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Determination of Vitamin D 3 Content in High, Low and Zero Fat Food Using High Performance Liquid Chromatography

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Abstract

Background: Vitamin D, has a significant role in bone metabolism and helps calcium absorption in the body. There are only few vitamin D assay methods available for zero fat food.

Aims of Study: (1) To develop an accurate and sensitive LC method for the quantification of vitamin D_3 in food by optimization of each step of the analytical method: Extraction, sample preparation, separation and detection. (2) To validate the developed method. (3) To apply the method to quantify the total vitamin D3 in food from several species.

Material and Methods: In this study, a rapid, simple, and economical reversed phase liquid chromatographic method was described for the determination of vitamin D_3, in some high, low and zero fat samples (milk products, cereal and chewing gum samples). The isolation of fat soluble vitamins includes a saponification step and an extraction step with petroleum ether and diethyl ether. The vitamin D_3 content of samples was determined by reversed phase liquid chromatography. Ultra violet-Visible (UV-VIS) detector and C18 column were used for this purpose.

Results: The linearity of standard curves of vitamin D_3 were 10-200 μg/ml expressed of the correlation coefficient $r^2 = 0.9992$). The detection (LOD) and quantification (LOQ) limits were 5.09 and 15.42 μg/ml, respectively. The accuracy was 101.37±4.37.

Conclusion: The described reversed-phase HPLC method is favorable compared with other published HPLC-UV methods (20 and 21) because of its stability-indicating nature, short run time and wide analytical range with outstanding linearity, accuracy and precision. The proposed method allows the determination of vitamin D_3 in a single chromatographic run and is suitable for the analysis of the stability of vitamin D_3. The obtained results from the assay of vitamin D_3 in commercial nutrition supplements confirmed that the method is appropriate for the routine analysis of various food samples.

Key Words: *Analytical measurement – Dietary supplements – Fortified foods – Infant – Vitamin D.*

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Introduction

VITAMIN D is very important fat soluble vitamin in human and animal diet. It exists in two forms, Vitamin D_2 and D_3. Vitamin D_3 (cholecalciferol) is synthesized endogenously from 7 – dehydrocholesterol after ultraviolet irradiation or is absorbed from the diet [1]. Vitamin D plays an important role in the maintenance of normal levels of calcium and phosphorus in the blood stream and is essential for the proper development and maintenance of bone [2]. Both vitamins D_2 and D_3 are biologically inactive. In humans, they are metabolized in the liver to calciferol (25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3, collectively known as 25(OH) D), Vitamin D that is not metabolized in the liver is stored in the adipose tissue and skeletal muscle, then released during vitamin D deprivation [3]. It has also been suggested that vitamin D_3 is more efficiently absorbed by the intestine than vitamin D_2 [4]. Vitamin D is soluble in lipids. Therefore the amount of fat in a meal may affect bioavailability with potentially higher vitamin D absorption from consumption in a higher fat food or meal [5].

Vitamin D deficiency is not only related to muscle weakness and osteomalacia, but has also been associated with cardiovascular disease, cancer, autoimmune diseases, diabetes mellitus and hypertension [6]. Furthermore, newborn babies may be affected in their normal growth and development, putting them at risk of long-term physical deficits [7].

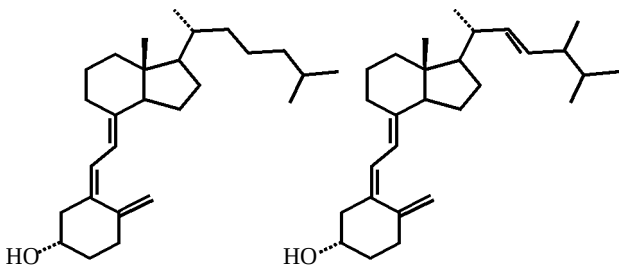
HPLC methods offer the best approach to accurate determination of vitamin D_3 content in foods and pharmaceuticals, as well as stability testing. In the last decade, high-performance liquid chromatography coupled to mass spectrometry has

become the technique of choice for vitamin D 3 determination in foods, feeds and pharmaceuticals [8].

