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Liquid chromatographic determination of geometrical retinol isomers and carotene in enteral feeding formulas

Rosa Maria Duarte Fávaro [a,*], Maria Helena Iha [a], Maria de Lourdes Pires Bianchi [b]

[a] *Seção de Bromatologia e Química, Laboratório de Ribeirão Preto, Instituto Adolfo Lutz, Rua Minas 877, Ribeirão Preto 14085-410, S.P., Brazil*
[b] *Departamento de Análises Clínicas, Toxicológicas e Bromatológicas Faculdade de Ciências Farmacêuticas de Ribeirão Preto—USP, Ribeirão Preto 14040-903, S.P., Brazil*

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Abstract

The objective of the present study was to evaluate a practical method for separation and determination of geometrical retinol isomers and carotene in enteral feeding formulas and to analyze 17 samples of commercial formulas. By using a normal-phase HPLC column and a mobile phase consisting of 1-octanol in *n*-hexane, seven isomers of retinol were separated and identified from the standard solution after photolysis. For evaluation of Vitamin A activity in these formulas, simultaneous determination of total carotene was performed. The data about linearity, recovery, accuracy and precision showed the reliability of analytical procedures. In the unsaponifiable portion of samples of commercial formulas, six retinol isomers were identified: (*E*)-retinol; (*Z*)-13; (*Z*)-9; (*Z*,*Z*)-9,13; (*Z*,*Z*)-11,13 and (*Z*)-7-retinol. (*Z*)-13/(*E*)-retinol ratios ranged between 3 and 37%. The range of total *Z*/(*E*)-retinol isomers fell between 5 and 42%. Despite the high concentration of *Z*-isomers observed in various commercial enteral feeding formulas, none of the samples presented Vitamin A activity below 90% of that specified on the label.

Keywords: Enteral feeding formulas; Retinol; Vitamin A; Carotenes

1. Introduction

Formulas for enteral nutrition meet all nutritional requirements specifically suited for nutritional support of subjects who cannot ingest or digest sufficient amounts of required foods. Many patients are totally dependent on the infusion of these preparations in order to receive their daily requirements of nutrients. At present, there are a large variety of commercial products exhibiting formulations specific for different diseases; their composition is based on protein (in the form of amino acids, peptides, or intact protein), carbohydrate (glucose, polymers or maltodextrins, disaccharides and oligosaccharides), fat (mono, di and triglycerides), minerals and vitamins.

Vitamin A is added to these formulas at different concentrations. In several clinical conditions, high vitamin concentrations contribute to medical treatment; in other cases, such as in chronic renal insufficiency, only the fulfillment of basal necessities is recommended. Lack, as well as excess of Vitamin A can damage the organism; therefore, a careful control of its administration is necessary.

HPLC is the method most frequently employed for the analysis of Vitamin A; estimation of total retinol or the separation of (*E*)-retinol and (*Z*)-13-retinol are the procedures most frequently utilized in food and drug analysis. However, the determination of total retinol only, can lead to underestimation of Vitamin A when *Z*-isomers are also present [1]. (*E*)-retinol possesses 100% Vitamin A activity. The activities of the *Z*-isomers range from 14% for (*Z*,*Z*)-11,13-retinol to 75% for (*Z*)-13-retinol [2].

The separation of geometric isomers of retinol in standard solutions in studies sometimes using the reverse-phase mode [3], but in general utilizing the normal-phase mode [4–7], has been demonstrated.

Utilizing normal-phase HPLC columns and *n*-hexane–isopropanol (99.6/0.4] as the mobile phase, Stancher and Zonta [8] separated isomers of retinol and 3-dehydroretinol (Vitamin A_2) in extracts of the unsaponifiable matter of fish samples, encountering (*E*)-; (*Z*)-13, (*Z*)-9 and

* Corresponding author. Fax: +55-16-6357994.
E-mail address: rmdfavaro@ial.sp.gov.br (R.M. Duarte Fávaro).

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(*Z*,*Z*)-9,13-retinol in all species studied. (*Z*)-11-retinol was observed only in the eye and liver of the sardine. Brinkmann et al. [5], using a narrow-bore column and 0.3% of 1-octanol in *n*-hexane, evaluated retinol isomers in the liver, liver-containing products, pasteurized UHT and fermented milk, found, in decreasing order (*E*)-retinol; (*Z*)-13-retinol, (*Z*)-9 and (*Z*,*Z*)-9,13-retinol. Traces of (*Z*)-7 and (*Z*,*Z*)-11,13 were observed in only a few foods. Considerable amounts of (*Z*)-11-retinol appeared in foods that had passed through a process of microbiological fermentation.

Studies of the different geometrical retinol isomers contents of enteral feeding formulas have not been found in the literature. Commercial enteral formulas go through sterilizing processes and prolonged storage, which could propitiate *E*-*Z* isomerization processes. Only Frias and Vidal-Valverde [9] have recently evaluated thiamine, tocopherols (*E*) and (*Z*)-13-retinol in five samples of enteral formulas; these authors made no reference to additional retinol isomers.

