Determination of Fat-Soluble Vitamins A, D3, and E in Infant Formula and Milk Powder Using High-Performance Liquid Chromatography with Photodiode Array Detection: Jordan Market as a Case Study

Alali, Feras*[+]

Pharmaceutical Sciences College of Pharmacy, QU Health, Qatar University, QATAR

Amayreh, Mohammad[•]

Department of Chemistry, Mut'ah University, Al-Karak 61710, JORDAN

Massadeh, Adnan

Department of Medicinal Chemistry and Pharmacognosy, Jordan University of Science and Technology, Irbid 22110, JORDAN

El-Alali, Abdullah

Department of Chemistry, Mut'ah University, Al-Karak 61710, JORDAN

ABSTRACT: *A new, simple, rapid, and sensitive reversed-phase High-Performance Liquid Chromatography-Photodiode Array (HPLC-PDA) method was developed and validated for the simultaneous analysis of fat-soluble vitamins A, D_3, and E. The method required a simple sample preparation step of saponification with aqueous KOH and extraction with n-hexane. The method was validated in terms of linearity, accuracy, precision, stability, detection limits, and recovery. The method has the advantage of simultaneous determination of vitamins A, D_3, and E in a short run time of 10 min. The method was applied successfully for the determination of vitamins A, D_3, and E in some infant formula and milk powder from the Jordanian market. Based on this study, vitamin Content in all brands was within 90.0-410% of the labeled value. Vitamin D3 content in the studied brands was within 100.0-850%, while vitamin E content in all brands was less than 48%. The results showed large variation and discrepancy of most vitamins' contents, which were not in good agreement with the manufacturer label value, though they were below toxic levels.*

KEYWORDS: *Fat-soluble vitamins; Infant formula; Milk Powder; HPLC-PDA.*

***** *To whom correspondence should be addressed.*

+ E-mail: feras.alali@qu.edu.qa

● *Other Address: Department of Chemistry, Faculty of Science, Al-Balqa Applied University,P.O.Box 19117, Al-Salt, JORDAN*

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INTRODUCTION

Human milk is commonly believed to be complete and perfect food for human infant, with breast feeding is the most important mode of delivery [1,2]. Moreover, breast milk may also protect infants against certain diseases; while it provides complete nutrition when feeding is successfully established [1-4].

Infant formulas are designed as nutritionally complete feeds to replace breast milk. This is may be for reasons of convenience, illness of the mother or inadequate supply of breast milk. These formulas are soy and cow's milk- based formulas to mimic the composition of mature breast milk. However**,** no formula can provide fully the whole benefits of breast milk [1,2,5].

According to the global standard for the composition of infant formula; infant formula must contains energy, proteins, lipids, carbohydrates, vitamins, and minerals [6].

Fortified food such as milk powder and infant formula are usually supplemented with vitamin A in the form of synthetic retinyl acetate or retinyl palmitate[7].The acetate ester of d-α-tocopheryl acetate (*RRR*-α-tocopheryl acetate) form is usually used in the fortification of foods[8,9]. However, vitamin D is added to food in the form of cholecalciferol. Milk continues to be the food of choice for vitamin D fortification, due to the presence of calcium [10].

The Recommended Dietary Allowance (RDA) of fat-soluble vitamins[11,12] are listed in Table 1.

The addition of vitamins to certain foods, fortification, is a common practice in most countries[13,14]. In other cases, vitamins are added to restore the vitamin content to that originally present before processing. Many studies were conducted to calculate the amount of fat-soluble vitamins added to milk powder and infant formula in comparison with the labeled values. Several studies in the USA showed that milk and infant formulas rarely contain the same amount of vitamin D stated on the manufacturer label; documented fortification errors in fortified milk products across the US milk industry were reported [15,16]. In other studies, hypervitaminosis D was shown to be resulted from drinking milk which is incorrectly and excessively fortified with vitamin D [17-19]. Thus, the fortification process must be carefully monitored.