Vitamin D chemistry:

Vitamin D exists in a number of forms, where the major physiologically relevant forms are vitamin D2 (ergocalciferol) and vitamin D 3 (cholecalciferol) (Fig. 1) [9]. Vitamin D 2 is produced by ultraviolet B (UVB) irradiation of the plant steroid ergosterol. Vitamin D 3 is synthesised in the skin of vertebrates through the action of UVB and 7-dehydrocholesterol. Vitamin D 2 is the less common form of vitamin D and has potentially lower bioavailability than Vitamin D 3 [10]. Vitamin D from sunlight or dietary sources is biologically inactive and is hydroxylated, via a two step process to 1,25-dihydroxyvitamin D (calcitriol), before it becomes metabolically active [9].

Vitamin D two major forms, cholecalciferol (vitamin D 3) and ergocalciferol (vitamin D 2).



Vitamin D3
Molecular weight:
384.64

Vitamin D2
Molecular weight:
396.65

Fig. (1): Different forms of vitamin D.

Material and Methods

Samples selection:

In January 2017 In January 2017, products were purchased for this pilot study from the local supermarkets in Cairo governorate, Egypt, the selection included the majority of the available brands, and all products were brought within the best-before date. To study the potential difference in vitamin D content between labeled and measured concentration, triple of products of the same brand but each with a different best-before date were brought.

According to the South African Food Data System (SAFOODS), full cream milk contains at least 3.3g of fat per 100ml of which 2.1 g is saturated fat. Low fat contains not more than 1.9g of fat per 100ml of which 1.2g is saturated. On the other hand fat-free or skim milk contains not more than 0.2g of fat per 100ml and only a trace of saturated fat.

Vitamin D fortified foods were classified in the following two product groups:

- High fat food samples: Were included in baby milk powder and creamy cooking cheese.
- Low and Skimmed food samples: Low and zero fat food samples were selected, low fat samples included yogurt and baby food based on cereals (cerelac). While, zero fat vitamin D 3 food supplement in chewable forms were include in baby gum.

Labeled vitamin D concentration:

The labeled vitamin D concentration (µg/100gm) was obtained by the manufacturer's label declaration for comparison with the analyzed vitamin D concentration.

It is, however, not always possible for food or supplements to contain the exact micronutrient level specified on the label, due to natural and processing variations, as well as changes during storage. But on the other hand substantial deviation from what is labeled could mislead the consumer and should be prevented [11]. The actual vitamin D concentration of fortified foods and dietary supplements may deviate from the label due to potential overages to cover losses during shelf life [12].

Chemical analysis (Reagents and materials):

All materials and reagents used in this study were pure laboratory chemicals. Potassium hydroxide pellets, sodium sulphate anhydrous, ascorbic acid were purchased from Merck. The organic solvents used include, ethanol, methanol, petroleum ether (40-60ºC), acetonitrileand diethyl ether peroxide free. All the used chemicals were of the highest purity available analytical grade and all solvents in this study were grade-HPLC and obtained from Merck, fluka, Fisher, sigma Aldrich Companies.

Experiments were carried out using a GBC HPLC system coupled to UV-VIS detector using single wavelength for this study, 264nm for Vitamin D3. The compounds were separated with reversed phase (250mm x4.6mm, 5µm) ACE 5 C18-300 column with a C 18 guard cartridge which maintained at room temperature. Mobile phase was (50%: 50%, v/v) methanol and acetonitrile HPLC grade and the flow rate was 1.00mL/min (isocratic elution). Total run time required was equal to 7 min.

Standard solutions:

Stocks and standards solution of Cholecalciferol (vitamin D 3): Stock solution was prepared by dissolving 1mg of cholecalciferol standard in 1ml

methanol. Serial standard solutions were prepared by using 20, 50, 100, 150 and 200 µl of the stock standard solution in 10ml measuring flask then complete to the mark by methanol. The stock and standard solutions were stored in brown flask with screw cap to avoid exposure to light and air at –4ºC in refrigerator.

