

Determination of Fat Soluble Vitamins A and E in Infant Formulas by HPLC-DAD

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<http://dx.doi.org/10.12944/CRNFSJ.4.Special-Issue-October.12>

(Received: August, 2016; Accepted: September, 2016)

ABSTRACT

A rapid, sensitive and inexpensive method has been developed for the simultaneous determination of fat-soluble vitamins A and E in infant formula (IF) samples. The isolation of fat-soluble vitamins includes a saponification step and an extraction step with petroleum ether. The qualitative and quantitative determination of vitamins was performed by High Performance Liquid Chromatography (HPLC) coupled to Diode Array Detector (DAD). More specifically, method development involved optimization of saponification time, testing of antioxidants, and number of extractions along with the extraction time applied. The method proved to be reliable as results agreed with the sample's labels and the permitted variation according to the EE law. The relative standard deviation decreased significantly after the optimization (%RSD 1.8 % for retinol and 3.1% for α -tocopherol).

Keywords: vitamin A, vitamin E, infant formulas, HPLC, saponification, extraction.

INTRODUCTION

Vitamins are organic compounds which are essential for human life, and are differentiated into fat and water soluble compounds. They are needed in relatively small amounts to sustain life and good health¹⁻⁴. The nutritional necessity of vitamins increases during pregnancy, lactation, and growth periods under the conditions of intensive work. Vitamin A and E are two of the fat soluble vitamins, which must be supplied in human nutrition, because they cannot be composed in the body and have many important biological functions. However, at times, dietary habits can create a deficiency of vitamins and provoke several chronic illnesses^{3,5}.

Vitamin A is essential for normal vision, growth, cellular differentiation, development and proper functioning of skin and mucous membranes.

It is defined as sum of trans-retinol or other retinoids as carotenoids^{1,6,7}. Vitamin E functions as a powerful biological antioxidant that protects the poly-unsaturated fatty acids of cell membranes from free radical damage. It also prevents oxidative damage to neonates exposed to higher oxygen levels than those in the intrauterine environment^{8,9}. It is defined as a collective group of related lipids called tocopherols (α - β -, γ - and δ -) and tocotrienols. Each form has its own biological activity but α -tocopherol possess the major antioxidant activity^{1,6,10-12}.

Infant formulas (IF), main source of feeding for infants, have to be able to provide sufficient contents of mentioned vitamins in infant diet as well as to prevent lipid oxidation. The most frequent forms of vitamin A and E used in infant formulas are retinol acetate or retinol palmitate and α -tocopherol acetate, respectively due to the fact

that they are more stable and less susceptible to degradation^{12,13}. The aim was to develop a simple, rapid, fit for purpose determination of these vitamins for IF routine analysis. Hence, a developed, optimized and validated HPLC-DAD method for simultaneous determination of retinol acetate as well as a-tocopherol acetate is presented.

MATERIALS AND METHODS

Chemicals used for sample preparations were of analytical reagent grade. Standards of retinol, retinol acetate, dl-a-tocopherol, a-tocopherol acetate, L-ascorbic acid, butylated hydroxyl-toluene (BHT) and sodium hydroxide were purchased from Sigma (St. Louis, MO, USA). Methanol, ethanol and petroleum benzene were obtained from Fisher (Waltham, MA, USA).

Experiments were carried out using an Agilent HPLC system (1200 infinity series) coupled to diode array detector (DAD) using dual wavelength for this study, 323 nm for Vitamin A and 292 nm for Vitamin E. The compounds were separated with a 5 μ m Agilent Zorbax Eclipse XDB 250 \times 4.6 mm C18 column fitted with a C18 guard cartridge which maintained at 35°C. Mobile phase was methanol HPLC grade and the flow rate was 1.00 mL/min (isocratic elution). Total run time required was equal to 20 min.

Commercial IF and specifically YIOTIS (logo is JOTIS) S.A baby foods Sanilac and Farine Lactee were tested. The powdered IF was dissolved in warm (60°C) deionized water (10% w/v) and was stirred until complete homogenization of the diluted sample.