Jordan market hosts varieties of powdered milk and infant formulas of different manufacturers and countries of origin. The Jordan Food and Drug Administration finds practical difficulty in analyzing vitamins in every batch and from all sources. Some brands are also smuggled into the country, which because of their cheaper prices, attract poor and middle class section of the society. Thus, this study was aimed to develop an accurate, sensitive, and simple analytical method utilizing a simple extraction procedure and reverse phase HPLC coupled with diode array detector (PDA) for simultaneous determination of the fat-soluble vitamins A, D_3 and E in infant formula, milk powder, and cereal food. The measured values were compared with the specified amounts on the manufacturer label. We believe this study will increase the public and government awareness of this particular vital issue.

EXPERIMENTAL SECTION

General

Analysis was performed using a Merck–Hitachi HPLC, (Merck–Hitachi, Tokyo, Japan) equipped with L-7150 isocratic pump, solvent degasser L-7612, autosampler L-7200, diode array detector L-7455, interface D-7000 and D-7000 HSM. The analytical chromatographic column was a reversed-phase C_{18}, 250 mm (length) × 4.6 mm (internal diameter) packed with 10 μm particles (Waters, Dublin, Ireland). Samples were dried using RE 200 rotary evaporator (Bibby, Liverpool, UK). Samples were sonicated using Eurosonic 22 Sonicator (Eurosonic Neumann GmbH, Kelten Germany). Digital micro pipettes (1000-100 μL and 100-10 μL) (witegLabortechnik GmbH, Wertheim, Germany). Glass syringes (100 and 10 μL) were obtained from Hamilton (Hamilton, Bonaduz, Switzerland). A filtration unit for mobile phase degassing was obtained from Schott Duran,

Samples were weighed using an AT Delta Range, Mettler Toledo analytical balance (Mettler Toledo, Switzerland).

L-(+)-ascorbic acid and potassium hydroxide pellets were obtained from Lonver House, England and Scharlau, Spain. Water was purified using Seradest S600, water purification systems D-5412, Ransbach, Baumbach. Methanol (HPLC grade), n-hexane (analytical grade), ethanol absolute (analytical grade), all were obtained from Scharlau Chemie S.A, Spain. Standard vitamin A (all-*trans*-retinol) 99.0% purity for HPLC

Table 1: Recommended Dietary Allowance of vitamin A, D3 and E(Units/day).

Age	Vitamin A(µg RE)[11]	Vitamin D (µg)[11]	VitaminE(mg/g) PUFA[12]
0-3 and 4-6 months	350	8.5-10	0.4
7-9 and 10-12 months	350	8.5-10	0.4
1-3 years	350	10	0.4

**RE, retinol equivalents; PUFA, polyunsaturated faty acids.*

analysis was purchased from Fluka Chemie GmbH, USA, vitamin E (dl-α-tocopherol) 98.0% purity for HPLC analysis from Applichem Biochemica Synthesis Services, Germany, and vitamin D_3 (cholecalciferol) 99.0% purity for HPLC analysis from ACROS, Europe.

Samples' collection

Two different batches of eight brands of infant formula, and three brands of full cream milk powder were collected randomly from Jordanian local markets. Brands of infant formula and full cream milk powder were cow's milk-based formula.

Chromatographic conditions

The optimized mobile phase was an isocratic blend of methanol and water (95:5, *v*/*v*) with a 2 mL/min flow rate, monitoring at 280 nm (optimum absorbance for the detection of vitamins A, E, and D_3, simultaneously), and a total analysis time of 10 min.