Saponification:

Saponification step of high fat food: Five grams of powdered or 5ml liquid sample weighted in 10ml measuring flask then complete to the mark by warm deionized water at 40ºC and mixed for 10min by vortex until complete homogenization occurred then 20ml 50% KOH solution and 1gram of ascorbic acid were added in 100ml measuring flask with covered foil and complete to the mark by ethanol with continuously shaking through over night in water bath at room temperature until the sample was completely saponified.

Saponification step of low and zero fat food: Five grams of powdered or 5ml liquid sample, 1 gram of rice bran oil (put in 100ml volumetric flask then add 20ml 50% KOH solution and one gram of ascorbic acid added and completed by ethanol to mark. The solution was covered by aluminum foil with continuously shaking through over night in water bath at room temperature until the sample completely saponified) and complete as the first saponification step.

Extraction step for high and low fat and non fat food: After shaking, the sample was transferred into 500ml separating funnel then add 50ml of (50%: 50%, v/v) diethyl ether: Petroleum ether, shake the mixture for 20min. The upper and organic layer was left in the same separating funnel. The aqueous layer was re-extracted with a further 50ml of the mixture and the mixture was shaken for 20 min in another separating funnel. The two organic layers were joined and washed twice by shaking 2min with 250ml of deionized water. The organic layer washed until no change in red pH paper. The organic layer was collected and evaporated by furnace at 40°C. For reversed phase chromatography the residue dissolved in 2 ml of methanol for determination of vitamin D_3 was directly filtered through 0.45 µm filter and 20 µl of extract was injected into the HPLC system. The vitamin D_3 concentration in the samples was calculated in µg/100g edible weight using peak area by comparison between standard and sample after injection.

Determination of fat crud:

0.5-2 g sample was weighted in 250ml volumetric flask, adding 10ml of distilled water, 1.5ml ammonium hydroxide was added then the flask was immersed on water bath at 70ºC for 15min then cooled. 10ml of concentrated hydrochloric acid was added. The flask was heated on hot plate for 5 min then cooled. 25ml petroleum ether, 25ml diethyl ether and 10ml ethyl alcohol 95% were added and mix them and leave for 24 hour. Solvent with fat was separated in beaker (known weight). The beaker was leaved in the oven at 50ºC. The beaker was put in desiccator until cool then the beaker weighted with fat.

- Weight of fat = Weight of beaker with fat – Weight of beaker
- % Crude fat = (Weight of fat / Weight of sample) x 100

Results

Table (1) shows the measured and labeled fat content in high fat food (represented by creamy cooked cheese and baby infant milk powder), low fat food (represented by yogurt and baby food based on cereals) and zero fat food (represented by baby gum).

Table (1): Determination of crud fat and comparison with label information.

Fat g/100 g	Label concentration g/100g	Found concentration g/100g	Mean	SD	RSD %	Recovery %
1- Fat in (creamy cooked cheese) cheese	25	24.08 24.14 24.75	24.32	0.37	1.52	97.29
2- Fat in baby infant milk powder	26.1	25.81 25.37 25.74	25.64	0.24	0.92	98.24
3- Fat in yogurt	3	2.83 2.59 2.70	2.71	0.12	4.44	90.22
4- Fat in baby food based on cereals	0.55	0.47 0.51 0.46	0.48	0.026	5.51	87.27
5- Fat in baby gum	0	0	0	0	0	0

The number of vitamin D fortified foods has increased. The food type with the greatest increase was yoghurt, Fortified skim milks and reduced fat milks [13]. Fortification can be achieved in multiple ways with varying efficiency. For example, vitamin D3 was added by: Addition of water-soluble emulsion, or crystalline liposoluble vitamin D [14]. Vitamin D3 was fortified into yoghurt in either a crystalline or emulsified form; both forms of vitamin D3 were stable in yoghurt during storage for the expected shelf lives of the products [15]. In an evaluation of increasing the level of vitamin D3 fortification in high temperature processed reduced fat milks and low-fat yoghurt serving, no loss of vitamin D3 during processing was found [16]. Vitamin D3 has also been shown to be stable in a non-fat food, with no change [17]. Study by [18] strongly recommend food industries to use opaque containers for storage of yogurt products or even any kinds of foodstuffs fortified with Vitamin D3 to reduce light-related degradation of the vitamin during the products shelf-life.

