Separation of *RRR*-α-Tocopherol by Chiral Chromatography

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Abstract

Background: α-Tocopherol can exist as eight possible stereoisomers due to the presence of three chiral carbons. Regulations and industry guidelines necessitate that dietary vitamin E intakes be based on the vitamin E activity of *RRR*-α-tocopherol. Food products fortified with synthetic *all-rac*-α-tocopherol or *all-rac*-α-tocopheryl acetate during manufacturing will require chiral separation of the α-tocopherol stereoisomers for accurate estimation of vitamin E activity. *Objective:* The development of an HPLC method utilizing a chiral column for the chromatographic separation of *RRR*-α-tocopherol from other α-tocopherol stereoisomers. *Method:* Normal phase liquid chromatographic separation using a polysaccharide-based chiral column with fluorescence detection of α-tocopherol stereoisomers. *Results:* The described chromatographic method achieves baseline resolution of *RRR*-α-tocopherol from its stereoisomers. Method selectivity, precision, and robustness were evaluated and acceptable performance was achieved. *Conclusions:* The chromatographic method was found to be suitable for application where both *RRR*-α-tocopherol content and total α-tocopherol content are required for routine compliance testing. *Highlights:* A robust and precise chromatographic method for the baseline resolution of *RRR*-α-tocopherol from its stereoisomers was achieved.

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Introduction

Vitamin E, the dominant mammalian *in vivo* lipid-soluble antioxidant, is composed of a group of tocopherol and tocotrienol compounds that are derivatives of a common six-chromanol moiety and that differ in the number and position of methyl groups on the ring structure. Each tocopherol can exist as eight possible stereoisomers due to the presence of three chiral carbons (Figure 1), whereas the tocotrienols have a single chiral carbon. The naturally occurring forms of vitamin E consist of four tocopherols (*RRR*-α-, *RRR*-β-, *RRR*-γ-, and *RRR*-δ-tocopherol) with a saturated phytyl side chain, whereas the four related tocotrienols (*R*-α-, *R*-β-, *R*-γ-, and *R*-δ-tocotrienol) have three non-conjugated double bonds in the isoprenoid side chain. In contrast to naturally occurring *RRR-α-tocopherol*, synthetic a-tocopherol consists of a mixture of eight possible stereoisomers (*RRR*, *RSR*, *RSS*, *RRS*, *SRR*, *SRS*, *SSR*, and *SSS*) in equal proportion and is designated as *all-rac*-α-tocopherol (1). Although natural and synthetic congeners have comparable bioavailability, the configuration of C-2 (*R* or *S*) is critical in determining *in vivo* biological activity, since the principal systemic α-tocopherol transport protein has significantly higher affinity for the 2*R* than the 2*S* stereoisomers, although the number and position of the chromanol ring methyl groups is also a factor (1–3).

Of the four naturally occurring tocopherol congeners, the most biologically active is α-tocopherol (α > β > γ > δ) and the activity of vitamin E is expressed relative specifically to the *RRR*-α-tocopherol stereoisomer. For dietary purposes, vitamin E activity is expressed either as International Units (IU) or as mg α-tocopherol equivalents (mg α-TE), where 1 mg α-TE is the activity of 1 mg of *RRR*-α-tocopherol.

As the human diet is generally plentiful with respect to vitamin E, clinical deficiency is rare in adult humans. However, primarily because of poor oxidative stability, vitamin E is selectively fortified to certain foods such as infant formula in the form of a synthetic racemic ester derivative, most commonly as *all-rac*-α-tocopheryl acetate. Pediatric infant formula will contain additional tocopherol congeners derived from both vegetable oil blends and bovine milk fat incorporated during manufacture. Regulations (4) and industry guidelines (5) necessitate that calculation of dietary vitamin E intakes be based on the vitamin E activity of *RRR*-α-tocopherol. It is apparent that, if synthetic *all-rac*-α-tocopherol or its acetate is used during manufacture, a compliant estimation of vitamin E content will be a significant analytical challenge, requiring chiral separation of the □-tocopherol stereoisomers for discrimination of biologically active forms (2, 6).