Standard solutions and samples preparation

Milk powder and infant formula samples were kept in a refrigerator at 4 °C to avoid temperature and light degradation. Portion, 2.50 g (±0.01) of each sample was accurately weighed and placed into 100 mL Erlenmeyer flask, a volume of 25 mL of hot water (50 °C) were added, followed by the addition of 0.8 g of ascorbic acid as antioxidant to avoid vitamins oxidation. The solution was swirled for 5 min to assure good mixing. A volume of 5 mL aqueous KOH (60% w/v) and 15 mL of absolute ethanol were added to the mixture. The Erlenmeyer flask was tightly closed to prevent air oxidation of vitamins during the saponification process, which depends on the saturation degree of ethanol vapor. The Erlenmeyer flask was covered by dark sheet to protect from light. All steps were carried out in dark place. The saponification step converts all the ester forms of vitamin A and vitamin E to parent alcohol (Fig. 1). The mixture was then sonicated for 30 min at 50 °C with frequent shaking, then it was left for 5 min in cold water, afterwards, the content was transferred to 250 mL separatory funnel. A volume of 25 mL of n-hexane were added, followed by vigorous shaking for one min and phase left to separate for 5 min; this step was repeated three times, ratio of sample-extraction solvent (w/v, g/mL)1:10. Organic phases were combined into 100 mL round-bottomed flask. The collected solvent was evaporated in a rotary evaporator at 50 °C under vacuum. The residues were then dissolved in 2 mL methanol, filtrated by 0.45 μm Teflon filter and transferred into 2 mL amber HPLC vials. An aliquot of 50 μL was injected into HPLC within 1 h. Three extraction replicates were prepared for each batch of samples.

Standard stock solutions of the vitamins: retinol (vitamin A) (500 μg/mL), cholecalciferol (vitamin D_3) (100 μg/mL), and α–tocopherol (vitamin E) (500 μg/mL) were prepared by accurately weighing 5, 10 and 50 mg of vitamins A, D_3 and E reference standards into 10, 100 and 100 mL volumetric flasks, respectively. In sequence, a volume of 2, 5 and 5 mL of absolute ethanol were added to each flask to aid solvation. Afterwards, each flask was filled to the mark using methanol. The standard stock solutions were stored in a refrigerator at -20 °C for a period of not more than one week for vitamins A and D_3 and two weeks for vitamin E. All standards were protected from light during the storage to minimize degradation.

Calculations

Vitamins A and D_3 content in milk powder and infant formula were calculated per 100 g milk powder using the following equation: Content (μg/100 g) = C × FV × Wl/Ws. While vitamin E content in milk powder and infant formula were calculated by the following equation: Content (mg/100 g) = C × FV × Wl/Ws ×1/1000; Where, *C:* is the sample's vitamin content (μg/mL) extrapolated