Calibration curve of vitamin D3:

The linearity of standard curves (Table 2) was expressed in terms of the determination coefficient (r^2) from plots of the integrated peak area versus concentration of the standard (µg/ml). These equations were obtained over a wide concentration range, in accordance with the levels of these compounds found in food samples. Linear equations were found, with satisfactory linearity (r^2 >0.99).

Table (2): Vitamin D3 Calibration curve analysis.

Concentration µg/ml	Beak area	Found concentration µg/ml	Recovery%
20	111	20.84827	104.2413
50	286	49.51563	99.03126
100	579	97.51298	97.51298
150	920	153.3734	102.2489
200	1197	198.7497	99.3 74 86

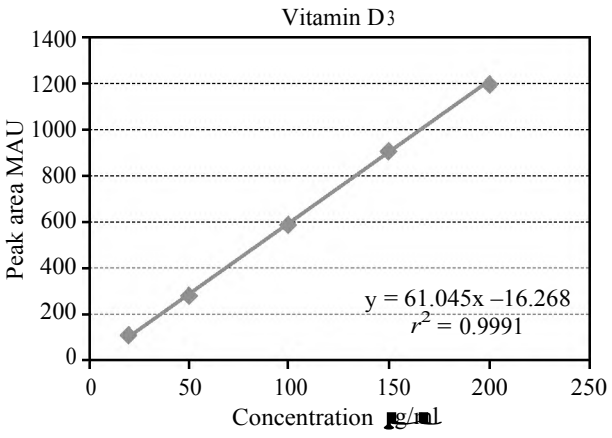


Fig. (2): Calibration curve of vitamin D3.

Validation of vitamin D3:

Validation studies were performed by measuring basic parameters such as precision, accuracy, linear region, limits of detection (LOD) and quantification (LOQ), and recovery.

Table (3): Validation sheet of vitamin D.

Parameter	Values
Accuracy	100.48±2.71
Slope	6.10
Intercept	–16.27
Linear Range	20-200 (µg/ml)
Correlation Coefficient (*r*)	0.9991
Standard Error	13.28
Variance	6.10
LOD	6.53
LOQ	21.76

Quality control chart of vitamin D3:

10 samples of standard reference material® 1849 a prepared by this method.

- Mean = 1.11 µg/100 g, SD = 0.085

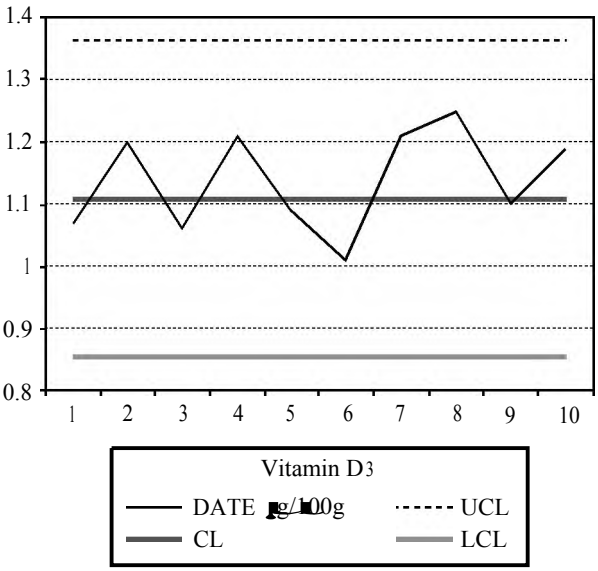


Fig. (3): Quality control chart of vitamin D3.

Vitamin D3 in Creamy cooked cheese:

Fig. (4) shows measured vitamin D3 concentration (µg per 100g) in creamy cooked cheese compared to the vitamin D content declared on the label, the measured vitamin D3 content ranged between 87.5% and 96.4% of the declared value.

