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Analysis of α-Tocopherol Stereoisomers in Fortified Infant Formula by Chiral Chromatography

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Abstract

Background: Direct measurement of the bioavailable α-tocopherol content presents a significant analytical challenge and requires chiral separation of the α-tocopherol stereoisomers. *Objective*: The objective of the study was to validate an analytical method for the analysis of α-tocopherol stereoisomers in infant formulas and dairy products. *Method*: Samples were saponified at elevated temperature and lipophilic components were extracted into an organic solvent, with subsequent chromatographic separation of the α-tocopherol stereoisomers achieved by HPLC with a chiral column and fluorescence detection. *Results*: The method was shown to be accurate, with spike recoveries of 91.9–108.8% for *RRR*-α-tocopherol and 90.1–104.7% for α-tocopherol, with no statistical bias against NIST 1849a certified reference material (P--value = 0.54) and an HPLC-UV analytical method (P-value = 0.48). Acceptable precision was confirmed, with repeatabilities estimated at 3.5% RSD_r (HorRat = 0.6) for *RRR*-α-tocopherol and 4.6% RSD_r (HorRat = 0.4) for α-tocopherol. *Conclusions*: A straightforward chiral chromatographic method for the analysis of stereoisomeric forms of α-tocopherol is described. In a single analytical run, the method can quantify: (i) the total α-tocopherol content; (ii) the nutritionally important *RRR*-α-tocopherol and/or 2R, 4′-*ambo*, 8′-*ambo*-α-tocopherol contents; (iii) the amount of *all-rac*-α-tocopherol, *all-rac*-α-tocopheryl acetate, or *all-rac*-α-tocopheryl succinate fortified into the product. *Highlights*: An accurate and precise chiral chromatographic method for the analysis of isomeric forms of α-tocopherol is described. The method is able to distinguish between natural and synthetic tocopherol sources. The method is accurate and precise and is suitable either for routine product compliance testing during product manufacture or as a possible reference method.

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Introduction

Vitamin E is a lipid-soluble antioxidant that plays a crucial role in human and animal reproduction. The predominant and most biologically active form of vitamin E is α-tocopherol, which is found in highest amounts in vegetable oils, nuts, dark green leafy vegetables, and egg yolk.

The three chiral carbons in α-tocopherol result in eight possible stereoisomers (*RRR*-, *RRS*-, *RSR*-, *RSS*-, *SSS*-, *SSR*-, *SRS*-, and *SRR*-α-tocopherol). Only *RRR*-α-tocopherol is found naturally, whereas synthetic *all-rac*-α-tocopherol derived from chemical synthesis consists of all eight possible stereoisomers in equal proportions (1, 2). In this study, the term α-tocopherol denotes a mixture of stereoisomers in unequal proportions as would be typically found in most infant formulas.

In humans, only the 2R stereoisomers are considered to have biological activity. Discrimination among the stereoisomers occurs in the liver via the α-tocopherol transfer protein, which preferentially binds only 2R-α-tocopherol (2R, 4′-*ambo*, 8′-*ambo*-α-tocopherol); the 2S-α-tocopherol (2S, 4′-*ambo*, 8′-*ambo* α-tocopherol) isomers possess low affinity for α-tocopherol transfer protein and are rapidly metabolized (2–4). The biological activity of *RRR*-α-tocopherol has been variably reported to be 1.36–2 times that of *all-rac*-α-tocopherol (5, 6)

In many countries, the allowable forms for fortification of vitamin E during infant formula manufacture are as *RRR*-α-tocopherol, *all-rac*-α-tocopherol, *RRR*-α-tocopheryl acetate, *all-rac*-α-tocopheryl acetate, *RRR*-α-tocopheryl succinate, or *all-rac*-α-tocopheryl succinate, with *all-rac*-α-tocopheryl acetate the most common form used. Dairy products, particularly infant formula, may also contain a number of different vitamin E congeners (α-, β-, γ-, and δ-tocopherol and tocotrienols) derived from vegetable oil blends and bovine milk fat. The calculation of dietary vitamin E intake in regulations (7) and industry guidelines (8) is currently based on the vitamin E activity of *RRR*-α-tocopherol. In Codex and other regulations, *RRR*-α-tocopherol is referenced as d-α-tocopherol and *all-rac*-α-tocopherol as dl-α-tocopherol (9).

Reporting of bioavailable vitamin E content is achieved by the conversion of quantities measured into biological units such as international units (IU) or tocopherol equivalents (TE). Direct measurement of the bioavailable vitamin E content therefore presents a significant analytical challenge, requiring chiral separation of the α-tocopherol stereoisomers for discrimination of the biologically active 2R forms (10–12).

Thus, this study describes a method utilizing chiral chromatography for the compliance testing of nutritionally important α-tocopherol forms in dairy foods, including infant formula, that are fortified with vitamin E.

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Methods

Apparatus

Specified weights and volumes calibrated with National Institute of Standards and Technology (NIST; Gaithersburg, MD) traceable calibration weights.

Apparatus

(a) *High-performance liquid chromatography (HPLC) system*.—Prominence HPLC system consisting of a LC-20AT pump, a SIL-20ACHT autosampler, a CTO-20AC column oven, a RF20Axs fluorescence detector, and a DGU-20A5R degasser unit (Shimadzu, Kyoto, Japan). Lab Solutions software Version 5.73 (Shimadzu) was used for instrument control and data processing.

(b) *HPLC column*.—Lux Cellulose-1, 250 × 4.6 mm (Phenomenex, Torrance, CA).

(c) *Guard column*.—Lux Cellulose-1, 3 × 4mm (Phenomenex).

(d) *In-line filter column*.—KrudKatcher Ultra, 4.6 mm id (Phenomenex).