The few reports of the chiral chromatographic separation of α-tocopherol stereoisomers are predominantly based on the derivatized methyl ethers, as recently reviewed (2, 6). Although derivatization provides certain resolution advantages, it has disadvantages of additional

manipulations prior to separation, the possibility of incomplete derivatization and increased test cost, and hence, for routine compliance testing, non-derivatization methods are preferred. Provided that *RRR*-α-tocopherol is chromatographically resolved from the other stereoisomers derived from supplemental synthetic α-tocopherol, complete separation of all eight stereoisomers is unnecessary. Such a chiral method is therefore able to distinguish between natural and synthetic sources of α-tocopherol (4).

RRR-α-tocopherol has previously been separated without the use of derivatization from 2-*ambo*-4'*R*, 8'*R*-α-tocopherol (7), or partially separated as 2*R*, 4'-*ambo*, 8'-*ambo*-α-tocopherol (8). However, to the best of our knowledge, no publication describes the separation of underivatized *RRR*-α-tocopherol from *all-rac*-α-tocopherol.

This study therefore describes an HPLC method utilizing a chiral column for the unambiguous chromatographic separation of underivatized *RRR*-α-tocopherol from its stereoisomers that is intended to have application to the compliance testing of vitamin E-supplemented foods, including infant formula.

Method

Apparatus

A Prominence HPLC system (Shimadzu, Kyoto, Japan) consisting of a CBM-20A control module, incorporating a Lux Cellulose-1 (250 × 4.6 mm, 3 µm) chiral column (Phenomenex, Torrance, CA), an RF-20Axs fluorescence detector, a SPD-M20A photodiode array detector, a DGU-20A5R degasser unit, two LC-20AT pumps, a SIL-20AC autosampler, and a CTO-20AC column oven was used. Lab Solutions software version 5.73 (Shimadzu)was used for instrument control and data processing.

Reagents

HPLC-grade ethanol, methanol, butanol, propan-2-ol, and hexane were supplied by Merck (Darmstadt, Germany) and HPLC grade 2,2,4-trimethylpentane (isooctane) was supplied by Thermo Fisher Scientific (Waltham, MA). *RRR*-γ-tocopherol and *RRR*-δ-tocopherol were supplied by Merck, and *all-rac*-α-tocopherol was supplied by Sigma-Aldrich (St. Louis, MO). A mixed tocopherol and tocotrienol standard, *RRR*-α-tocopherol, and *RRR*-α-tocopherol were supplied by Chromadex (Los Angeles, CA).

Standards

Stock standard solutions were prepared by dissolving approximately 50 mg of each tocopherol in 25 mL of ethanol, transferring 2.5 mL of each solution into separate 50 mL volumetric flasks, and diluting to volume with ethanol. The concentrations of these stock solutions were determined by measurement of the UV absorbance at appropriate wavelengths and calculated against published extinction coefficients (1).

Chromatographic Conditions

A standard solution of *all-rac*-α-tocopherol (1 µg/mL) was injected at a flow rate of 1.0 mL/min with fluorescence detection (λ_{ex} = 292 nm, λ_{em} = 330 nm). In order to optimize resolution, various mobile phase compositions, column temperatures, and injection volumes were evaluated. Optimal conditions were achieved with a mobile phase of isooctane: ethanol: propan-2-ol (100:0.06:0.06 v/v/v), and a column temperature of 50 °C.

Method Selectivity

Chromatographic selectivity was further evaluated with (1) a mixed tocopherol and tocotrienol standard, and (2) whole milk powder and infant formula sample extracts with dual UV (200–330 nm) and fluorescence detection.

Method Precision

Replicate injections (n = 15) of *all-rac*-α-tocopherol (1 µg/mL prepared in isooctane) were performed over 5 days with the mobile phase prepared fresh each day.

Method Robustness

The robustness of the chromatographic method was assessed by conducting a seven-factor Plackett-Burman trial (9, 10), with evaluation of the factors that were deemed to potentially influence chromatographic performance, at levels likely to occur during routine method application. A combination of eight mobile phases and method configurations was used with the following factors: environmental temperature (16 and 24 °C), mobile phase moisture (“wet” and “dry”), polar modifier (0.11 and 0.13%), flow rate (0.95 and 1.05 mL/min), injection volume (25 and 50 µL), column oven temperature (48 and 52 °C), and a dummy factor. An *all-rac*-α-tocopherol standard (1 µg/mL) was analyzed under these eight conditions and chromatographic parameters calculated.