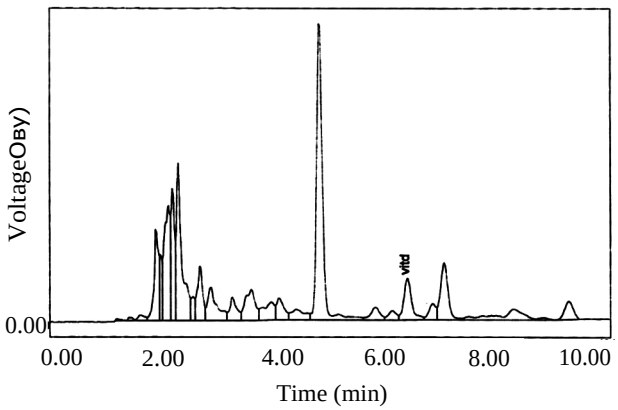


Fig. (4): Vitamin D_3 in creamy cooked cheese.

Vitamin D_3 in Baby infant milk powder:

Fig. (5) shows measured vitamin D3 concentration (g g per 100g) in baby infant milk powder compared to the vitamin D content declared on the label, the measured vitamin D_3 content ranged between 91.5% and 96.2% of the declared value.

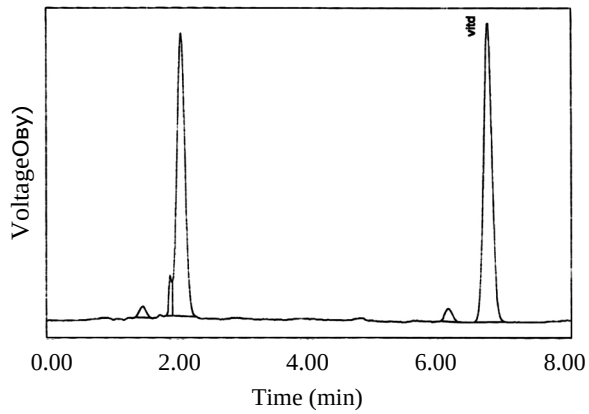


Fig. (5): Vitamin D_3 baby in infant milk powder.

Vitamin D_3 in Yogurt:

Fig. (6) shows measured vitamin D_3 concentration (g g per 100g) in yogurt compared to the vitamin D content declared on the label, the measured vitamin D_3 content ranged between 82.3% and 89% of the declared value.

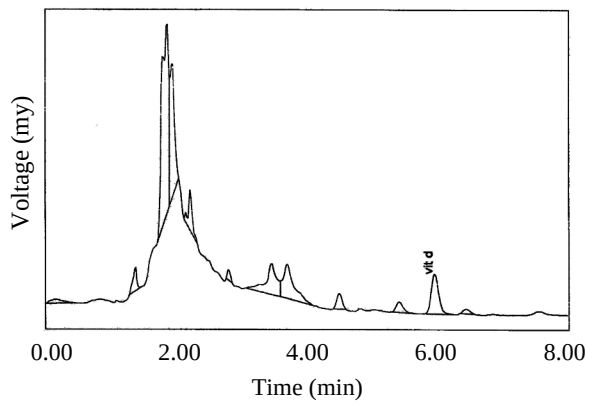


Fig. (6): Vitamin D_3 in yogurt.

Vitamin D_3 in Baby food based on cereals:

Fig. (7) shows measured vitamin D_3 concentration (g g per 100g) in yogurt compared to the vitamin D content declared on the label, the measured vitamin D_3 content ranged between 91.4% and 96.4% of the declared value.

