoon* bread was calculated using equation 3. True retention time was measured on the second day of bread production and also after six months storing the bread samples in -20ºC freezer (Equation 3).

Results

The outcomes of the changing variables in saponification step are listed in Table 1 (b). Ethanol addition before adding potassium hydroxide solution and heating the samples in tight containers were diagnosed as the suitable conditions giving rise to better homogenization of the sample, followed by ease in separation of the resulting saponified mixture. The results of the varied parameters in the extraction steps are listed in Table 2 (b). As indicated in this table, distilled water addition following extraction with diethyl ether and Petroleum-Ether, adding ethanol at each step of washing and using SPE column were considered as superior conditions in this step, leading to production of an appropriate extract. In the evaporation step, it was found that the position of out dried sediment in the evaporation vessel affects its later resolving efficiency in methanol. Uniform deposition of sample at the bottom of the vessel resulted in high yielded resolving material in methanol. Through the chromatography measurement of vitamin D_3, mobile phase shift from pure methanol to 5:95 water: methanol mixture provides the method more differentiation power within the range of vitamin D_3 retention time, resulting in more accurate chromatogram. The effect of using SPE micro columns, type and ratio of solvents used for SPE and also mobile phase used for HPLC on the peak of vitamin D_3 were compared in Figure 2 and Figure 3.

According to Table 2 and Fig. 2 and 3, using the cleanup solid phase, changing the type and ratio of solvent that was used in Micro-column and using water-methanol mixture (95:5), led to clear peak spacing in the chromatogram.

The amount of vitamin D_3 levels in the fortified dough and fresh bread samples were obtained as 42µg/Kg (1680 IU/Kg) and 56 µg/Kg (2240 IU/Kg), respectively. Using the obtained data, true retention yielded as 83.4% in the bread, one day after production and it increased to 92.24% after six months freezing.

Equation 3:

$$\%\text{True retention} = \frac{gvitDper100gramofcookedfoodmultipliedbytheamountofcookedfood}{gvitDper100gramofrawfoodmultipliedbytheamountofrawfood} * 100$$

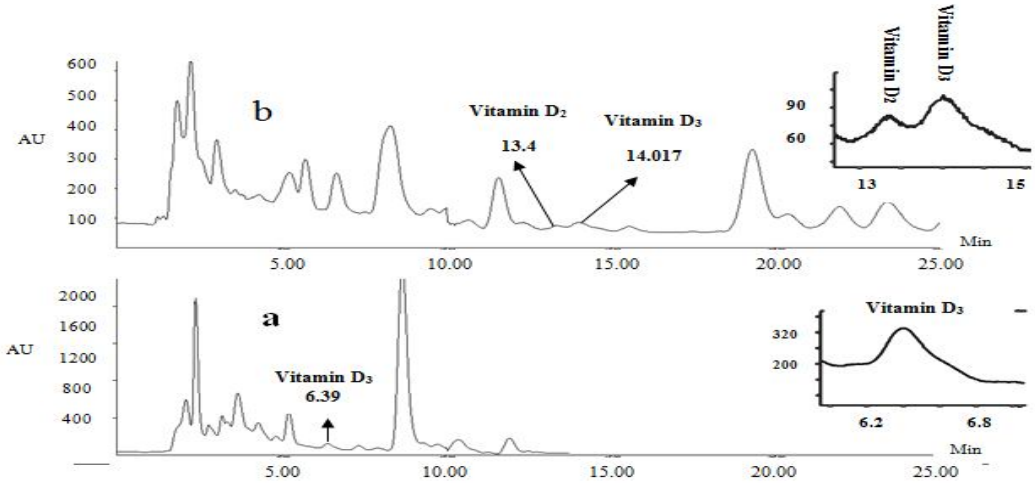


Fig2. a) The chromatogram generated by a test sample without passing the samples through SPE and using methanol as mobile phase revealed a peak of vitamin D_3 after 6min, **b)** The chromatogram generated by a test sample after passing the samples through SPE and using 95% methanol and 5% water as mobile phase revealed a peak of vitamin D_3 after 14min.

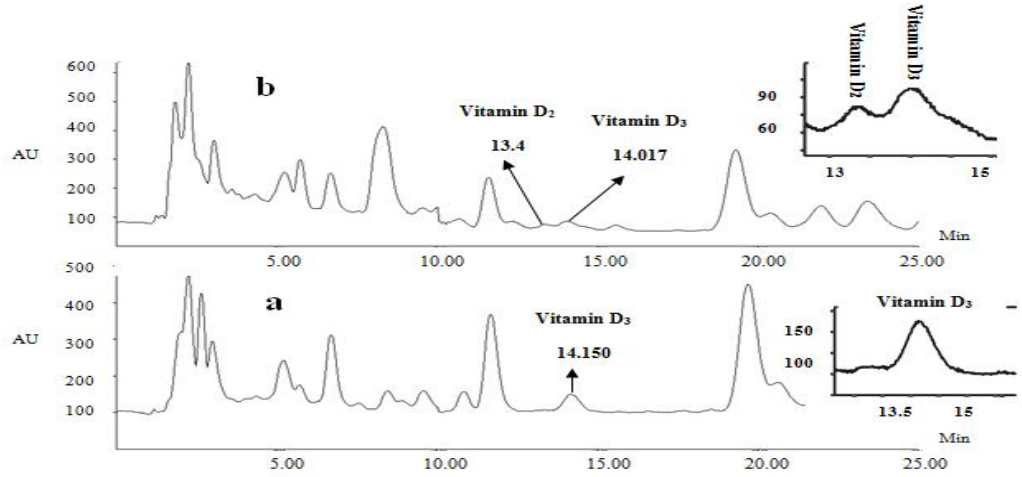


Fig3. a) The chromatogram of a test sample using SPE and conditions recommended in standard NO.13579 results in no differentiation between vitamin D_2 and Vitamin D_3. **b)** The chromatogram of a test sample using SPE and conditions adapted by Jäpelt.[5] Revealed a clear differentiation between Vitamin D_2 and vitamin D_3.