The mobile phase reservoir was placed in temperature controlled water baths at 16 and 24 °C as a proxy for environmental temperature. "Wet" mobile phase was made by adding equal volumes of water and isooctane to a separating funnel; after mixing thoroughly and allowing the phases to separate, the stopper was opened and excess water was removed. "Dry" mobile phase was made by adding anhydrous sodium sulfate to isooctane, which was then decanted into a separating funnel, filtered drop-wise through anhydrous sodium sulfate, and then filtered and stored over a molecular sieve.

Critical effects were identified by statistical analysis using a *t*-test, whereby a calculated *t*-value based on the effect, and an estimation of the standard error, were compared with a critical value ($\alpha = 0.05$). Graphical interpretation was achieved by constructing a half-normal plot; non-significant effects typically fall on a straight line through zero, whereas significant effects deviate from the straight line. The standard error estimate was used to calculate the margin of error (ME), which was plotted on the half-normal plot to identify the limit above which effects were deemed to be significant (11, 12).

Results and Discussion

Method Development

Preliminary analyses of *all-rac*-α-tocopherol on the Lux Cellulose-1 column using hexane: propan-2-ol mobile phases indicated that propan-2-ol concentrations of < 0.25% v/v and temperatures of > 35 °C were necessary to achieve nominal separation of some of the a-tocopherol stereoisomers. The repeatability of the chromatographic performance with respect to resolution and retention was sensitive to both the column temperature and the low polar modifier concentration in the mobile phase. Therefore, to maintain a stable temperature throughout the HPLC system during routine use, a number of precautions were taken: a pre-mixer with the volume set at 2.6 mL was mounted inside the column oven; a pre-column warming coil (PEEK; 152.4 × 0.0127 cm id) was added to the column; the length of tubing outside of the instrument modules was minimized and sheathed in a tube of flexible PVC hose.

Chromatographic parameters obtained for the resolution of *RRR*-α-tocopherol from its stereoisomers under different conditions are summarized in Table 1 and illustrate performance improvements when hexane was substituted with isooctane. The use of propan-2-ol as polar modifier gave increased resolution of the *RRR*-α-tocopherol peak, whereas the use of ethanol significantly reduced overall retention time. The optimal mobile phase of isooctane–ethanol–propan-2-ol (100:0.06:0.06 v/v/v) was chosen to accommodate the positive attributes of both ethanol and propan-2-ol. Column temperature is a significant variable that influences both retention time and resolution, by affecting

the eluent viscosity and the kinetics of the retention mechanism. Typical chromatograms of an *all-rac*-α-tocopherol standard and infant formula sample obtained under optimized conditions are illustrated in Figures 2 and 3. Baseline separation of the *RRR*-α-tocopherol stereoisomer is achieved with partial separation of the other α-tocopherol stereoisomers, with identity confirmed by retention time and predicted peak area ratios of 2:2:2:1:1.

The column used in this study exploits a modified polysaccharide (cellulose tris-3,5-dimethylphenylcarbamate) chiral selector coated to underlying silica and, consistent with most chiral separations reported, is used under normal phase conditions with an isocratic binary non-polar hydrocarbon and alcohol modifier as eluent (6). Variability in retention time in conventional normal phase HPLC is due to the strong dependence of the retention on the content of highly polar mobile phase constituents, water in particular (13). Even when water is not deliberately added to the mobile phase, trace quantities will always be present, even in non-polar solvents. Additionally, equilibration of a generic normal phase column can be lengthy because of the hygroscopic nature of silica. However, in contrast, performance stability of the modified cellulose column was achieved after 10 column volumes of the optimized mobile phase.

Method Selectivity

Many food products, particularly those with added vegetable oils, will contain significant amounts of the other tocopherol congeners (*RRR*-β-, *RRR*-γ-, and *RRR*-δ-tocopherol), and to a lesser extent, tocotrienol congeners. The analysis of a mixed tocopherol and tocotrienol standard under the described conditions confirmed that these other congeners eluted after a-tocopherol, therefore eliminating these as possible chromatographic interferences.

An evaluation of a 3D contour plot of UV absorbance versus wavelength versus retention time for whole milk powder and infant formula extracts showed no evidence of interfering peaks eluting at the retention time of the a-tocopherol stereoisomers, and the UV spectrum of the a-tocopherol stereoisomer peaks showed no qualitative difference when compared to the UV spectrum of an α-tocopherol standard, demonstrating the selectivity of the chromatographic separation and detection mode for these matrices.