Enteral formulas are enriched by minerals and vitamins, Vitamin A being added in the form of (*E*)-retinyl palmitate or (*E*)-retinyl acetate. Some formulas are also enriched with carotenes, predominantly β-carotene, the carotene with the highest Vitamin A activity. In our evaluation of Vitamin A activity in formulas, evaluation of total carotene levels (as β-carotene) was also performed.

Our objective in this study was to propose and evaluate a practical method for the separation and determination of the geometric isomers of retinol and carotene in commercial enteral feeding formulas, studying them in regard to these substances.

2. Experimental

2.1. Instruments and chromatographic conditions

The HPLC system consisted of a solvent pump (model LC-10AD*VP*), a diode array detector (SPD-M 10A*VP*), a column oven (CTO-10A*VP*), a System controller (SCL-10A*VP*), a degasser (DGU-14A), a software (CLASS *VP* version 5.02) all from Shimadzu Instruments, Japan, and a Rheodyne L.P. injector with a 20 μl loop from Rheodyne, USA.

A guard column (Shin-pack-G-SIL-4 mm × 4 mm) was connected to the analytical column (Shin-pack CLC-SIL-M-250 mm × 4.6 mm), both from Shimadzu Instruments, Japan. The mobile phase consisted of *n*-hexane-1-octanol (96:4 (v/v)) and the flow rate was 0.6 ml/min. Separations were carried out at 35 °C in a column oven.

2.2. Standards and chemicals

The standards used were all-*trans*-retinol, 13-*cis*-retinol, all-*trans*-retinyl palmitate, all-*trans*-retinyl acetate and β-carotene from Sigma, St. Louis, MO, USA. Methylene chloride, *n*-hexane, 2-propanol (Em Science, Gillstown, USA) were of HPLC grade. Ethanol, 1-octanol, potassium hydroxide pellets, ascorbic acid and sodium chloride (Merck, Darmstadt, Germany) were of analytical-reagent grade.

2.3. Photolysis

Pure standards used to determine concentrations in the samples, were (*E*)-retinol, (*Z*)-13-retinol and β-carotene. Pure standards of the other retinol isomers are not available. For their obtainment, 4 ml of (*E*)-retinol in ethanol (170.0 μg/ml) and 2 ml of (*Z*)-13-retinol in ethanol (190.0 μg/ml), were placed in a cuvette, immediately sealed following removal of oxygen with a nitrogen flux. The solution was exposed to direct sunlight for 30 min; untoward heating was avoided by placing the cuvette in an icebath between successive exposures to sunlight. By this procedure, it was possible to obtain a solution containing (*E*)-retinol, (*Z*)-7; (*Z*)-9; (*Z*,*Z*)-9,13; (*Z*)-13; (*Z*,*Z*)-11,13 and (*Z*)-11-retinol. Literature reports showed that authors performed photolysis using only (*E*)-retinol; however (*Z*,*Z*)-9,13-retinol was obtained in considerably high quantity by the addition of (*Z*)-13-retinol, prior to exposure to light. Peak identification was achieved by comparison of the absorption spectra and the maximum absorption wavelength (λ_{max}) obtained, with those reported in the literature.

2.4. Standard solutions

Stock solutions of (*E*)-retinol, (*Z*)-13-retinol and β-carotene were prepared and their actual concentrations confirmed using known extinction coefficients of each vitamin [10]. Working solutions were prepared by pooling suitable volumes of each stock solution followed by dilution with *n*-hexane to obtain concentrations ranging from 0.68–339.9 μg/100 ml for (*E*)-retinol, 0.71–213.0 μg/100 ml for (*Z*)-13-retinol and 0.85–428.4 μg/100 ml for β-carotene.

2.5. Samples of enteral feeding formulas

Seventeen different commercial enteral feeding formulas of five brands imported by Brazilian firms, were studied; 11 were in liquid form and 6 in powder form, for reconstitution. Samples were supplied by laboratory representatives or purchased in specialized pharmacies. Macronutrients contained in the samples studied were, according to label: proteins from 3 to 10 g/100 ml; lipids from 0.7 to 10.0 g/100 ml and carbohydrates from 9.1 to 21 g/100 ml. All contained vitamins and minerals.

2.6. Alkaline saponification and extraction

These steps were performed as previously described [11], with the following modifications. Liquid samples were homogenized and powders reconstituted according to labeling. One milliliter of a solution of NaCl 1% (w/v), 0.05 g

of ascorbic acid, 9 ml of ethanol, 2 ml of a KOH solution (10 g KOH in 10 ml water) and 3 ml of the enteral formula were placed in a tapered tube. When necessary, in order to obtain a better dispersion of the homogenate, 4–6 ml of NaCl 1% were added. Alkaline digestion was performed in a water-bath at 65 °C for 45 min with stirring. Tubes were cooled in an ice-bath and 15 ml of NaCl 1%, added. Vitamin A and carotenes were extracted with three 6 ml portions each, of hexane. The organic phase was evaporated under nitrogen and the residues dissolved in 2 or 3 ml of hexane and a sample injected into the chromatograph. All procedures were performed in a room whose glass windows were protected with black insul-film to avoid penetration of direct sunlight. Amber glass flasks or flasks covered with black paper were utilized.