Discussion

In this study, some parameters in the national standard document No.13579 were changed in order to recommend a specific method for determination of Vitamin D_3 in flat bread in the both extraction and quantification stages.

In the saponification step, previous studies mainly have focused on the effect of varied temperature and time with the most confirming 70-85°C and 25 to 30 min conditions (8-12).

However, some studies stated that using high temperature water bath can negatively affect the saponification process(13, 14). Evaporation of solvent at high temperature; similar to what was observed in the present study could be involved. Changing the order of KOH and Ethanol addition ended up to the desired condition in our study which was not reported elsewhere.

In the extraction step, water addition resulted in biphasic extract with clear cut at interface. Formation of intractable emulation layer in this step was considered as a common problem in this step; Katherine M. Phillips *et al*, concluded that substitution of hexane with a more polar solvent such as ethyl ether and petroleum ether, detained emulsion formation using the wide range of food matrices. Regarding the type of solvents used in the extraction step, more studies could be addressed that accomplished vitamin D_3 measurement using the same solvent combination used in the present study (9, 14-17).

During the washing step, ethanol addition to the distilled water used for washing solvents, which had a significant impact on separation which confirmed the results of Katherine M. Phillips *et al*, who mentioned the positive impact of ethanol in stabilizing the solution state of solvents.(8) In the post extraction treatment, the results of this study in terms of using micro-column, confirmed the results of Qian and Sheng in that solid phase extraction helped distinguishing two types of vitamin D_2 and D_3 (5, 14).

As another changing variable in this study, changing the ratio, type and amount of solvent that was used in SPE had significant effects on the performance of this treatment. Following the reference standard method document No.13579, we obtained very weak results. While by adapting the instruction used by Jäpelt, more accurate results were obtained (7). Differences in the polarity of the used solvents in the two tested protocols may gave rise to the different results. In the reference standard method, 6% diethyl ether in heptane was recommended as a rinsing solvent. In the increased in polarity, 20% diethyl ether in heptane was used as the collective solvent in the given protocol. While as the modified condition in the present study, gradual increase in polarity may lead to more specific conditions for determining the vitamin D_3 in the purified form. Gradual increase in polarity of the collective solvent in washing pure form of vitamin D_3 from micro column was stated elsewhere (9).

New studies in the field recommend micro-extraction as a comparable method to the classic assay which was used in the present study.

In HPLC determination of vitamin D_3, it was shown in this study that substitution of the mobile phase methanol: water (95:5) instead of the pure methanol, led to better separation of vitamin D_2 and D_3 peaks, though, the vitamin D_3 retention time became longer. Using 6% to 7% water in methanol as HPLC mobile phase, similar results, as well as proper recovery results were reported by other studies in vitamin D_3 measurements in fortified bread (3, 14, 18). Generally bread is not considered as a source of vitamin D_3, for instance in the relative publications as well as nutrient component tables of food, no detectable vitamin D_3 is reported for different kinds of breads (3, 19, 20). However, although in very small amount, vitamin D_2 as a plant source of vitamin D exist in many types of bread which could be from the yeast (21, 22). Structural similarity between vitamin D_2 and D_3 could interfere with the vitamin D_3 calculation as D_2 can appear in the chromatogram with the same retention time as vitamin D_3, when the applied protocol does not allow to differentiate the two component from together. Investigating the vitamin D groups in foods, Mattila reported that vitamin D_2 may appear along with vitamin D_3 peak leading to false positive results.(14, 23) The observed peak in fig.3 (a) at 14 min correspond to 0.07 ppm vitamin D_3 which is higher than the fortified levels, while using optimize condition, differentiation of vitamin D_2 and Vitamin D_3 was obtained. Examining the first appeared peak in fig.3 (b) at 13.4, show that it belongs to vitamin D_2 which corresponds to 0.014 ppm. This confirms the results of Mattila in that using SPE column a clear differentiation was observed with about 2 minutes time difference in vitamin D_2 and vitamin D_3 retention time. True retention time of vitamin D_3 in *Taftoon* bread in our study (83.4%) was comparable to the results of the study carried out by Jakobsen on wheat bread and rye bread with the retention of vitamin D_3 as 85%, and 73% respectively. Increased value of retention time after freezing in this study could also be due to moisture loss. However, this result shows that added vitamin D_3 adequately maintained in the bread during freezing and can be quantified with the modified protocol used in this study.

Conclusion

Application of some changes in the general vitamin D_3 measurement method which was previously introduced as a national standard protocol can result in a more efficient assay with adequate accuracy for vitamin D_3 and vitamin D_2 quantification in *Taftoon* bread. Using this modified protocol, vitamin D_3 revealed to be quite stable with high retention in *Taftoon* bread even after freezing.

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