Method Precision

Replicate injections of the *all-rac*-α-tocopherol standard over 5 days demonstrated acceptable precision estimates for retention time (mean, 33.8 min; RSD_{iR}, 3.3%), capacity factor (mean, 10.7;

RSD_{iR}, 3.7%), resolution of *RRR*-α-tocopherol peak with preceding peak (mean, 1.8; RSD_{iR}, 5.6%), and resolution of trailing peak with *RRR*-α-tocopherol peak (mean, 1.6; RSD_{iR}, 6.3%).

Method Robustness

The chromatographic parameters obtained for the resolved *RRR*-α-tocopherol peak from the eight experiments using combinations of the seven factors are presented in Table 2. The derived half-normal plots are presented in Figure 4 and graphically distinguish significant effects from insignificant effects for each parameter, with the ME value establishing the decision criteria (11).

Unsurprisingly, the retention time of *RRR*-α-tocopherol a-tocopherol was considerably influenced by the flow rate, the column oven temperature, and the concentration of polar modifier. Most importantly, changes in the percentage of polar modifier had minimal effect on the resolution of the *RRR*-α-tocopherol peak, confirming the robustness of the described chiral chromatographic method. Although the column temperature did significantly influence the resolution between the *RRR*-α-tocopherol peak and the trailing peak, this factor is easily controlled by using a column oven with a precision of ± 0.1 °C.

There was no evidence that moisture in the mobile phase affected either the retention or the selectivity of the *RRR*-α-tocopherol peak. Although the variation in the column temperature influenced theoretical plate number the most, it was not statistically significant. Other parameters, such as environmental temperature and injection volume, did not affect either the retention or the selectivity of the *RRR*-α-tocopherol peak

Conclusions

This study illustrates the successful application of a polysaccharide-based chiral column in normal phase mode with fluorescence detection for the separation of underivatized, biologically active *RRR*-α-tocopherol from other α-tocopherol stereoisomers. Although resolution of the 2*S* stereoisomers of α-tocopherol is not as efficient as for the methylated derivative, the unequivocal resolution of non-methylated *RRR*-α-tocopherol will facilitate the development of enhanced methods for the nutritional analysis of vitamin E-supplemented foods.

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Table 1. Chromatographic parameters of the separation of *all-rac*-α-tocopherol on the Lux Cellulose-1 column

Mobile phase	T, °C[a]	Tr, min[b]	k[c]	Rs(P)[d]	Rs(T)[e]
hexane–butan-1-ol	40	35.0	11.0	1.4	1.2
(99.875:0.125)	50	27.7	8.5	1.4	1.0
hexane–propan-2-ol	40	34.8	11.0	1.4	1.3
(99.875:0.125)	50	27.3	8.4	1.2	1.1
hexane–ethanol	40	28.6	8.8	NR[f]	1.6
(99.875:0.125)	50	24.3	7.4	0.7	1.3
isooctane–butan-1-ol	40	44.2	14.2	2.0	1.0
(99.875:0.125)	50	34.9	11.0	1.3	1.1
isooctane–propan-2-ol	40	47.2	15.2	1.8	1.5
(99.875:0.125)	50	34.3	10.8	1.9	1.6
isooctane–ethanol	40	35.4	11.2	1.4	1.4
(99.875:0.125)	50	29.7	9.2	1.6	1.3

[a] T = Temperature

[b] Tr = Retention time

[c] k = Capacity factor

[d] Rs(P) = Resolution of *RRR*-α-tocopherol peak with preceding peak

[e] Rs(T) = Resolution of trailing peak with *RRR*-α-tocopherol peak

[f] NR = Not resolved

Table 2. Results obtained from eight runs performed for robustness evaluation

Experiment	Tr, min[a]	N[b]	Rs(P)[c]	Rs(T)[d]
X1	28.5	15934	1.81	1.51
X2	33.8	15729	1.92	1.32
X3	34.6	15783	1.88	1.45
X4	33.8	16732	1.93	1.55
X5	30.8	17286	1.80	1.65
X6	37.5	15920	1.91	1.43
X7	31.4	15173	1.88	1.44
X8	30.7	15911	1.88	1.59