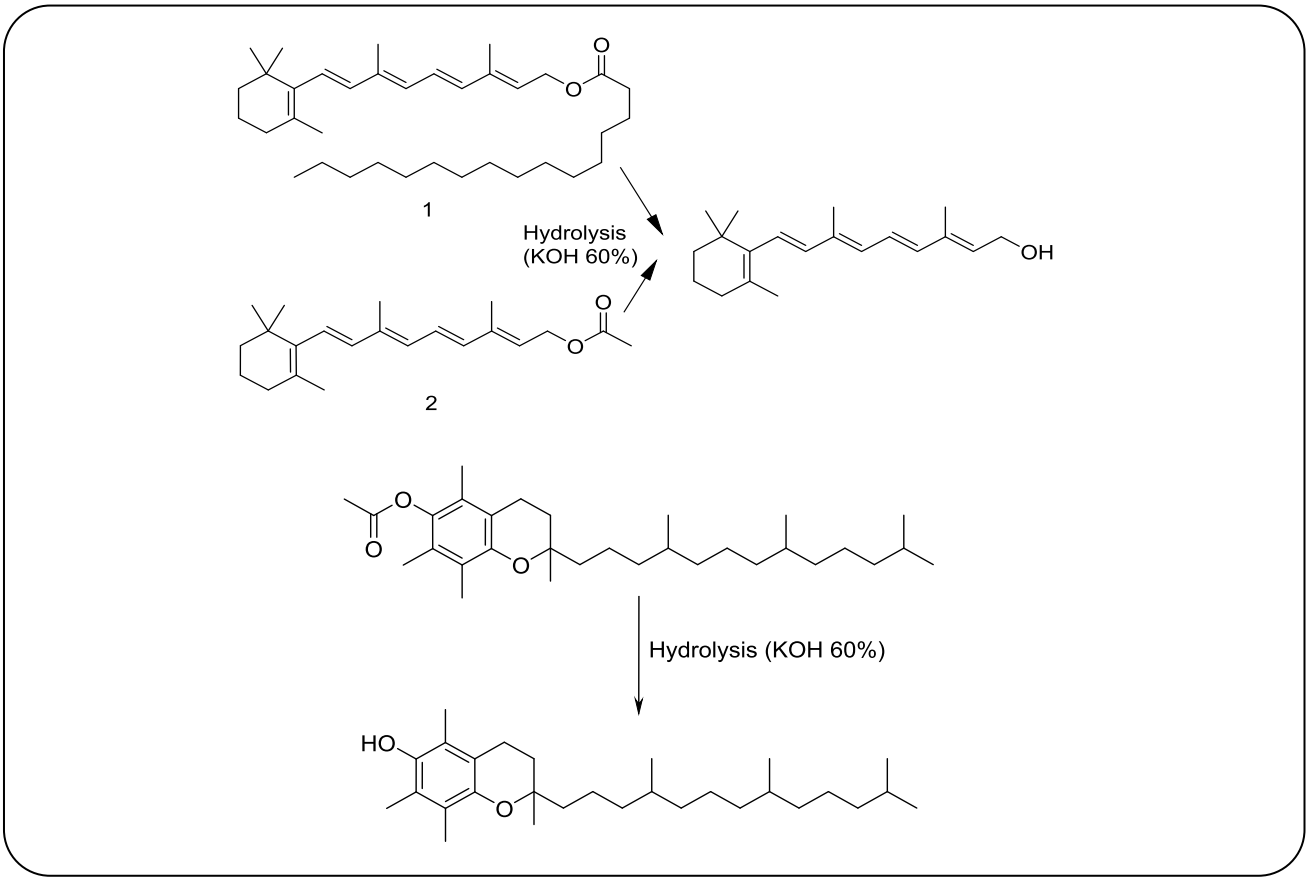

Fig. 1: Hydrolysis of vitamin A palmitate (1, A) and vitamin A acetate (2, A) and vitamin E acetate (B).

from calibration curve's linear regression; *FV:* is the final volume of the sample extracted (mL); *Wl*: is the weight of milk powder (which is equal to 100 g); *Ws:* is the weight of portion sample (g) (which is equal to 2.5 g).

The values obtained from the equations were normalized by the % recovery measured for each vitamin. The recovery for vitamins A, D_3 and E were 95.33, 73.77 and 83.47%, respectively. Vitamin E was calculated as α-tocopherol, so the value in mg was multiplied by 1.2 to account for other vitamins that are present which gives an approximation value for total vitamin E activity as mg of α-tocopherol equivalents [9].

RESULTS AND DISCUSSION

Development and validation of HPLC-PDA method for vitamins A, D3 and E analysis

Method development

The main aim of the chromatographic system developed was to obtain better resolution, faster and simultaneous analysis of fat-soluble vitamins A, D_3 and E using reversed phase liquid chromatography. A reverse phase and isocratic system of methanol-water (95:5 *v/v*) using HPLC-PDA was adapted. Flow rate of 2 mL/min and a run time of 10 min were set as a result of optimization process. In order to improve baseline level, an injection volume of maximum 50 μL was used. The three vitamins were investigated for maximum absorption using PDA. A compromise wavelength of maximum absorption at 280 nm for the three vitamins was selected. Identification of vitamins in the samples depended on the retention times of standard vitamins. Standard vitamins and milk powder samples were first injected into HPLC to check the resolution and retention time variability (Fig. 2 and Fig. 3).