2.7. Quantitative analysis

Concentrations of (*E*)-retinol, (*Z*)-13-retinol and β-carotene were determined using an external standard method. The concentration of other isomers was calculated from the knowledge of (*E*)-retinol as proposed by Stancher and Zonta [12] and Brinkmann et al. [5]. All isomer peaks were detected and integrated at 325 nm, a wavelength for maximal absorbency of (*E*)-retinol. Two correction constants were applied in the calculations: k_1 representing the ratio of the specific extinction of the *Z*-isomers against (*E*)-retinol and k_2 representing the ratio of the peak area measured at the maximum absorption wavelength, against that at 325 nm. The constant k_1 was calculated making use of literature data on the specific extinction [5,13] and k_2 was calculated utilizing data obtained in the laboratory (average of the values obtained by the injection of various standards following photolysis).

2.8. Linearity, recovery, precision, accuracy and determination limits

The linearity study was carried out over the range of 0.68–339.9 μg/100 ml for (*E*)-retinol, 0.71–213.0 μg/100 ml for (*Z*)-13-retinol and 0.85–428.4 μg/100 ml for β-carotene. Recovery by the method employed, was measured by adding three different concentrations (Table 3) of (*E*)-retinol, (*Z*)-13-retinol and β-carotene to a vitamin-free enteral formula, freshly prepared in our laboratory with special ingredients for enteral formulas (protein, oligosaccharides, triglycerides, mixture of minerals). The analytical procedures were the same as those previously described.

Intra-assay precision (within-day precision) was evaluated in the above described spiked samples of the vitamin-free enteral formula. In these assays, precision was evaluated only for (*E*)-retinol, (*Z*)-13-retinol and β-carotene, since other isomers were not present. In another part of the study, intra-assay precision was evaluated using a vitamin-containing commercial formula; in this case, it was possible to evaluate the intra-assay reproducibility of (*Z*)-9 and (*Z,Z*)-9,13-retinol, as well. Inter-assay precision (between-day precision) of (*E*), (*Z*)-13, (*Z*)-9; (*Z,Z*)-9,13-retinol and β-carotene was determined in a vitamin-containing commercial formula, for 4 consecutive days. All assays were determined by analyzing six replicates. The precision of the method was calculated as the relative standard deviation (R.S.D.), and analysis of variance (ANOVA). The accuracy was calculated as the percent deviation of observed concentrations from the theoretical concentration and by the *t*-test, comparing given concentrations with the real value. All statistical tests were considered significant at the 5% level. The determination limits were evaluated at the lowest level of contamination performed for the determination of recovery, precision and accuracy.

2.9. Evaluation of "spontaneous" isomerization

In order to evaluate possible isomerization occurring during saponification, extraction and chromatographic procedures, the following additional assays were performed: (a) A sample of a freshly prepared vitamin-free enteral formula was contaminated with (*E*)-retinyl palmitate (101.1 μg retinol/100 ml formula); since the standard contained 5.4% of the (*Z*)-13- retinol, this amount must be maintained after all analytical procedures; (b) Two different samples of commercial enteral formulas were mixed with respectively, a standard of (*E*)-retinyl palmitate (sample 1), and of (*E*)-retinyl acetate (sample 2) and their content of *Z*-isomers verified prior to, and following addition of the standard; (c) To evaluate as well the influence of a possible isomerization of (*E*) and (*Z*)-13-retinol (the isomers which always appear at higher concentrations), on the content of the other isomers, a second commercial enteral formula containing vitamins was contaminated with (*E*)-retinyl palmitate and with (*Z*)-13-retinol, and its content of isomers determined prior to and following the addition of the standards. The difference between the results was evaluated utilizing Student's *t*-test. The percentage of the (*Z*)-13-isomer contained in the (*E*)-retinyl palmitate and acetate standards was evaluated as previously described [14,15].

3. Results and discussion

The literature presents information about the determination and distribution of the various geometric isomers of retinol in some foods [5,8,12,15]. However, we could not find such information for enteral feeding formulas, which are considered a special class of products, formulated and processed with nutrients in different forms and proportions. The method proposed by the AOAC [16] for the analysis of Vitamin A in milk and milk-based infant formulas, only separates and quantifies (*E*) and (*Z*)-13-retinol, following saponification of 20–40 ml of milk at room temperature for 18 h. The few existing studies on the diverse isomers of Vitamin A in foods perform the saponification at room