[a] Tr = Retention time

[b] N = Theoretical plate number

[c] Rs(P) = Resolution of *RRR*-α-tocopherol peak with preceding peak

[d] Rs(T) = Resolution of trailing peak with *RRR*-α-tocopherol peak

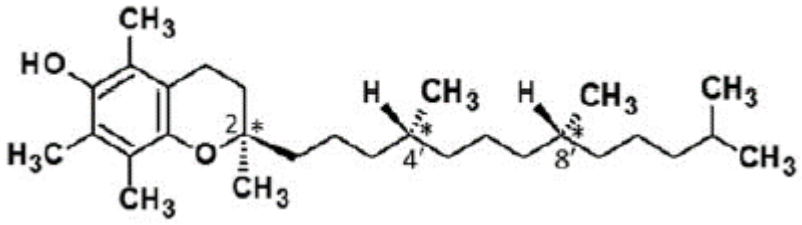

Figure 1. Structure of *RRR*-α-tocopherol (chiral centers marked with * located on carbons 2, 4' , and 8')

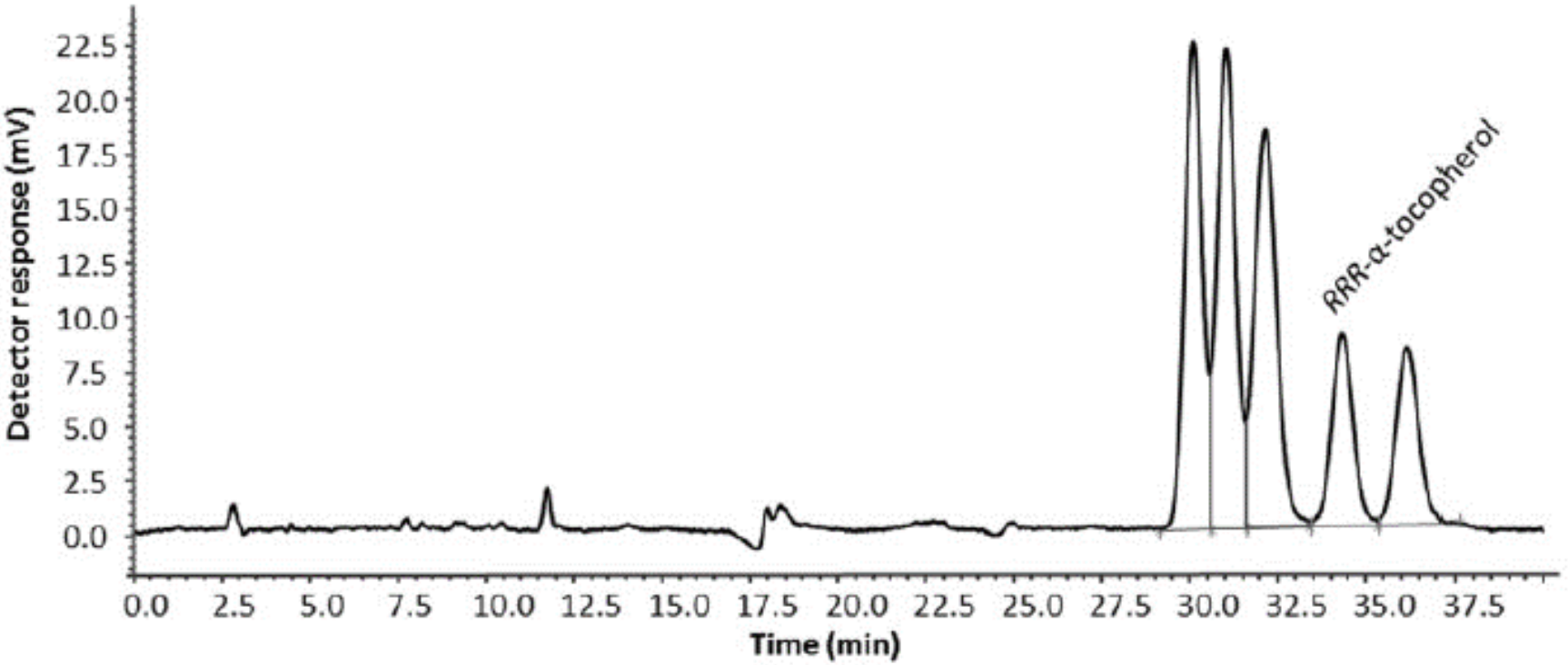

Figure 2. Chromatogram of *all-rac*-α-tocopherol standard (1 µg/mL) illustrating baseline separation of *RRR*-α-tocopherol from the seven other stereoisomers (peak area ratio = 2:2:2:1:1, 0 dec.pl.); mobile phase = isooctane–ethanol–propan-2-ol (100:0.06:0.06, v/v/v), flow rate = 1.0 mL/min, fluorescence λ_{ex} = 292 nm, λ_{em} = 330 nm, column oven 50 °C