(e) *Micropipettes, calibrated*.—Research plus, 100–1000 µL and 1–10 mL (Eppendorf, Hauppauge, NY).

(f) *Centrifuge*.—Gerber Universal (Gerber, Effretikon, Switzerland).

(g) *Vortex mixer*.—Genius 3 (IKA, Wilmington, NC).

(h) *Boiling tubes*.—Pyrex 70 mL with screw-top cap (Sigma-Aldrich, St. Louis, MO).

(i) *Analytical balance, calibrated*.—Mettler-Toledo AE260 analytical delta range (± 0.1 mg; Columbus, OH).

(j) *Solvent dispensers, calibrated*.— Dispensette 1.0–10 mL, 2.5–25 mL (Brand, Wertheim, Germany).

(k) *Plate shaker*.— Fitted with HPLC vial adaptor (Grant Instruments, Royston, UK).

(l) *Cryogenic vials*.—2 mL Nalgene (ThermoFisher, Waltham, MA).

(m) *Graduated cylinders*.—100 and 1000 mL.

(n) *Volumetric flasks*.—25, 50, and 100 mL.

(o) *Glass solvent bottles*.—500 mL and 1 L.

(p) *HPLC injection vials*.—Amber 1 mL with Teflon-coated caps.

(q) *Water bath*.—At 70 ± 2 °C.

Reagents

(a) *Isooctane (2,2,4-trimethylpentane)*.—HPLC grade (Thermo-Fisher Scientific, Waltham, MA).

(b) *Isopropanol (propan-2-ol)*.—HPLC grade (Merck, Darmstadt, Germany).

(c) *Hexane*.—HPLC grade (Merck).

(d) *Diisopropyl ether (2-[(propan-2-yl)oxy]propane)*.—Reagent grade (Merck).

(e) *Ethanol*.—Reagent grade (Merck).

(f) *Water*.—HPLC grade (Barnstead, Dubuque, IA).

(g) *RRR-α-tocopherol InChIKey = GVJHHUAWPYXKBD-IEOSBIPESAN*.—Purity ≥ 97% (Chromadex, Los Angeles, CA).

(h) *all-rac-α-tocopherol InChIKey = GVJHHUAWPYXKBD-UHFFFAOYSAN.*—Purity ≥ 96% (Sigma-Aldrich, St. Louis,MO).

(i) *Potassium hydroxide.*—Reagent grade (Sigma-Aldrich).

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Solutions

(a) *Potassium hydroxide solution (50%, m/v).*—In a 500 mL glass solvent bottle, dissolve 200 g potassium hydroxide in 400 mL water.

(b) *Extraction solution (hexane—diisopropylether, 75:25, v/v).*—To a 1 L glass solvent bottle, add 750 mL hexane and 250 mL diisopropylether.

(c) *Mobile phase (isooctane—ethanol—isopropanol, 100:0.06:0.06, v/v/v).*—To a 1 L glass solvent bottle, add 1000 mL isooctane, 0.6 mL ethanol, and 0.6 mL isopropanol.

Samples

Method performance was evaluated using a range of dairy products including sliced cheese, liquid whole milk, an unfortified whole milk powder (WMP), NIST 1849a certified reference material (CRM), and a selection of vitamin-fortified infant formulas derived from the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) kit, including a low-fat adult nutritional powder, a milk-based toddler formula powder, an infant elemental powder, a soy-based infant formula powder, a milk-based child formula powder, and a milk-based infant formula powder fortified with fructooligosaccharide and galactooligosaccharide. An unfortified skim milk powder (SMP), which had almost no milkfat (≤ 0.1%), was used as a blank matrix.

Standards

A stock standard was made by transferring the contents of a 1 g vial of *RRR*-α-tocopherol primary standard into a 100 mL volumetric flask with ethanol, dissolving, and then making to volume. Aliquots (~1 mL) were transferred into cryogenic vials and stored in a freezer at less than ~10 °C prior to use. A working standard was made by adding 0.5 mL stock standard to a 50 mL volumetric flask and diluting to volume with ethanol. The concentration of the working standard was determined

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spectrophotometrically by measuring the absorbance at 292 nm ($E^{1\%}_{1cm}$: 75.8 g/mol.cm) (13) against ethanol.

An unfortified WMP was used for preparation of the matrix-matched external standard by adding a 2.0 ± 0.02 mL aliquot of the working standard solution to 0.5 ± 0.05 g WMP, with an unspiked 0.5 ± 0.05 g WMP used as a blank.

Sample Preparation

Approximately 0.5 ± 0.05 g solid powder or cheese, or 4 ± 0.2 mL liquid milk, was weighed accurately into a 70 mL glass boiling tube, 4 ± 0.2 mL water was added, and then the sample was vortex mixed for 30 s. To the sample, 10 mL ethanol and 2 mL potassium hydroxide solution were added and the tube was again vortex mixed for 30 s. The boiling tubes were placed in a water bath at 70 °C for 10 min and vortex mixed every 5 min. After cooling to room temperature in a cold water bath, 20 ± 0.2 mL extraction solution was added and the samples were placed on a horizontal shaker for 10 min. To each tube, 25 mL water was added; the tubes were inverted ten times and then placed in a centrifuge at 270 × g for 15 min. A 0.5 mL aliquot of the upper layer was transferred to an HPLC vial, with the vial being evaporated to dryness under nitrogen on a heating block at 40 °C. The samples were reconstituted in 0.5 mL mobile phase ready for analysis.

Instrumental Analysis

(a) *Column temperature.*—50 ± 0.5 °C.

(b) *Chiller temperature*.—15 °C.

(c) *Injection volume*.—20 µL.