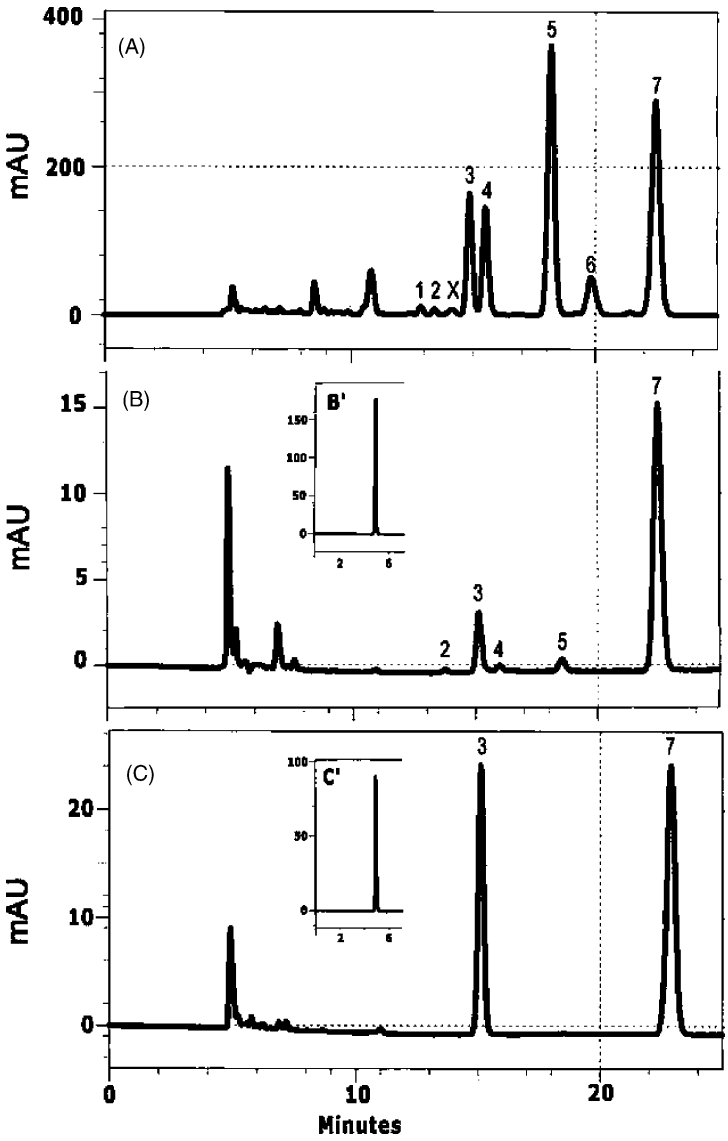

Fig. 1. Chromatograms obtained from: (A) a standard solution of (*E*)-retinol, (*Z*)-13-retinol after photoisomerization; (B and B′) an enteral feeding formula sample-chromatogram at 325 and 450 nm (above); (C and C′) from a standard solution of (*E*), (*Z*)-13-retinol and β-carotene-chromatogram at 325 and 450 nm (above), stationary phase 5 μm silica gel column 250 × 4.6 mm; mobile phase *n*-hexane:1-octanol (96:4); temperature 35 °C; flow-rate 0.6 ml/min. Detection wavelengths 325 nm for retinol isomers and 450 nm for β-carotene. Peaks: (1) (*Z*)-11-retinol; (2) (*Z*,*Z*)-11,13-; *X*: no. retinol spectra; (3) (*Z*)-13; (4) (*Z*,*Z*)-9,13; (5) (*Z*)-9; (6) (*Z*)-7; (7) (*E*)-retinol; β-carotene above.

temperature for 16 h [5,8,12]. In this study, saponification of the samples was performed for 45 min at 65 ± 1 °C, followed by extraction in the same tube in which the alkaline digestion had been performed.

Fig. 1 shows the chromatogram obtained following photoisomerization of the standard of (*E*)-retinol in the presence of (*Z*)-13-retinol. Separation of isomers: (*Z*)-11; (*Z*,*Z*)-11,13; (*Z*)-13; (*Z*,*Z*)-9,13; (*Z*)-9; (*Z*)-7 and (*E*)-retinol was observed. The time retention of β-carotene was of 4.89 ± 0.02 min (average of 17 determinations). Since we performed the separation at 35 °C (the temperature of the column's oven), we observed that the retention times of (*E*)-retinol and isomers were lower than those obtained by Zonta and Stancher [7], utilizing a (Si 60, Merck) column and a mobile phase of *n*-hexane-1-octanol 96:4, similar to that utilized in the present study, but at room temperature. Brinkmann et al. [5] also achieved a good separation of these isomers utilizing a narrow-bore column and *n*-hexane-1-octanol (99.7:0.3). In the present study, we observed, like Brinkmann et al. [5], that elution of (*Z*,*Z*)-9,13-retinol occurred prior to that of (*Z*)-13-retinol, thus differing from the observation of Zonta and Stancher [7].

Fig. 1 also shows the chromatogram of a sample of an enteral feeding formula, chosen because it was the only formula presenting levels of (*Z*,*Z*)-11,13-retinol above the established limit of quantification. This formula presented high concentration of β-carotene; therefore, a pronounced peak at 450 nm (B′) could be observed. Since commercial

Table 1
Absorption maxima of retinol isomers measured in *n*-hexane-1-octanol (96:4) compared with literature data

Isomer	Maximum absorption wavelength (nm)		
	This work	[5]	[7]
(*Z*)-11	318–320	322	322
(*Z*,*Z*)-11,13	314	314	312
(*Z*)-13	328	328	328
(*Z*,*Z*)-9,13	324	324	–
(*Z*)-9	322	322	323
(*Z*)-7	314	314	322
(*E*)-retinol	325	325	326

enteral formulas presented very low amounts of carotenes (Table 7), and when enriched, β-carotene is the main form utilized, in the present study total carotenes were determined as β-carotene.