Method validation

The method was validated in terms of linearity, precision, recovery, detection limits, and stability according to the Food and Drug Administration (FDA) guidelines for analytical method validations [20].

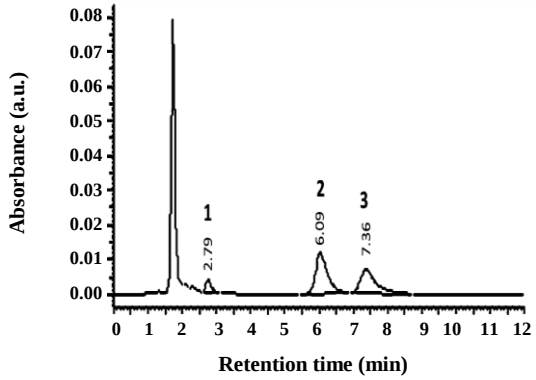

Fig. 2: A chromatogram for the standard solutions of fat-soluble vitamins: vitamin A (1) (2.79 min):5.0µg/mL, vitamin D3 (2) (6.09 min):4.0µg/mL and vitamin E (3) (7.36 min): 3.0µg/mL; retention time varied within 0.5 min.

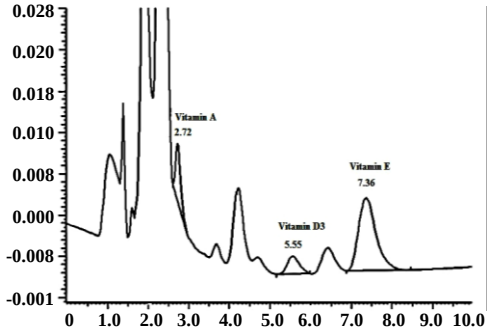

Fig. 3: A chromatogram of fat-soluble vitamins in milk powder: vitamin A (2.72 min), Vitamin D3 (5.55 min), and vitamin E (7.36 min).

Linearity

Reference standard solutions for each vitamin were prepared. Triplicate injections of each preparation from the reference standard were made. Measuring peak height, a linear calibration curve was constructed for each with regression coefficient (r^2) values of 0.9983, 0.9998 and 0.9984 in the ranges of 0.5-50 μg/mL, 0.05-10 μg/mL and 10-200 μg/mL for vitamins A, D_3 and E, respectively , as shown in Table 2.

In order to check the accuracy, three quality control points at 4, 20, and 40 µg/mL for vitamin A, 2, 4 and 8 µg/mL for vitamin D_3, and 40, 70 and 120 µg/mL for vitamin E were injected each month for a period of five months. The accuracy for each vitamin was found to be within 15% during this period.

Precision

The inter- and intra-day precision was tested by successive injections with 5 replicate determinations for two different concentrations of each vitamin. The repeatability is expressed as relative standard deviation (*RSD*) as shown in Table 3.

Recovery

Recovery was tested using a blank of an ultra-pure water (deionized water) which was spiked with a known concentration at 30.0, 20.0 and 15.0 μg/mL for vitamin A, 10.0, 5.0 and 2.0 μg/mL for vitamin D_3, and 40.0, 30.0 and 20.0 μg/mL for vitamin E, respectively. These known concentrations were subjected to the following procedure: saponification, extraction with n-hexane, evaporation and analysis by HPLC. Water as a blank was used since milk powder matrix without fat-soluble vitamins were not available. The average recoveries for vitamins A, D_3 and E were 95.33±1.25, 73.77±1.91 for and 83.47±4.86, respectively.

Detection limits

The detection limits (DL) for vitamins A, D_3 and E were found to be 0.1, 0.01, and 0.1 μg/mL, respectively. The Quantitation Limits (QL) were determined by choosing the lowest quantifiable concentration on the calibration curve. Thus, the QL for vitamin A, D_3 and E were found to be 0.3, 0.03, and 0.3 μg/mL, respectively.