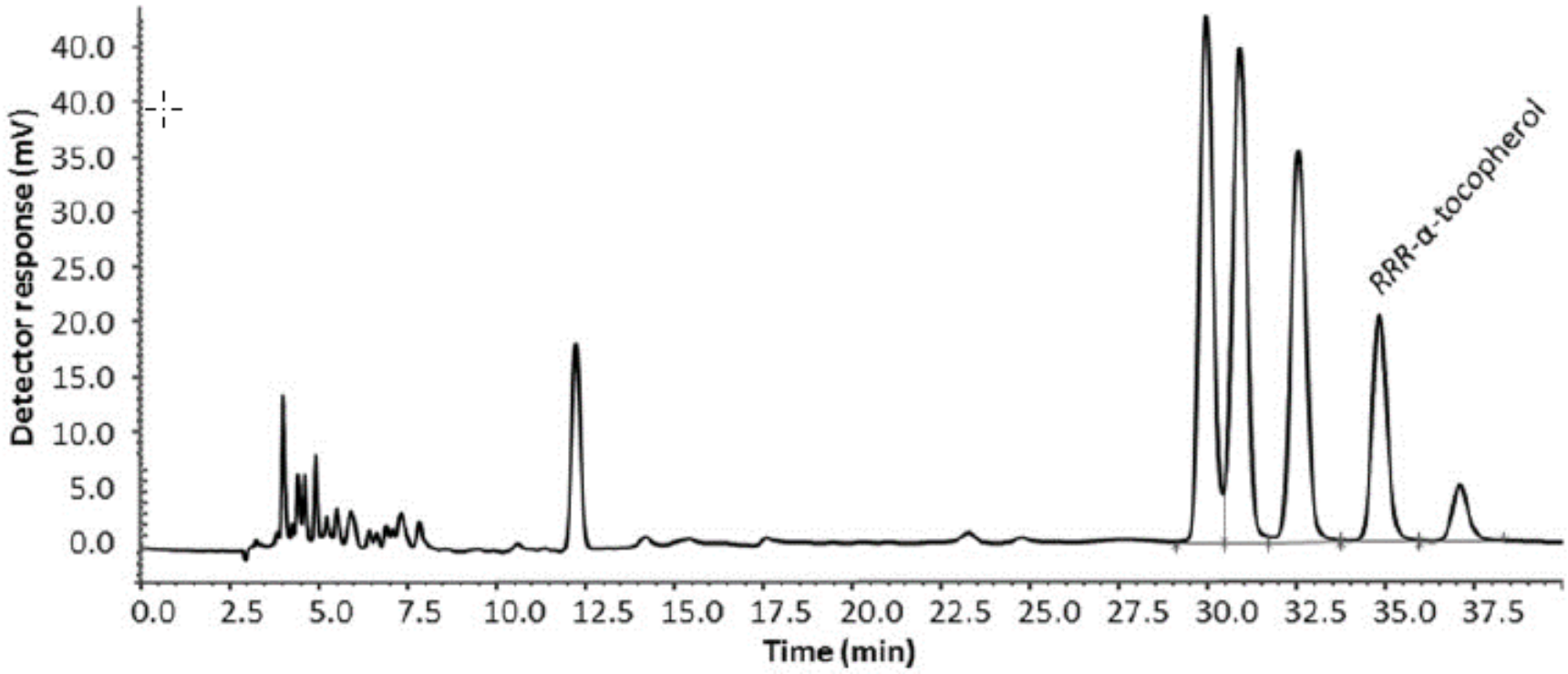

Figure 3. Chromatogram of infant formula sample illustrating baseline separation of *RRR*-α-tocopherol from the seven other stereoisomers; mobile phase = isooctane–ethanol–propan-2-ol (100:0.06:0.06, v/v/v), flow rate = 1.0 mL/min, fluorescence λ_{ex} = 292 nm, λ_{em} = 330 nm, column oven 50 °C