(d) *Flow rate.*—1.0 mL/min.

(e) *Run time*.—60 min.

(f) *Detection*.—Fluorescence: λ_{ex} = 292 nm; λ_{em} = 330 nm.

Calculations

The concentrations of α-tocopherol in various forms are given by the following equations:

$$\mathrm{RRR}-\alpha-\mathrm{Toc}=\frac{\mathrm{A_{RRR}}}{\mathrm{A_S}-\mathrm{A_U}}\times\frac{2\times\mathrm{C_S}}{\mathrm{M_S}}\times\frac{100}{1000} \quad (1)$$

$$\mathrm{RSR}-\alpha-\mathrm{Toc}=\frac{\mathrm{A_{RSR}}}{\mathrm{A_S}-\mathrm{A_U}}\times\frac{2\times\mathrm{C_S}}{\mathrm{M_S}}\times\frac{100}{1000}=\mathrm{RSS}-\alpha-\mathrm{Toc}=\mathrm{RRS}-\alpha-\mathrm{Toc} \quad (2)$$

$$2R-\alpha-Toc=(RSR-\alpha-Toc\times 3)+RRR-\alpha-Toc \qquad (3)$$

$$\alpha-Toc=\frac{A_{Tot}}{A_S-A_U}\times\frac{2\times C_S}{M_S}\times\frac{100}{1000} \qquad (4)$$

$$\alpha-TocE=(RSR-\alpha-Toc\times 8)\times\frac{Mr\ (\alpha-TocE)}{Mr\ (\alpha-Toc)} \qquad (5)$$

where: *RRR*-α-Toc = concentration of *RRR*-α-tocopherol (mg/hg); α-Toc = concentration of *all-rac*-α-tocopherol (mg/hg); *RSR*-α-Toc = concentration of *RSR*-α-tocopherol (mg/hg); α-TocE = concentration of *all-rac*-α-tocopheryl ester (mg/hg); 2R-α-Toc = concentration of 2R, 4′-*ambo*, 8′-*ambo*-α-tocopherol (mg/hg); A_{RRR} = peak area of *RRR*-α-tocopherol in sample (no units); A_{RSR} = peak area of *RSR*-α-tocopherol in sample (no units); A_{Tot} = sum of peak areas of α-tocopherol stereoisomers in sample (no units); A_S = peak area of *RRR*-α-tocopherol in spiked WMP (no units); A_U = peak area of *RRR*-α-tocopherol in unspiked WMP (no units); 2 = volume of standard in spiked WMP (mL); C_S = concentration of *RRR*-α-tocopherol in spiked WMP (µg/mL); M_S = mass of sample (g); 100 = mass conversion factor (mg/g to mg/hg); 1000 = concentration conversion factor (mg/hg to mg/hg); M_r (α-TocE) = molecular mass of α-tocopheryl ester (g/mol); M_r (α-Toc) = molecular mass of α-tocopherol (g/mol).

Results and Discussion

The principal intention was to develop a method that was capable of selectively quantifying both *RRR*-α-tocopherol and total α-tocopherol. However, it was confirmed that the method could also indirectly estimate the concentrations of added α-tocopheryl acetate and the nutritionally important 2R forms, the latter either as individual values or collectively as the sum of *RSR*-, *RSS*-, *RRS*-, and *RRR*-α-tocopherol. This affords great utility in that this method is capable of reporting many different aspects of the α-tocopherol content in dairy products for nutritional information (*RRR*-α-tocopherol, 2R, 4′-*ambo*, 8′-*ambo*-α-tocopherol), product formulation (*all-rac*-α-tocopheryl ester), and compliance testing (α-tocopherol and/or *RRR*-α-tocopherol) in a single analysis.

Method Optimization

The analytical method combines a sample preparation, which is modified from that described previously (14), with a chiral chromatographic separation that has been reported recently (15). The analytical run time had to be optimized to ensure that late-eluting tocopherols, primarily γ-tocopherol, would not compromise integration of the targeted α-tocopherol peaks (Figures 1 and 2).

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An in-line filter was incorporated into the HPLC system immediately prior to the fluorescence detector to avoid flow cell blockages, as fluorescence detectors have a much lower maximum allowable pressure than UV detectors.

Method Validation

Linearity was assessed by spiking SMP samples (n = 8) with different volumes of a standard mixture containing 0–184 µg *all-rac*-α-tocopherol (0–73.8 mg/hg), of which one-eighth (0–23.1 µg) is *RRR*-α-tocopherol (0–9.2 mg/hg), and then analyzing using the described method. Linearity of the method was evaluated by plotting the instrument response as peak area against the concentrations of *all-rac*-α-tocopherol and *RRR*-α-tocopherol, with analysis by least-squares regression (Figures 3 and 4). Visual inspection of the linear regression line and residual plots, as well as the regression equation and the correlation coefficients, was used to demonstrate a linear relationship between instrument response and analyte concentration over the expected sample concentration range. The value of the calibration correlation coefficient (r^2) for both *RRR*-α-tocopherol and *all-rac*-α-tocopherol was 0.9997 with the residual plots showing no discernible pattern.

Accuracy of the method was demonstrated in three ways: (i) as bias against a CRM; (ii) as bias against an HPLC-UV compliance testing method for α-tocopherol (13) and against an Official Method 2012.10 (16, 17) for α-tocopheryl acetate (n = 8); (iii) as spike recovery of known amounts of *all-rac*-α-tocopherol.