Stability

Stability data of standard vitamins A, D_3 and E showed that the standard solution of vitamin A has a noticeable degradation by 10% after 6 h, 15.50% after 16 h and 42.60% after 24 h at room temperature. Vitamin D_3 showed a noticeable degradation of 12.80% after 6 h, 16.8% after 16 h and 22.09% after 24 h at room temperature. Vitamin E showed no significant degradation after 6 h, 8.28% after 16 h and 29.70% after 24 h (Table 4).

The slight increase in *RSD* values observed for vitamins A, D_3 and E can largely be attributed to the delicacy of vitamins analysis. *RSD* values were 2.4-19.70, 6.14- 39.76 and 0.34-41.36 for vitamins A, D_3 and E, respectively. Relative standard deviations data were found to be comparable to the *RSD* values reported

Table 2: Linearity parameters for vitamin (A, D_3 and E).

Vitamin	Concentration range (μg/mL)	b*	R^2 §
A	0.5-50	1623	0.9983
D_3	0.05-10	1352.2	0.9998
E	10–200	149.26	0.9984

**slope, §regression value.*

Table 3: Precision of the HPLC method for determination of vitamins A, D3 and E in infant milk and milk powder.

Vitamin	Con. (µg/mL)	RSDs %
A	30	0.86-4.21
	20	0.64-6.84
D_3	8	0.56-3.97
	4	0.79-3.19
E	160	0.17-3.16

Table 4: Stability data of standard solutions of vitamins A, D3 and E kept at room temperature.

Time intervals (hour)	Vitamin A (30 μg/mL)*	Vitamin D_3 (2 μg/mL)*	Vitamin E (40 μg/mL)*
0	30.45	1.72	41.66
3	27.57	1.66	41.24
6	27.40	1.50	40.96
16	25.73	1.44	38.21
24	17.49	1.34	29.29

**Initial concentration*

by other workers in the world. *RSD* values of 3.88-38.46 and 3.45-52.83 for vitamins D_3 and E, respectively, were reported [21]; *RSD* values of 2.88-39.13 and 3.33-37.78 for vitamins D_3 and E, respectively[22]; *RSD* values of 3.27-7.16 and 5.50-10.20 by applying the AOAC method, 6.69-9.23 and 2.81-3.65 by applying the proposed method for vitamins A and E, respectively [23].

Samples analysis

The collected samples of infant formula, milk powder, and cereal food from the local market in Jordan were analyzed using the developed and validated method. Each run was carried out in triplicate and the results obtained are given in Tables 5 and 6.

In our study, only 1 of 8 brands (12.5%) of infant formula, and 1 of the 3 brands of milk powder (33.33%) contained 80-120% of the labeled amount of vitamin A. Two of the 8 brands (25%) of infant formula and one of the three brands (33.33%) of milk powder contained less than 80% of the amount stated on the label. Two of the 8 brands (25%) of infant formula and none of the 3 brands of milk powder contained more than 300% of the amount stated on the label (Tables 5 and 6).

For vitamin D_3, none of the 8 brands of infant formula, and 2 of the 3 brands (66.67%) of milk powder contained 80-120 percent of the labeled value. None of the 8 brands of infant formula, and none of the 3 brands of milk powder contained less than 80% of the value stated on the label. Six of the 8 brands (75%) of infant formula, food and none of the 3 brands of milk powder contained more than 300% of the value stated on the label (Tables 5 and 6).

For vitamin E, none of the 8 brands of infant formula, and none of the 3 brands of milk powder contained 80-120% of the labeled value. All the brands infant formula and milk powder contained less than 60% of the labeled value (Tables 2 and 3).

Table 5: A comparison between vitamin A, D3 and E measured content (mean ± SD) with label values for the samples tested.