The chromatogram referring to the standard solution containing known amounts of (*E*), (*Z*)-13 and β-carotene, is shown on Fig. 1. The identification of peaks referring to other isomers was achieved by comparison of the spectra and the maximum absorption wavelengths obtained in this work, with literature data (Table 1). Stancher and Zonta [7], Brinkmann et al. [5] also applied this type of peak confirmation. Spectra of (*E*); (*Z*)-7; (*Z*)-9; (*Z*,*Z*)-9,13; (*Z*)-13 and (*Z*,*Z*)-11,13-retinol obtained, are presented on Fig. 2.

The most common procedures employed to obtain geometric isomers of retinol are: (a) illumination of a standard of all-*trans*-retinol with white light for 1–2 h; (b) illumination of retinal followed by reduction to the corresponding isomers of retinol by $NaBH_4$. According to Nöll [4], the second one is the better option, but it is not possible to isolate (*Z*)-7-retinol by this procedure due to the instability of this isomer. Brinkmann et al. [5] achieved better results by illuminating (sunlight), a standard solution of retinyl palmitate for 2 h followed by saponification, rather than illuminating the standard of (*E*)-retinol directly. By this procedure, they were able, as in the present study, to obtain 7 isomers but only obtained a small quantity of (*Z*)-7-retinol. To obtain higher amounts of (*Z*)-11-retinol, they utilized the reaction with iodine. In the present study, we observed that exposure of (*E*)-retinol to direct sunlight for 30–40 min was ideal, since high amounts of (*Z*)-7 and (*Z*)-9-retinol were formed; longer time periods led to loss of the more labile isomers. We also observed that addition of (*Z*)-13-retinol to the all-trans-retinol standard prior to exposure to light propitiated a considerably higher formation of (*Z*,*Z*)-9,13-retinol. The amount of (*Z*)-11-retinol formed was not sufficient to furnish an adequate absorption spectrum. The identification of this isomer was performed using the maximum absorption wavelength closest to that observed by other authors (Table 1), and observing the increase of the peak corresponding to (*Z*)-11-retinol following addition of an alcoholic solution of iodine to the standard [5,17]. (*Z*)-11-retinol was not detected in the samples studied; for this reason we did not invest more time searching for information on this aspect (Table 2).

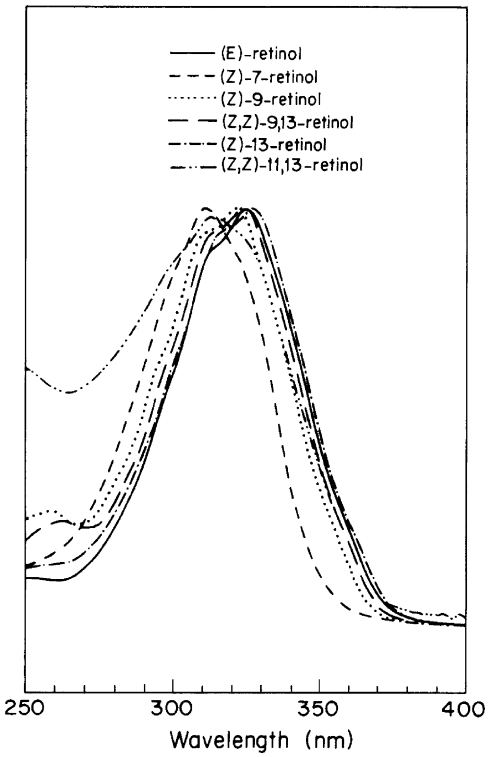

Fig. 2. Spectra of retinol isomers.

Table 2
Correction constants ($k_1 \cdot k_2$) obtained, compared with literature data

Isomer	This work	[5]	[8]
(*Z*)-11	–	1.631	1.56
(*Z*,*Z*)-11,13	2.177	2.272	2.21
(*Z*)-13	1.105	1.107	1.10
(*Z*,*Z*)-9,13	1.342	–	–
(*Z*)-9	1.280	1.279	1.31
(*Z*)-7	1.140[a]	–	–
(*E*)-retinol	1.000	1.000	1.00

[a] This value represents only k_2 (dats obtained in the laboratory).

Tables 3–8 summarize the data obtained for the validation of the method. The linearity of the standard curves (10 data points; 3 replicates) was demonstrated over a wide concentration range, an important factor specially for (*E*)-retinol, since the (*Z*)-isomers are always found at proportionally lower concentrations and will be calculated using the same analytical curve (excepting (*Z*)-13). The regression lines and correlation coefficients were respectively, $y = 3.396 \times 10^{-6}x + 0.0043$ and 0.9964 for (*E*)-retinol; $y = 3.505 \times 10^{-6}x + 0.0014$ and 0.9996 for (*Z*)-13-retinol, and $y = 2.030 \times 10^{-6}x + 0.0048$ and 0.9996 for β-carotene.