The saponification step was carried out as follows: in a 15 mL plastic centrifuge tube were added: 2mL of homogenized sample (10% w/v), 2.5mL of ethanolic solution of 0.2% w/v ascorbic acid and 1mL of aqueous solution of 50% w/v KOH. To prevent degradation of vitamins A and E, the tube was purged with nitrogen for 20 seconds after any use. The tube was placed in a water bath at 80°C for 10 min and afterwards at an ice bath in order to cool down. The sample was centrifuged at 4000 rpm for 4 min and the upper phase was transferred into a transparent glass vial.

Extraction step was accomplished as follows: 800 μ l of petroleum ether containing 1% w/v BHT was added in the transparent vial and was stirred with magnetic stirrer for 20 min. At the end of the extraction time the sample was left for 2-3 min so the two phases could be separated. The upper organic phase was removed and transferred to a plastic Eppendorf tube (first extraction). Same procedure was repeated and the second organic phase was transferred into the same Eppendorf tube (second extraction). The sample was then evaporated with nitrogen to dryness and was then reconstituted with 150 μ l of methanol. 20 μ l of this reconstituted sample solution was injected directly into the HPLC system for analysis.

RESULTS

The isolation of fat-soluble vitamins was divided into three main parts: sample preparation, saponification and extraction. The method was based to previous work³ and was optimized in all three main parts.

Optimization of sample preparation step

We tested to weigh 1, 2, 5 and 25 g of IF in order to prepare 10%w/v homogenized sample. The optimum amount of the sample was 5g IF as 25g IF proved to be difficult to be homogenized and 1g is considered to be a minimum amount of mass. Based on different official methods 5g IF were chosen to be weighed as this amount is a representative quantity for sample testing. We have to underline that there is a significant inhomogeneity of vitamins into the IF powder¹⁴. Furthermore, higher repeatability was achieved when 5g were processed, with % Relative Standard Deviation for triplicate analysis (%RSD, n =3) to be the lower ones for both vitamins A and E (13% and 29% respectively) compared to the rest masses tested. For these reasons the amount of 5g was selected as the optimum.

Optimization of saponification step

Time of saponification and presence of ascorbic acid were investigated. It was found that 5 minutes of saponification was not always the required time for total saponification as part of vitamins and specifically of retinol acetate remained unsaponified. For this reason longer saponification times such as 10, 20, 30, 40, and 50 min were tested. An optimum

of 10 minutes of saponification was applied as a compromise between complete saponification and avoidance of vitamins degradation as it is shown at Table 2. As far the ascorbic acid role, it proved to be crucial because of preventing oxidation and degradation of vitamins during this step.

Optimization of extraction step

Time of extraction combined with the number of extractions was optimized. Although in the aforementioned work³ only one extraction of 20 min extraction was proposed, we observed that the extraction of vitamins for this time was insufficient as indicated in Table 3. We tried two extractions of 5, 10, 15 and 20 min and three extractions of 15 and 20 min each. Among the two extractions those of 20 min were preferred as they provided better results for both vitamins and specifically for a-tocopherol. As about the three extractions, we preferred those of 15 min because for extractions of 20 min degradation of vitamins A and E was observed. Furthermore, two extractions of 20 min were chosen due to the fact that the results show better reproducibility and lower % RSD (14% for Vitamin A and 32% for Vitamin E).

Table 1: Comparative results of optimization of mass weighed of IF

Sample mass g	Vitamin A		Vitamin E	
	$\mu\text{g}/100\text{g}$	%RSD	$\text{mg}/100\text{g}$	%RSD
2	268	30	0.81	54
5	420	13	3.57	29
25	540	21	3.87	46

After optimising the method a significance difference in the amounts of vitamins was observed and at the same time %RSD was reduced significantly (1.8 % for retinol and 3.1 for a-tocopherol). As can be seen in Figure 1, the final results obtained are to some extent identical.

The method was validated by spiking retinol acetate and a-tocopherol acetate in three concentration levels in blank samples (IFs without vitamins added) and at the same time was checked the linearity of spiked samples. Table 5 shows the results of % recoveries obtained.