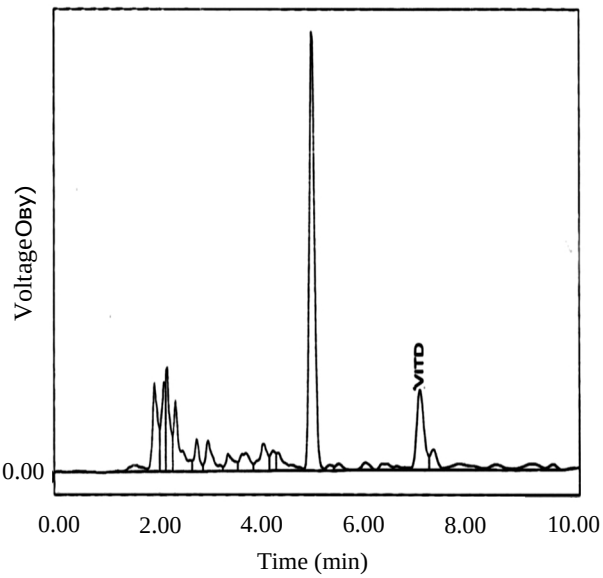


Fig. (7): Vitamin D_3 in baby food based on cereals.

Vitamin D_3 in Baby gum:

Fig. (8) shows measured vitamin D_3 concentration (g g per 100g) in herba land kids gummy compared to the vitamin D content declared on the label, the measured vitamin D_3 content ranged between 93.5% and 96.5% of the declared value.

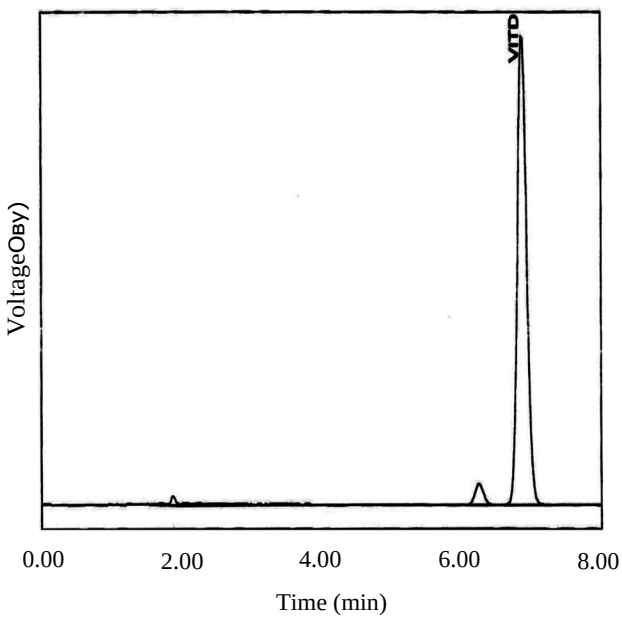


Fig. (8): Vitamin D_3 in baby gum.

Table (4): Overview of labeled and measured vitamin D 3 contents in fortified foods and dietary supplements.

Vitamin D3 µg per 100 g	Label concentration µg/100g	Found concentration µg/100g	Mean	SD	RSD %	Recovery %
1- Vitamin D3 in creamy cooked cheese	2.5	2.18 2.32 2.41	2.32	0.116	5	92.8
2- Vitamin D3 in baby infant milk powder	10	9.62 9.58 9.15	9.45	0.26	2.76	94.5
3- Vitamin D3 in yogurt	3	2.67 2.47 2.52	2.55	0.104	4.08	93.33
4- Vitamin D3 in baby food based on cereals	5	4.61 4.57 4.82	4.67	0.134	2.88	90.59
5- Vitamin D3 in baby gum	170	165 159 164	154	3.53	2.96	

Discussion

The measured vitamin D concentrations were compared with the labeled values. The products for which the measured vitamin D value deviated significantly from the declared value were re-analysed

Figs. (4-8) and Table (4) show the concentrations of vitamin D 3 in food samples (creamy cooked cheese, baby infant milk powder, yogurt, baby food based on cereals and baby gum, it was found to be 2.32±0.12, 9.45±0.26, 2.55±0.10, 4.67±0.13 and 154±3.53 µg/100g respectively and label vitamins content were 2.5, 10, 3, 5 and 170. The recoveries rang 90.6%-94.5%. The relative standard deviations were 2.8-5%.

In this study, some parameters were changed in order to recommend a specific method for determination of Vitamin D 3 in high, low and zero fat content food 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 70-85ºC and 25 to 30min conditions **[19]**.

However, some studies stated that using high temperature water bath can negatively affect the saponification process **[20,21]**. In this study, warm deionized water at 40ºC and mixed for 10min. was applied.