The forms of Vitamin A utilized in enteral preparations are (*E*)-retinyl palmitate or acetate, both retinol esters. Retinyl palmitate was the form utilized in the majority of the tests described; however, the recovery (at the concentration level), and the evaluation of isomerization were also performed with

Table 3
Recovery by the described method of (*E*)-retinol, (*Z*)-13-retinol and β-carotene

Vitamins/precursors, concentration (μg/100 ml)	*N*	Recovery (%)	R.S.D. (%)
(*E*)-retinyl palmitate (as retinol)			
1.76[a]	6	96.1 ± 7.2	7.49
60.35	6	99.9 ± 2.8	2.77
241.39	6	99.4 ± 1.9	1.91
(*Z*)-13-retinol			
1.63[a]	6	96.2 ± 4.9	5.09
24.84	6	99.0 ± 2.3	2.36
99.37	6	97.4 ± 1.9	1.95
β-carotene			
2.27[a]	6	94.1 ± 3.8	4.04
68.60	6	98.9 ± 3.0	3.08
274.40	6	98.9 ± 2.1	2.12
(*E*)-retinyl acetate (as retinol)			
122.00	6	95.7 ± 2.4	2.5

[a] Established quantification limit; *n*: number of determinations; R.S.D.: relative standard deviation.

retinyl acetate in order to observe the similarities between the results obtained with either form.

Recovery was over 90% for all vitamins and precursors, at all levels evaluated. Relative standard deviation (R.S.D.) demonstrated the slight variation between the results of the six replicates tested (Table 3). The within-day and between-day precision of the method for the determination of (*E*)-retinol; (*Z*)-13, (*Z*)-9; (*Z*,*Z*)-9,13-retinol and β-carotene is summarized in Tables 4 and 5. R.S.D. values of less than 10% were obtained for retinol isomers and β-carotene. The analysis of variance ($\alpha = 5\%$) showed that there was no statistically significant difference between results of the analysis over 4 days-period. In addition, the

Table 4
Analysis of within-day precision and accuracy of the method for the analysis of (*E*), (*Z*)-13-retinol and β-carotene

Vitamins/precursors, concentration (μg/100 ml)	*n*	Determined concentration (μg/100 ml)	R.S.D. (%)	*E* (%)
(*E*)-retinyl palmitate (as retinol)				
1.76[a]	6	1.7 ± 0.1	5.9	3.48
60.35	6	60.3 ± 1.7	2.8	1.65
241.39	6	240.1 ± 4.5	1.9	0.41
(*Z*)-13-retinol				
1.63[a]	6	1.6 ± 0.1	6.2	1.84
24.84	6	24.6 ± 0.6	2.4	0.96
99.37	6	97.4 ± 2.0	2.1	1.98
β-carotene				
2.27[a]	6	2.2 ± 0.1	4.5	3.08
68.60	6	67.9 ± 0.9	1.3	1.02
274.40	6	271.4 ± 5.9	2.2	1.09

[a] Established quantification limit; *n*: number of determinations; R.S.D.: relative standard deviation; *E*: deviation from nominal value. No significant differences between the added and determined concentrations, verified by the *t*-test ($P > 0.05\%$).

Table 5
Analysis of the precision of the method for the analysis of geometrical retinol isomers and β-carotene in enteral feeding formulas

Vitamins/ precursors	Within-day ($n^a = 6$)		Between-days ($n^b = 4$)	
	Determined concentration (μg/100 ml)	R.S.D. (%)	Determined concentration (μg/100 ml)	R.S.D. (%)
(*E*)-retinol	64.4 ± 1.88	2.92	60.1 ± 2.88	4.79
(*Z*)-9-retinol	4.8 ± 0.32	6.72	4.5 ± 0.17	3.74
(*Z*,*Z*)-9,13	3.1 ± 0.28	8.82	3.0 ± 0.13	4.29
(*Z*)-13-retinol	14.8 ± 0.25	1.67	14.1 ± 0.50	3.55
β carotene	34.1 ± 0.54	1.57	33.5 ± 0.41	1.22

[a] Number of determinations.
[b] Number of assays; R.S.D.: relative standard deviation. No significant differences in the results of the analysis over a 4 day-period (ANOVA, $\alpha = 5\%$), were observed.

methods were accurate since deviation from the theoretical value was also in the 10% range (Table 4). The *t*-test ($\alpha = 5\%$) showed that there were no significant differences between the real and the determined concentrations. The established limit of quantification was 1.76 μg/100 ml for (*E*)-retinol, 1.63 for (*Z*)-13 and 2.27 for β-carotene. Precision, accuracy and recovery obtained with these concentration levels are presented in Tables 3 and 4.