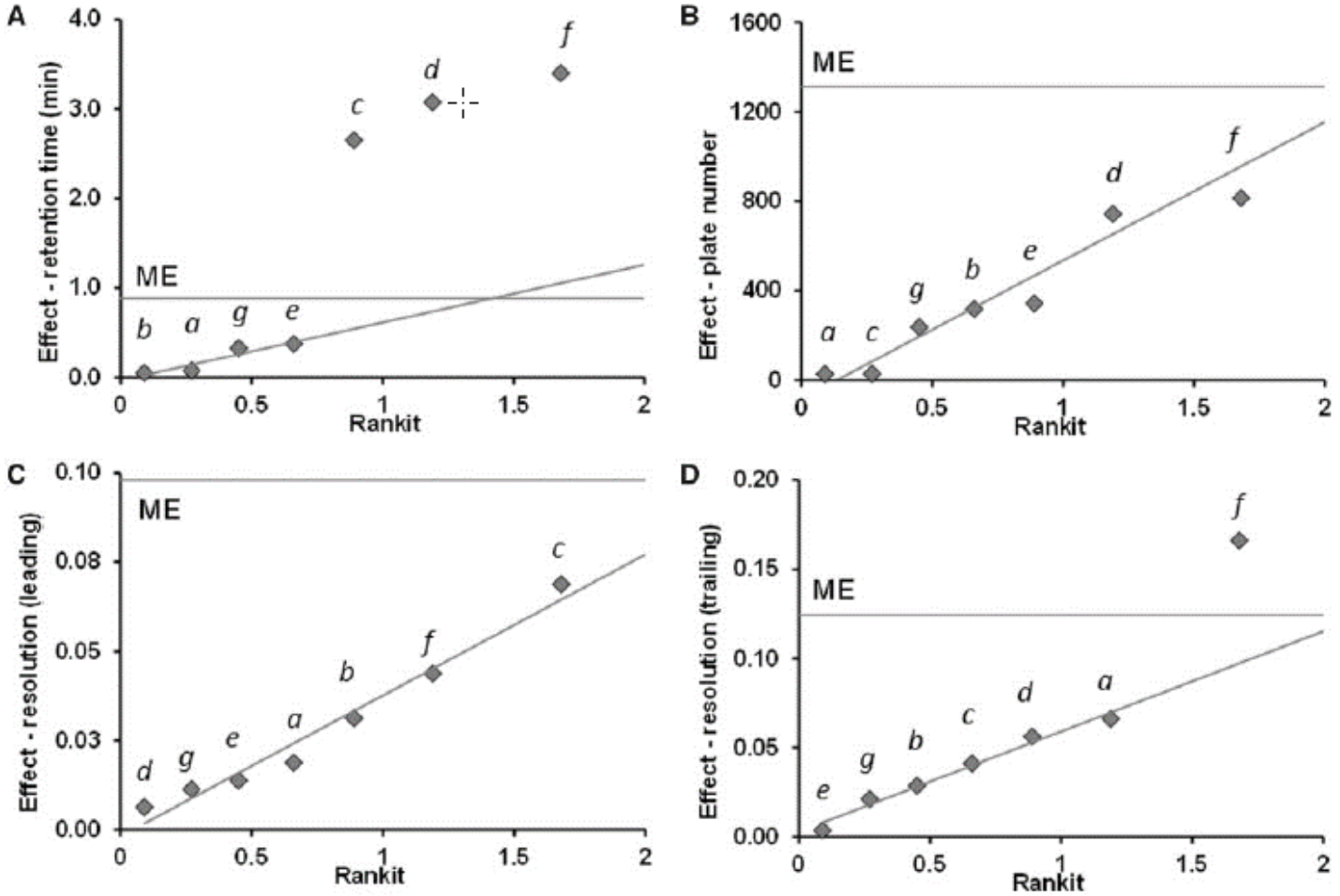

Figure 4. Half-normal plots for chromatographic robustness: (A) retention time, (B) theoretical plate number, (C) resolution of *RRR*-α-tocopherol peak with preceding peak, (D) resolution of trailing peak with *RRR*-α-tocopherol peak; a = environmental temperature, b = mobile phase moisture, c = polar modifier, d = flow rate, e = injection volume, f = column oven temperature, g = dummy factor, ME = margin of error