Bias against a CRM was evaluated by replicate testing of NIST 1849a CRM over five different days (18). No bias between the measured results and the certified value for α-tocopherol was found (P-value = 0.54, α = 0.05, n = 13). An estimation of bias of the described chiral method against the HPLC-UV method for α-tocopherol in dairy products also showed no evidence of bias (P-value = 0.48, α = 0.05, n = 31), with the analytical equivalence of the two methods further illustrated in a Bland-Altman plot (Figure 5). For the determination of α-tocopheryl acetate, no statistical bias between the described chiral method and Official Method 2012.10 was found (P-value = 0.93, α = 0.05, n = 8), indicating the validity of the indirect calculation for determining its content.

Recovery was evaluated by spiking a range of dairy product samples at 0, 50, and 100% of a nominal concentration (18.2 mg/hg) and comparing the recovery with that of a low-fat SMP sample spiked at the same concentration. The recovery values measured were 91.9–108.8% (mean = 100.8%, standard deviation = 5.5%, n = 18) for *RRR*-α-tocopherol and 90.1–104.7% (mean = 96.2%, standard deviation = 4.1%, n = 18) for α-tocopherol (Table 1). The recovery estimates were within the 85–100% range for samples at the 0.01% (10 mg/hg) concentration level specified in the Official Methods of Analysis (19).

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Precision was estimated by the analysis of duplicate pairs of 16 samples over three different days. Repeatability was estimated to be 3.5% RSD*r* (HorRat = 0.6, n = 16) for *RRR*-α-tocopherol and 4.6% RSD_r (HorRat = 0.4, n = 16) for total α-tocopherol. Intermediate precision was evaluated on a sample matrix tested on five different days (n = 13) and was estimated to be 4.7% RSD_{iR} (HorRat = 0.4, n = 8) and 7.8% RSD_{iR} (HorRat = 0.7, n = 8) for *RRR*-α-tocopherol and α-tocopherol, respectively. The HorRat values demonstrated that the precision was within the expected range specified in the AOAC Official Methods of Analysis (20).

A seven-factor Plackett–Burman ruggedness trial (21, 22) that evaluated the measured concentrations of *RRR*-α-tocopherol and α-tocopherol was performed on a milk-based infant formula using the following factors: sample weight (0.6, 0.4 g), potassium hydroxide solution volume (2.2, 1.8 mL), extraction solvent volume (21, 19 mL), shaker time (20, 10 min), centrifuge time (6, 4 min), vial mixer time (6, 4 min), and a dummy factor; binary levels on either side of the optimized values were selected for each variable. Half-normal plots graphically distinguish significant effects from insignificant effects for each parameter; of the factors tested, only one factor, the volume of extraction solution, showed a significant effect (Figure 6). This was unsurprising given the importance of the volume being the same for both samples and standards, although it did indicate that an accurately calibrated and precise volume of extraction solvent must be dispensed.

The method detection limit (MDL) is the smallest amount of an analyte that can be quantified with reliability, estimated from the precision of the complete method applied to a sample containing low levels of analyte (23). The MDL was estimated by testing eight replicates of a 50:50 mixed WMP and SMP sample. The values for the MDL and the method limit were calculated as 0.06 and 0.23 mg/hg, respectively, demonstrating that the described method is suitable for application to fortified infant formulas, as these typically contain α-tocopherol concentrations that are generally at least two orders of magnitude higher.

The described method fulfils the requirements of the applicability statement in the Standard Method Performance Requirements (SMPR) for vitamin E (Official Method 2011.010, 24), with respect to the range of adult and pediatric formula products evaluated and with respect to the required analytes, including *RRR*-α-tocopherol, *all-rac*-α-tocopherol, and their esters, and is able to report the quantities of α-tocopherol and esters separately. An assessment of the method performance against the vitamin E method performance requirements illustrates that the method meets or exceeds all the criteria. The method limit of 0.23 mg/hg in a powder equates to 0.03 mg/hg in a reconstituted product, i.e., much lower than the 0.2 mg/hg SMPR limit, with the linear range demonstrated to be 0–9.2 mg/hg reconstituted final product. The spike recovery of 90.1–108.8% for both *RRR*-α-tocopherol and α-tocopherol is within the specified range of 90–110%. The repeatability, estimated at 3.5% and

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4.6%, is well below the lowest limit of 6% RSD_r and the intermediate precision of 4.7% and 7.8%, although only indicative of reproducibility, is much less than the lowest limit for reproducibility of 16% RSD_R.

The results obtained for the various forms of α-tocopherol in a range of infant formulas and dairy products are given in Table 2. The calculations of 2R, 4′-*ambo*, 8′-*ambo*-α-tocopherol and α-tocopheryl esters are possible only for infant formulas fortified with *all-rac*-α-tocopherol or *all-rac*-α-tocopheryl esters. For formulas fortified with *RRR*-α-tocopherol or *RRR*-α-tocopheryl esters, only a single peak is obtained, limiting the results to *RRR*-α-tocopherol.

Conclusions

Chiral analysis is a necessary tool to measure the bioavailable isomers of α-tocopherol. A straightforward chiral chromatographic method for the analysis of isomeric forms of α-tocopherol in infant formula is described. The method is capable of distinguishing between natural and synthetic tocopherol sources. The method was single laboratory validated and found to be accurate, precise, and robust for the intended application of the analysis of dairy products fortified with *all-rac*-α-tocopherol, *all-rac*-α-tocopheryl acetate, or *all-rac*-α-tocopheryl succinate. This method is intended for use in high-throughput laboratories as part of routine product compliance release during product manufacture, although the validation data show that this method could also be suitable as a reference method.