Another important aspect speaking in favor of the trustworthiness of our data is that the proposed analytical procedures do not evoke the formation of (*Z*)-isomers, or that if so, it is insignificant. Some authors have demonstrated that provided care with oxygen and light is taken during analysis, isomerization of the (*E*)-retinol into (*Z*)-isomers either does not occur, or is insignificant [1,8,15]. However, Steurle [18] showed that minerals added to an animal ration, catalyze isomerization during saponification of the sample. Table 6 shows that there was no significant difference ($P > 0.05$), between the percentage of form (*Z*)-13 relative to the (*E*) form contained in the standard of (*E*)-retinyl palmitate, prior to and following its addition to a sample of the enteral formula free of Vitamin A, and analyzed by the methodology here proposed. (*Z*)-9 and (*Z*,*Z*)-9,13-retinol appeared in insignificant amounts, probably originating from the standard itself. Table 7 shows that three different samples of commercial enteral feeding formulas presented similar concentrations ($P > 0.05$), of geometric isomers in the (*Z*)-form, prior to and following addition of standards of (*E*)-retinyl palmitate or (*E*)-retinyl palmitate plus (*Z*)-13-retinol or (*E*)-retinyl acetate. Had the analytical procedures evoked the isomerization of the (*E*) or (*Z*)-13 form, the isomer contents would have increased following enrichment. Panfili et al. [1]

Table 6
Percentage of (*Z*)-13-retinol relative to (*E*)-retinol, in vitamin-free enteral formulas, enriched with a retinyl palmitate standard ($n = 6$).

(*E*)-Retinyl palmitate standard	5.4 ± 0.3
Enteral formula enriched with 140 μg/100 ml of formula	5.4 ± 0.4

Values do not differ statistically according to the *t*-test ($P > 0.05$).

Table 7
(*E*)-retinol and geometric retinol isomers concentrations (μg/100 ml) in samples of enteral formulas, before and following addition of (*E*)-retinyl palmitate standard or (*Z*)-13-retinol plus (*E*)-retinyl palmitate or (*E*)-retinyl acetate

Standard	*n*	Isomers						
		All-*trans*	13-*cis*	9-*cis*	9,13-di-*cis*	7-*cis*	11-*cis*	11,13-di-*cis*
(*E*)-palmitate								
Before addition	6	132.8 ± 5.8a	29.1 ± 1.2a	6.4 ± 0.6a	3.0 ± 0.4a	nd	nd	tr
Following addition	6	187.0 ± 6.5b	30.1 ± 1.8a[a]	6.6 ± 0.6a	3.1 ± 0.5a	nd	nd	tr
(*E*) + (*Z*)-13								
Before addition	10	63.3 ± 0.6a	14.8 ± 0.5a	4.8 ± 0.1a	3.2 ± 0.2a	nd	nd	nd
Following addition	10	104.2 ± 3.6b	29.2 ± 2.2b	4.5 ± 0.2a	3.2 ± 0.3a	nd	nd	nd
(*E*)-acetate								
Before addition	6	59.7 ± 1.5a	22.6 ± 0.7a	4.0 ± 0.2a	2.0 ± 0.3a	nd	nd	tr
Following addition	6	116.3 ± 3.8b	21.9 ± 0.8a[a]	4.1 ± 0.2a	2.3 ± 0.4a	nd	nd	tr

Values in the same column of the same subdivision of the table, indicated by the same letter, do not differ statistically among each other, according to the *t*-test ($P > 0.05$).

[a] After subtraction of the quantity of (*Z*)-13-isomer contained in the (*E*)-retinyl palmitate standard (ratio (*Z*)-13:(*E*) = 5.4%) and in the (*E*)-retinyl acetate standard (ratio (*Z*)-13:(*E*) = 4.0%); nd: not detected; tr: below the quantification limits.

detected (*Z*)-13-isomer of retinol in UHT milk, but found no signs of *Z*-isomers in fresh milk even after sample saponification at 70 °C, thus reinforcing our data.

Tables 8–10 summarize the data obtained from the analysis of 17 samples of enteral formulas (*E*)-retinol always appeared at highest concentration, followed by (*Z*)-13; (*Z*)-9; (*Z*,*Z*)-9,13 and (*Z*,*Z*)-11,13-retinol, respectively (Table 8). This sequence of concentrations of retinol isomers has also been observed in UHT milk following conventional and microwave heating, and manufactured products containing liver, liver and fish [5,8]. (*Z*)-11-retinol was not detected in any of the samples studied. (*Z*)-11-retinol has been detected in considerable amounts in foods going through microbiological fermentation, and in liver containing processed food, possibly due to the presence of enzymes, which promote the formation of this isomer [5]. In the case of commercial enteral formulas, microbiological fermentation should not occur, and enzymes would hardly be present in such formulas.

Table 8
Content of retinol isomers (μg/100 ml) and carotene (μg/100 ml) in commercial enteral feeding formulas

Isomers	Liquid ($n = 11$)	Powder ($n = 6$)
E-retinol	94.3 (35.4–169.2)	114.1 (87.8–206.1)
(*Z*)-13	20.3 (4.7–62.5)	10.8 (3.7–56.6)
(*Z*)-9	4.2 (tr – 12.2)	2.7 (tr – 11.2)
(*Z*,*Z*)-9,13	2.4 (tr – 6.5)	tr (tr – 6.4)
(*Z*,*Z*)-11,13	One sample = 1.9	
	Three samples = tr	Two samples = tr
	Seven samples = ND	Four samples = ND
(*Z*)-11	ND	ND
(*Z*)-7	One sample = tr	
	Ten samples = ND	ND
β-carotene	201.5 (110.0–753.2)	–

Values are presented as medians and range of variation. Only preparations enriched in β-carotene were considered (six liquid diets); in preparations not enriched with this substance, the total carotene content was below the established quantification limit, except in two samples (2.6–5.8 μg/100 ml); tr: traces; ND: not detected.