DISCUSSION

The experimental results showed the applicability of the method for the determination of fat-soluble vitamins A and E in powdered IF. Method proved to be ideal for routine analysis and for measuring a large number of IF samples

Table 2: Comparative results of optimization of saponification time

Time of saponification min	Vitamin A		Vitamin E	
	$\mu\text{g}/100\text{g}$	% RSD	$\text{mg}/100\text{g}$	% RSD
5	524	24	4.97	17
10	591	15	6.35	8.2
20	531	4.5	5.85	3.5
30	710	15	4.56	20
40	690	9.3	4.28	9.1
50	706	6.6	4.35	1.5

Table 3: Comparative results of optimization of number and time of extractions

Number and time of extractions	Vitamin A		Vitamin E	
	$\mu\text{g}/100\text{g}$	%RSD	$\text{mg}/100\text{g}$	%RSD
1 extraction x 20 min	200	26	1.00	42
2 extractions x 5 min	290	26	1.26	25
2 extractions x 10 min	342	5.4	2.51	6.4
2 extractions x 15 min	334	15	1.80	100
2 extractions x 20 min	483	14	7.69	32
3 extractions x 15 min	629	23	5.12	108
3 extractions x 20 min	523	39	4.60	93

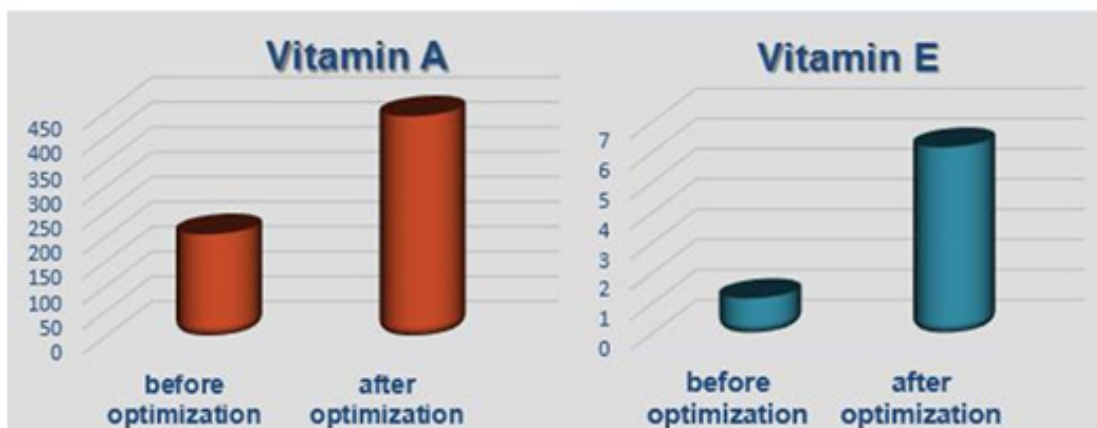


Fig. 1: Vitamin A and E before and after the optimization of the method

Table 4: Comparative results of IF before and after the optimization of the method

Before the optimization				
	Vitamin A		Vitamin E	
	$\mu\text{g}/100\text{g}$	%RSD	$\text{mg}/100\text{g}$	%RSD
	208	23.6	1.21	38
After the optimization				
	Vitamin A		Vitamin E	
	$\mu\text{g}/100\text{g}$	%RSD	$\text{mg}/100\text{g}$	%RSD
	544	1.8	6.21	3.1

Table 5: Recoveries (%) of Vitamin A and E from Spiked IF

	Added Concentration	Measured Concentration	Recovery (%)
Vitamin A	250	315	126
($\mu\text{g}/100\text{g}$)	500	583	117
	750	799	107
Vitamin E	2.2	2.1	98
($\text{mg}/100\text{g}$)	4.4	3.8	86
	6.6	5.3	80

per day. After the optimization of the method we concluded that the mass of powder (IF) weighed for the preparation of the homogenized sample plays an important role because of the inhomogeneity of the vitamins into the IF powder. In addition, the number of extractions combined with the stirring time improved the quantities of vitamins extracted and the selection of appropriate glass bottles stabilized the extraction conditions. Finally, it is important to refer that the use of two different antioxidants (L-ascorbic acid and BHT) combined with the use of aluminum foil contributes effectively to the protection of vitamins throughout the duration of the experiment.

Future experiments will employ the use of enzyme α -amylase and the method will be applied in other type of foods such as instant rice baby food, cereals, chocolate, cacao etc. Accuracy and precision of method developed will be tested in 1849a Infant Formula Standard Reference Material.

ACKNOWLEDGEMENTS

The authors thank JOTIS S.A, Greece for providing the financial and technical support of this research.

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