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Table 1. Spike recovery for a range of dairy products

Sample	RRR-α-tocopherol		α-tocopherol	
	50%[a]	100%[b]	50%[c]	100%[d]
Low-fat adult nutritional powder	97.7	100.9	94.6	96.8
Milk-based toddler powder	108.3	108.8	96.5	99.8
Infant elemental powder	100.0	106.1	94.7	91.8
Soy-based IF[e] powder	95.2	104.0	94.0	100.6
Milk-based child formula powder	107.7	91.9	102.6	92.2
FOS/GOS[f] milk-based IF powder	96.3	101.8	90.1	96.8
Liquid milk	106.6	103.1	91.9	104.7
Cheese	93.2	100.8	91.2	98.2
WMP[g]	92.8	99.3	94.4	100.0

[a] 50% RRR-α-tocopherol spike = 1.1 mg/hg product as is
[b] 100% RRR-α-tocopherol spike = 2.2 mg/hg product as is
[c] 50% α-tocopherol spike = 8.7 mg/hg product as is
[d] 100% α-tocopherol spike = 17.3 mg/hg product as is
[e] IF = Infant formula
[f] FOS = Fructooligosaccharide; GOS = galactooligosaccharide
[g] WMP = Whole milk powder

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Table 2. Results for each α-tocopherol stereoisomeric form for a range of dairy products.

Sample	Results, mg/hg				
	RRR-α-Toc	2R-α-Toc	Bioactive α-Toc[a]	α-Toc	α-TocA
NIST 1849a[b]	20.7	20.7	20.7	20.7	ND[c]
	21.0	21.0	21.0	21.0	ND
Milk-based infant formula	5.5	15.3	17.5	29.4	28.7
	5.8	16.2	18.6	31.1	30.5
Low-fat adult nutritional powder	2.3	8.3	9.7	17.2	17.6
	2.5	9.0	10.5	19.0	18.8
Milk-based toddler powder	2.0	2.7	2.9	4.1	2.3
	1.9	2.7	2.9	4.0	2.4
Infant elemental powder	2.7	7.7	8.8	13.7	14.6
	2.8	7.9	9.1	15.2	15.0
Soy-based IF powder	4.1	7.7	8.5	13.3	10.5
	4.2	8.2	9.1	13.6	11.4
Milk-based child formula powder	12.9	17.4	18.4	24.0	13.1
	13.1	18.9	20.3	25.0	16.9
FOS/GOS milk-based IF powder	2.2	3.8	4.2	6.2	4.6
	2.3	3.5	3.7	6.6	3.3
Liquid milk[c]	1.1	1.1	1.1	1.1	ND
	1.1	1.1	1.1	1.1	ND
Cheese[c]	1.2	1.2	1.2	1.2	ND
	1.2	1.2	1.2	1.2	ND
WMP[c]	0.5	0.5	0.5	0.5	ND
	0.6	0.6	0.6	0.6	ND

[a] Results as RRR-α-tocopherol equivalents [individual stereoisomers multiplied by their bioactivity factors from Eitenmiller, Landen & Ye (2008)] (13)

[b] Product fortified with RRR-α-tocopheryl acetate

[c] Unfortified products with no added vitamin E

RRR-a-Toc = RRR-α-tocopherol, 2R-α-Toc = 2R, 4'-*ambo*, 8'-*ambo*-α-tocopherol, α-Toc = *all-rac*-α-tocopherol. a-TocA = *all-rac*-α-tocopheryl acetate

ND = Not detected

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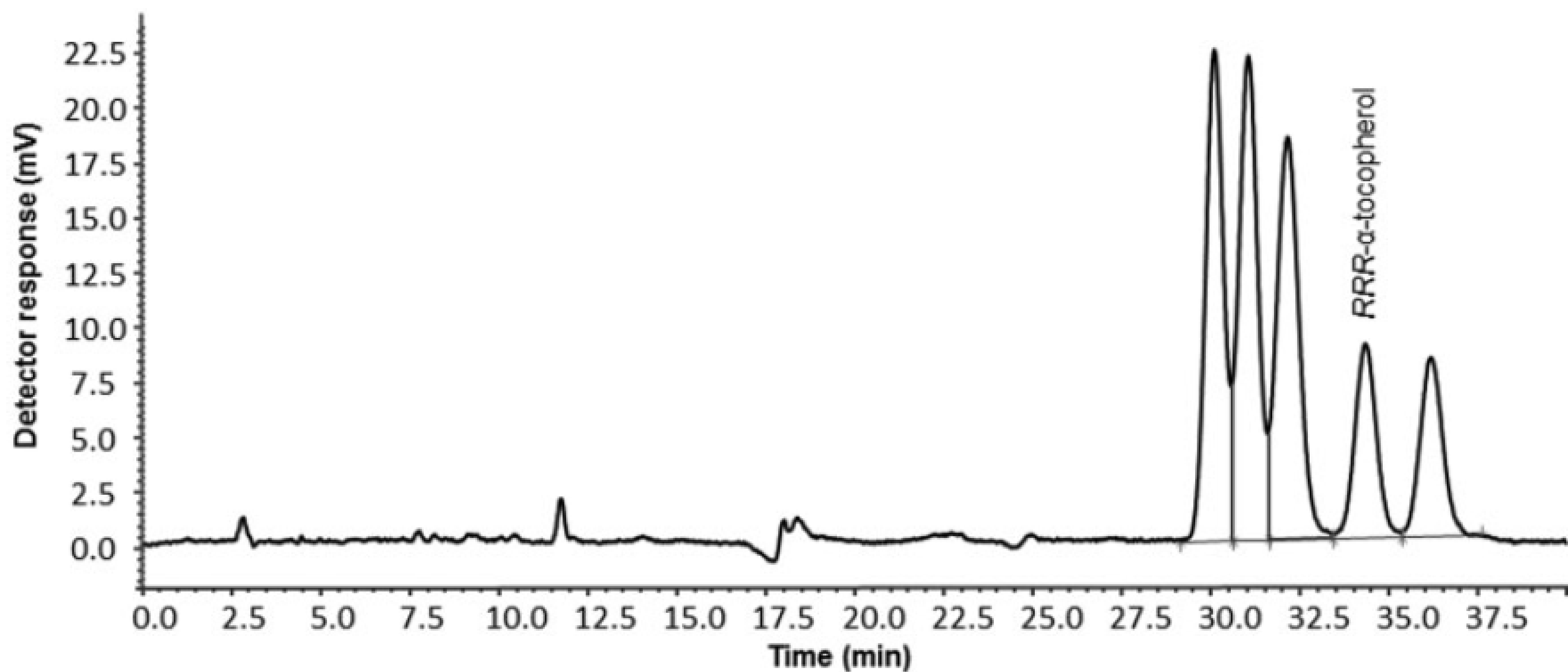