The percentage of *Z*-isomers relative to the *E*-isomers in the 11 samples of liquid formulas was, in increasing order: 13, 23, 23, 24, 27, 27, 38, 40, 41, 42 and 42%, and in the 6 samples of powdered formulas was: 5, 8, 9, 15, 17 and 36% (Table 9). The percentage of *Z*-isomers was higher in the liquid formulas, probably due to the instability of retinol in aqueous media, which appears to exert an influence, despite the protection by the microcapsules which envelop this vitamin for greater stability and hydromiscibility. Frias and Vidal-Valverde [9] determined thiamine, tocopherols, (*Z*)-13 and (*E*)-retinol in five samples of commercial enteral samples in powder form, observing (*Z*)-13-retinol/(*E*)-retinol ratios between 7 and 8%.

Panfili et al. [1], found up to 33.5% of (*Z*)-13-retinol in sterilized milk. Brinkmann et al. [5] found up to 43% total isomers relative to the (*E*)-isomer in liver containing, manufactured infant food. Stancher and Zonta [19], studying parmesan, taleggio, and montasio cheeses, observed respectively 18, 47 and 56% of (*Z*)-13 relative to (*E*)-retinol. The values observed for the distribution of the geometric isomers of retinol in this study of commercial enteral formulas, are similar to the values observed in foods going through high temperature processing and prolonged cure (cheeses). The amount of (*Z*)-isomers in the enteral formulas is probably also dependent on the presence of these isomers in the synthetic (*E*)-retinyl-palmitate or (*E*)-retinyl-acetate utilized for the preparation of the formulas. Another aspect to be considered is the period of storage. Woolard and Indyk [15], showed an increased amount of *Z*-isomers in Vitamin A-enriched powdered milk during storage, while Kim et al. [20] observed that storage had little effect on the formation of *Z*-isomers in fortified corn flakes. The here evaluated samples had remained for 3–6 months on the shelf, at room temperature. The validity of liquid enteral formulas varies between 10 and 15 months, and of powders between 15 and 24 months, at room temperature.

Table 9
Relation between concentrations of (*Z*)-13 and (*E*)-retinol and between total isomers and (*E*)-retinol, in enteral feeding formulas

Ratio	Liquid ($n = 11$)	Powder ($n = 6$)
(*Z*)-13/(*E*)-retinol	0.23 ± 0.08 (0.13–0.37)	0.12 ± 0.08 (0.03–0.27)
Total isomers/(*E*)-retinol	0.31 ± 0.10 (0.13–0.42)	0.15 ± 0.10 (0.05–0.036)

Values are shown as means ± standard deviation and range; *n*: number of samples.

Table 10
Percentage of total Vitamin A[a] found in the 17 samples of enteral formulas, relative to the statements on the labels

Percentage to statement on the label	*n*
90–100	2
101–150	8
151–200	6
201–210	1

[a] Calculations performed according to the biological activity (2) of each vitamin isomer precursor—(*E*)-retinol: 100%; (*Z*)-13: 75%; (*Z*)-9: 19%; (*Z*,*Z*)-9,13: 21%; β-carotene: 16.7%; *n*: number of samples.

Frias and Vidal-Valverde [9] determined (*E*)- and (*Z*)-13-retinol in five samples of commercial enteral feeding formulas and found results which compared favorably with label declarations. Chase Jr. et al. [21] quantified total retinyl palmitate in four samples and found Vitamin A activity below the declared ones in three. However, these three products only listed on their labels the total activity of Vitamin A, including the contribution of β-carotene added to these formulas, which was not determined by the method proposed by these authors. In the present study, considering β-carotene content in the enriched samples, we observed that despite the high content of *Z*-isomers observed in the various samples studied, none presented Vitamin A activity below 90% of that stated in their labels, as shown on Table 10.

4. Conclusions

In previous studies of analytical methods destined to determine the various isomers of retinol in food, authors only determined precision and accuracy for (*E*)-retinol, assuming that further isomers presented the same values. In the present study, we made a complete validation for (*E*)-retinol, (*Z*)-13-retinol and β-carotene, compounds which contribute most to Vitamin A activity, and determined as well, the intra- and inter-assay reproducibility for (*Z*)-9 and (*Z*,*Z*)-9,13-retinol, compounds which appear in considerable concentration in the majority of samples. The procedures utilized propitiate the performance of analyses in a practical and safe way, which includes the obtention of a standard containing seven isomers. Six retinol isomers were identified in the analyzed samples of commercial enteral feeding formulas. The determination of the retinol isomers in these formulas is of importance for the more precise evaluation of Vitamin A activity and also by contributing to knowledge about the different chemical forms of the nutrients which constitute these formulas, whose use by persons in a critical health condition is becoming ever more frequent.

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