In the extraction step, Formation of intractable emulation layer in this step was considered as a common problem in this step, the 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.

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 **[20]** who mentioned the positive impact of ethanol in stabilizing the solution state of solvents. In the post extraction treatment, the results of this study in terms of using micro-column, confirmed the results **[23]** stated that solid phase extraction helped distinguishing two types of vitamin D2 and D3.

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.

Recent 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 was (50%: 50%, v/v) methanol and acetonitrile instead of the pure methanol, led to better separation of vitamin D 2 and D3 peaks, though, the vitamin D 3 retention time became longer.

Structural similarity between vitamin D 2 and D3 could interfere with the vitamin D 3 calculation as D2 can appear in the chromatogram with the same retention time as vitamin D 3, when the applied protocol does not allow todifferentiate the two components from together. Investigating the vitamin D groups in foods, **[22]** reported that vitamin D2 may appear along with vitamin D 3 peak leading to false positive results. The observed peak in Figs.

(4-8) 7min correspond to vitamin D_3 which is higher than vitamin D_2 at 6min. 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. 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.

Comparing the claimed and experimentally obtained values of vitamin D_3, less than 18% difference was observed for all samples. These results are satisfactory considering the vitamin D content tends to decrease with time and changes in storage conditions.

Recommendations:

Based on the obtained result, application of some changes in the general vitamin D_3 measurement method can result in a more efficient assay with adequate accuracy for vitamin D_3 in fortified food.

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تحديد محتوى فيتامين د٣ فى الأطعمة الغنية ، المنخفضة والخالية من الدهون بإستخدام كروماتوجرافيا السوائل عالية الأداء

أجريت هذه الدراسة لأستحداث طريقة أكثر دقة وسهولة من الطرق السابقة لتقدير فيتامين د٣ بإستخدام كروماتوجرافيا السوائل عالية الأداء فى الأغذية المدعمة بفيتامين د٣.

وقد شملت الدراسة أختيار أغذية عالية الدهن والتى شملت حليب الأطفال المجفف والجبن الدسم المطبوخ، أغذية منخفضة الدهن والتى شملت الزبادى وأغذية الأطفال التى أساسها الحبوب. كما شملت الدراسة أيضاً تقدير فيتامين د فى الأغذية خالية الدهن والمدعمة بفيتامين د٣ كطريقة جديدة وقد تم أختيار لعقة الأطفال الخالية من الدهن والمدعمة بفيتامين د٣ لهذه الدراسة.

فى هذه الدراسة تم تعديل فى بعض مراحل التحديد الكمى لفيتامين D3 فى الأغذية عالية ومنخفضة الدهون كالتالى :

مرحلة \التصبن : تم تقليل درجة الحرارة والوقت ليصبح ٤٠م والمدة ١٠دقائق بدلاً من ٧٠-٨٥م ولمدة ٣٠ دقيقة فى الطرق السابقة.

مرحلة \الأستخلاص : تم استبدال الهاكسين بمذيب أكثر قطبية وهو الأثير البترولى أو الأثير الأيثيلى.

- تم أيضاً تغير نسبة ونوع وكمية المذيب المستخدم فى مرحلة الأستخراج الصلب حيث تم أستخدام الميثانول مع اسيتونيتريل بنسبة ١:١ بدلاً من الميثانول لوحده فقط.
- فى مرحلة الغسيل : تم إضافة الإيثانول إلى الماء المقطر المستخدم فى غسل المذيبات.

ولقد ساهمت هذه التعديلات فى أستخدام هذه الطريقة لتقدير فيتامين د٣ فى الأغذية الخالية من الدهون. كما أدى إلى فصل أفضل من قمم فيتامين D_2 و D_3، كما أن هذه التعديلات أصبح مدة فصل فيتامين د٣ من ٦-٧ دقائق بدلاً من ١٢-١٥ دقيقة.

على ضوء هذه النتائج فإن هذه الطريقة مناسبة للتحليل الروتينى للعينات الغذائية المختلفة لتحديد فيتامين D3.