Figure 1. Chromatogram of *all-rac*-α-tocopherol standard (1 µg/mL); mobile phase = isooctane-ethanol-propan-2-ol (100:0.06:0.06, v/v/v), flow rate = 1.0 mL/min, fluorescence λ_{ex} = 292 nm, λ_{em} = 330 nm, column oven 50 °C.

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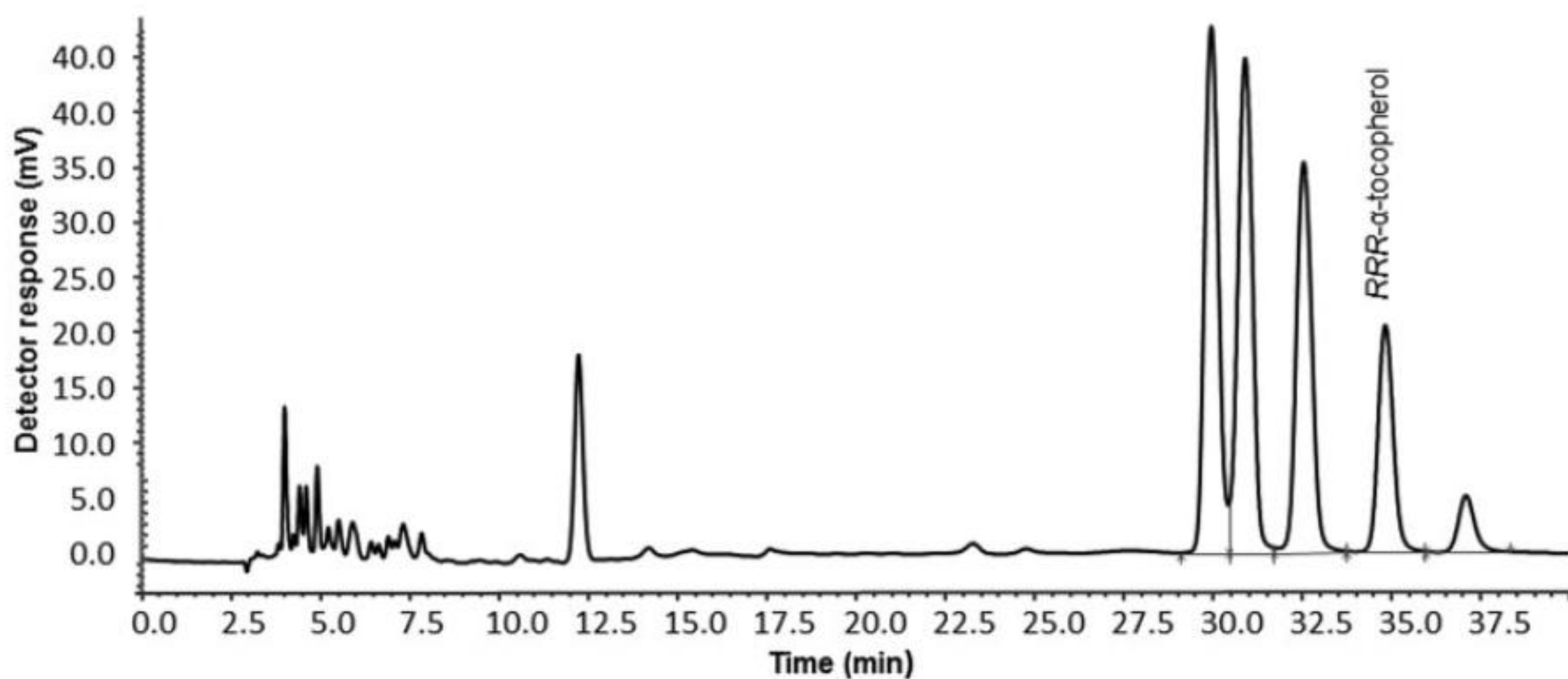

Figure 2. Chromatogram of infant formula sample; mobile phase = isooctane-ethanol-propan-2-ol (100:0.06:0.06, v/v/v), flow rate = 1.0 mL/min, fluorescence λ_{ex} = 292 nm, λ_{em} = 330 nm, column oven 50 °C.