Sample Name	Vitamin A		Vitamin D_3		Vitamin E	
	Measured value (μg/100g)[¥]	Label value (μg/100g)	Measured value (μg/100g)[¥]	Label value (μg/100g)	Measured value (mg/100g)[¥]	Label value (mg/100g)
Infant formula						
A	2500±100	480	40±4	8.2	1.72±0.16	4.6
B	430±100	473.10	67±31	7.88	1.68±0.11	10.74
C	2400±100	520	47±11	7.5	1.07±0.18	4.03
D	370±100	600	28±3	10	0.63±0.33	4.03
E	300±100	470	25±7	12	0.69±0.64	4.56
F	✠	540	38±7	7.75	4.12±0.09	13.42
G	✠	570	45±5	8.75	0.55±0.18	3.36
H	250±100	510	38±5	9.75	0.33±0.11	4.56
Full Cream Milk Powder						
A	700±100	630	13±6	12.5	—[§]	—[▫]
B	1800±100	630	14±1	12.5	< 0.96	—[▫]
C	320±100	540	16±15	5.75	< 0.96	3.36

[¥]Measured values are the average values derived from triple experiments, each run in triplicate for each batch of samples.
✠Can't be observed due to fatty acid overlapping; [§]Not added; [▫]No labeled value.

Table 6: The percentage of vitamins in infant formula, cereal food, and milk powder in comparison with the labeling values.

Sample Name	Vitamin A	Vitamin D_3	Vitamin E
Infant Formula			
A	520.0	485.0	37.0
B	90.0	848.0	16.0
C	470.0	630.0	27.0
D	160.0	280.0	16.0
E	64.0	200.0	15.0
F	—*	490.0	31.0
G	—*	510.0	16.0
H	49.0	390.0	7.2
Full Cream Milk Powder			
A	110.0	100.0	—[§]
B	290.0	110.0	—[§]
C	60.0	280.0	17.0

There were a significant variation in the vitamins content of the studied brands, infant formula and milk powder. For vitamin A, the highest and lowest measured values compared to the manufacturer labeled values fall in the range 34.52-544.20% (Tables 5 and 6). This is still far away from the toxic level which is 25 times of the Recommended Daily Allowance (RDA). For vitamin D_3, the vitamin content was significantly higher than the label amount in all brands of infant formula. The range of vitamin D_3 was 103.60-1177.60% of the label values. Vitamin D_3 content measured in our study is still far below the toxic level which amount to 50 times the RDA. A factor to worry about regarding higher values of vitamin D_3 is the amount of infant formula consumed by the infants.

Vitamin E content in all brands was significantly below the labeling value with a range of 7.24-58.48% (Tables 2 and 3).

The significant differences in vitamin content observed for the infant formula with respect to the stated values might be due to an error in the fortification process or incorrect labeled values. Companies report usually the added amount of each vitamin and not the total content (added and natural). Also, differences in fortification procedures may have a large impact upon vitamin content [14, 15]. It has been reported that adherence to label claim decrease with decreasing fat content. In partial, this may be due to the method and stage of vitamin addition prior to processing [24]. For vitamin A, this difference might be also due to variable natural content of vitamin A in the samples [22]. Regarding vitamin E, iron and copper ions which are added as a supplement to milk powder may act as catalytic centers to oxidize vitamin E [9,25]. Also, the long time required to saponify retinyl palmitate may destroy some of the α-tocopherol [26]. Removal of fat from cow's milk decreases vitamin E content of the resulting dairy products [27].

CONCLUSIONS

A simple and rapid HPLC method coupled with PDA detection was developed and validated for simultaneous analysis of vitamins A, D_3 and E. The method was applied successfully for analysis of vitamins A, D_3 and E in samples of infant formula and milk powder that were obtained from local Jordan markets. The results showed high variablity in vitamins' content among different samples and among different batches of the same samples. Morover, there was disagreement between the vitamins' content found and those typed in the manfugacturerer label, though they were still beyond the toxic levels. Errors in the fortification process, incorrect label value and fatty acid content may contribute to some significant differences in vitamins' content in infant formula and milk powder. In light of the findings of the current study, it is highly recommended that heath authorities must analyze fortified infant products for their content of vitamins A, D_3, and E.

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