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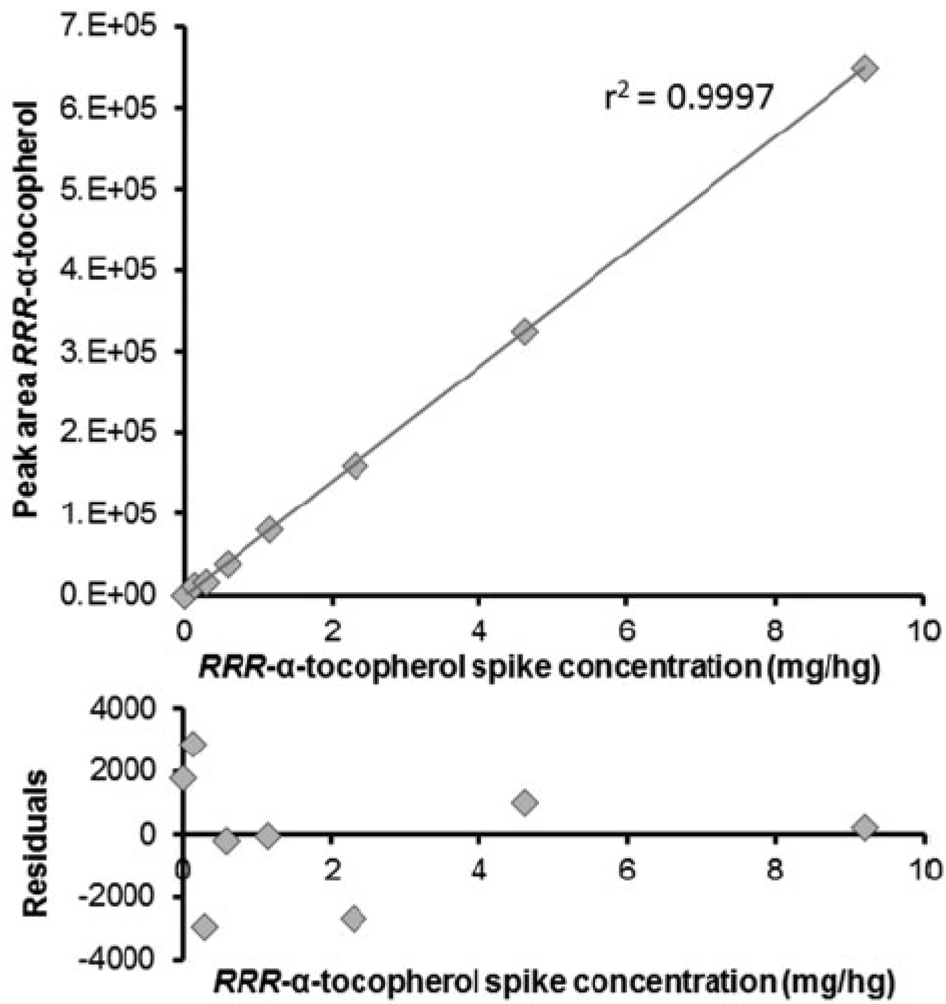

Figure 3. Linear regression and residual plots of detector response against RRR-α-tocopherol concentration in the spiked skim milk powder sample.

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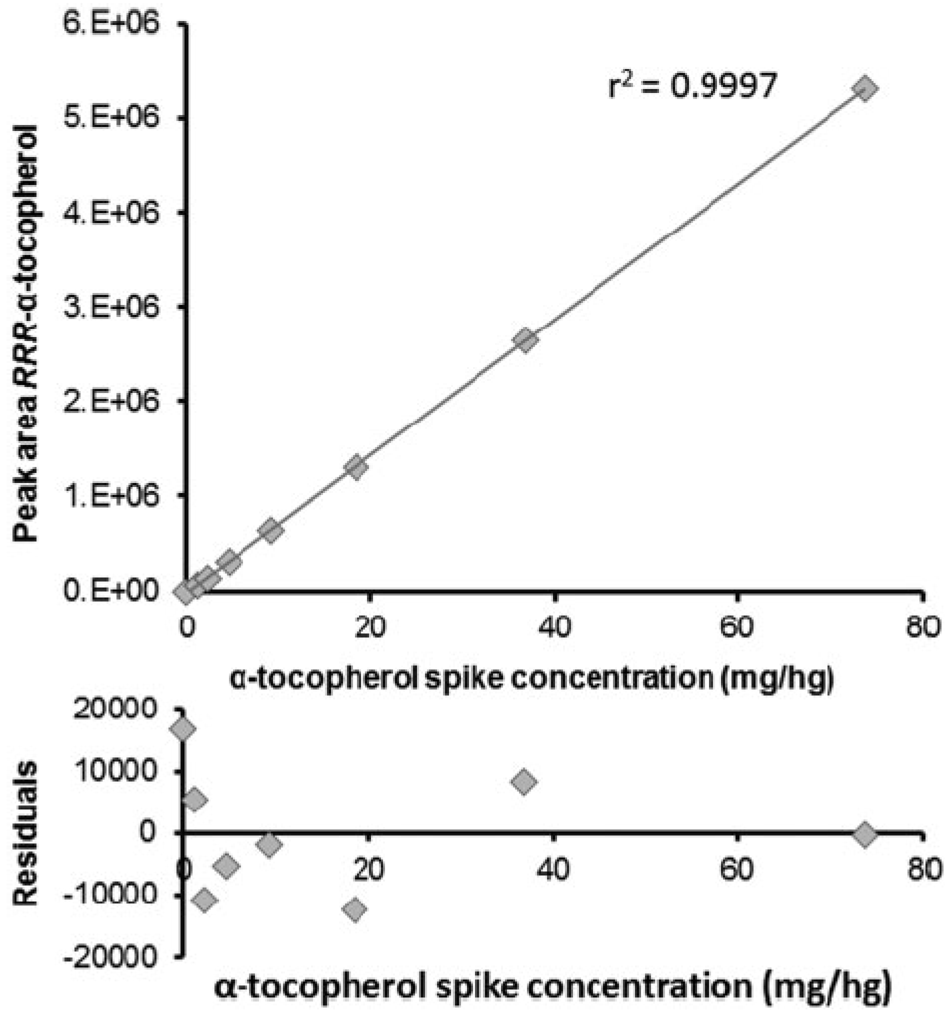

Figure 4. Linear regression and residual plots of detector response against α-tocopherol concentration in the spiked skim milk powder sample

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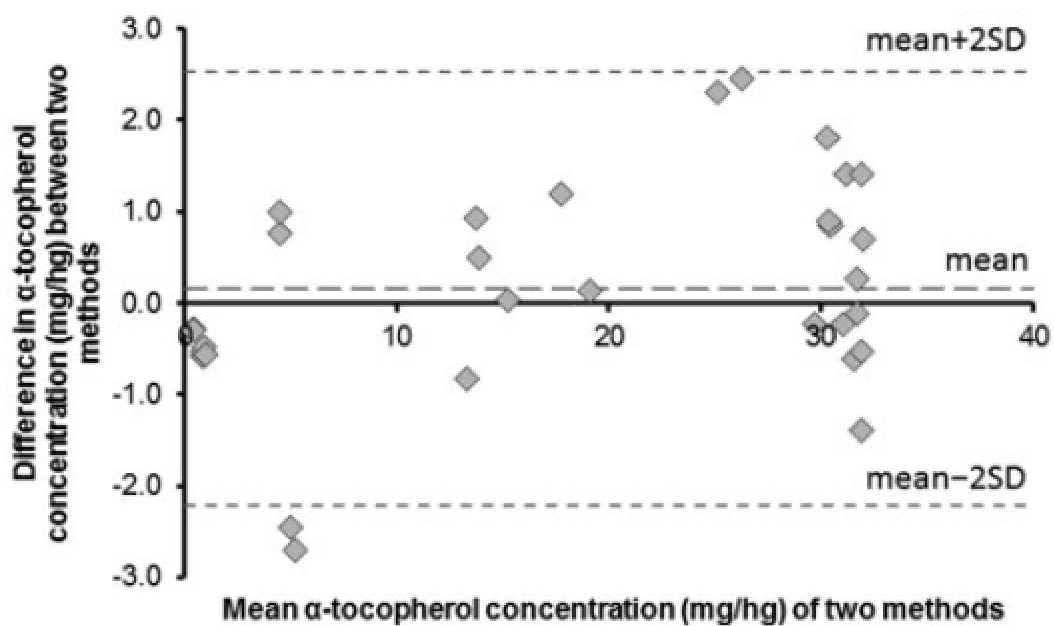

Figure 5. Bland-Altman plot for α-tocopherol, comparing the chiral chromatographic method with an HPLC-UV method (12)

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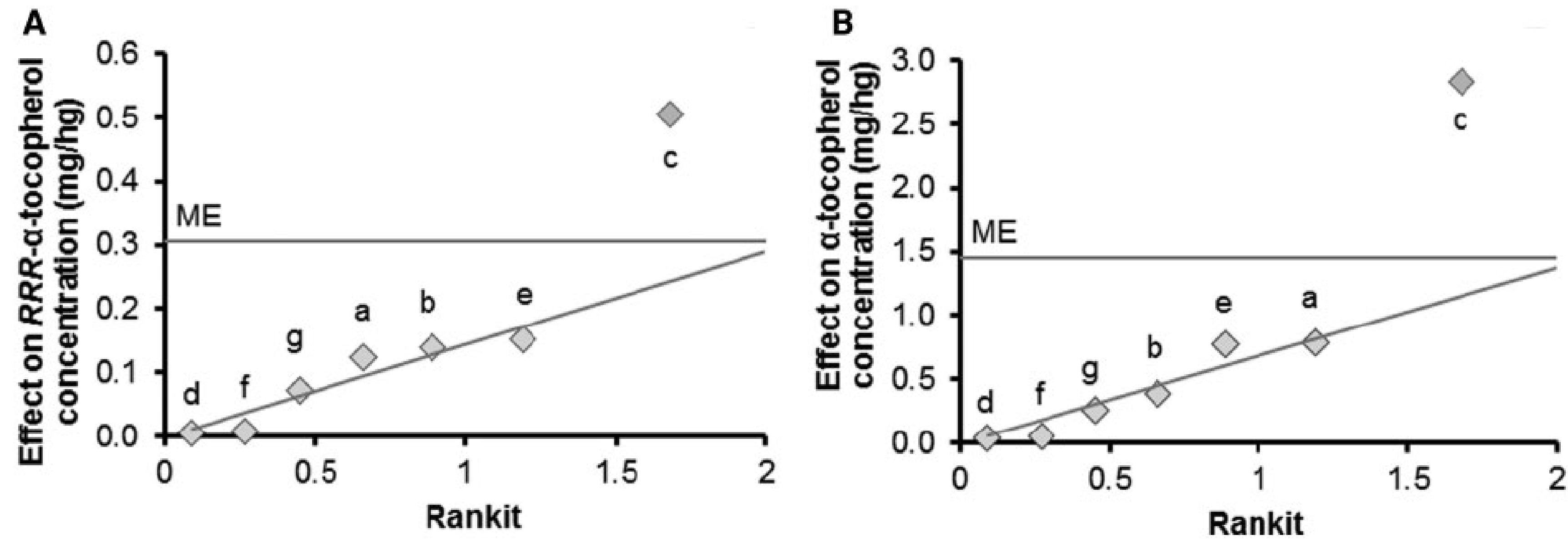

Figure 6.Half-normal plots for ruggedness trial of the measured concentrations of (A) = *RRR*-α-tocopherol (mg/hg) and (B) = α-tocopherol (mg/hg). a = sample weight (0.6, 0.4 g), b = potassium hydroxide solution volume (2.2, 1.8 mL), c = extraction solvent volume (21, 19 mL), d = shaker time (20, 10 min), e = centrifuge time (6, 4 min), f = vial mixer time (6, 4 min), g = a dummy factor, ME = margin of error