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Research Article

Rapid Separation of All Four Tocopherol Homologues in Selected Fruit Seeds via Supercritical Fluid Chromatography Using a Solid-Core C18 Column

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Tocopherol separations employing the same Kinetex™ C18 column via supercritical fluid chromatography (SFC) and reversed-phase liquid chromatography (RP-LC) were compared. The application of the SFC system with UV diode array detection (DAD) resulted in rapid separation of all four tocopherol homologues with a total analysis time below 2 min. The RP-LC approach could not separate the isomers β and γ. The developed SFC-DAD method was precise, accurate, and most importantly more environmentally friendlier compared to the RP-LC method due to the 125-fold decrease in methanol consumption. The present study illustrated the selectivity differences between LC and SFC and how the C18 column can be used for tocopherol characterization. The optimized SFC method was successfully applied for the tocopherol determination in the seeds of nine different fruit species.

1. Introduction

Tocopherols (Ts), four homologues (α, β, γ, and δ), are biomolecules with a lipophilic nature of great biological importance due to the unique physicochemical properties, among others, antioxidant properties, and vitamin E activity [1]. The presence of vitamin E in a daily diet is essential for the proper function of physiological human systems such as vascular, neural, reproductive, and musculoskeletal [2]. In the year 2005, the recommended daily allowance (RDA) of vitamin E for adult women and men has been raised from at 8 and 10 mg, respectively, to 15 mg for both [3]. One of the richest sources of tocopherols is the conventional, as well as unconventional, seeds and their oils [4–6]. In recent years, unconventional seeds resources, for instance, recovered from by-products of the fruit industry have received greater attention [5, 6]. Since the profile and concentration of tocopherols in the plant material depends on many factors, for instance, genotype and species [7], the routine analysis of tocopherols composition in the samples is required.

The liquid chromatography (LC), including both reversed phase (RP) and normal phase (NP), is the most common technique for tocopherols determination. The RP-LC is favored over the NP-LC because of some advantages such as column stability and/or reproducibility of retention times. Nowadays, by using both NP-LC and RP-LC and an appropriate column such as silica (Si), diol (Diol), and amino (NH_3) for NP-LC [8] and pentafluorophenyl (PFP or F5), C30, naphthalene (πNAP), and planar pyrene (5PYE) for RP-LC [9–13], which allows for isomers β and γ separation, all tocopherols can be determined. Tocopherol homologues are often determined by RP-LC with a C18 column to obtain rapid separation. Unfortunately, such an approach has one major disadvantage, it does not allow for the β and γ isomers separation [14, 15]. With the exception of the one report, a successful separation of all tocopherol

and tocotrienol homologues was obtained by applying the PerfectSil Target ODS-3 column (modified C18 phase); however, longer analysis times were achieved (60 min) [16]; generally, the C18 column used in RP-LC does not allow for β and γ separation. Therefore, when the tocopherols are determined by RP-LC with a C18 column, the two isomers (β and γ) coelute and are represented as a sum of both forms because of the same retention time. Unfortunately, the results are frequently interpreted as the γ-T because of a common occurrence and high concentration of this homologue in the plant material as opposed to β-T [17]. Nevertheless, such interpretation of the results, when the presence of β-T cannot be excluded in the tested sample, is incorrect. Recent studies show simultaneously that the rareness of β homologue occurrence in the plant world may be underestimated, mainly due to improper methodology [18]. The aspect of the separation of all tocopherol homologues is particularly important when the plant material is tested for the first time.

Although supercritical fluid chromatography (SFC) with supercritical carbon dioxide (CO_2) was discovered earlier than LC, it has been abandoned for many years, because of poor precision and reproducibility of this analytical technique. In the last decade, there has been a breakthrough for the SFC system which has enabled to obtain comparable precision and reproducibility as in the case of the LC system. Currently, the SFC provides a meaningful advantage on the LC due to uses low viscosity a CO_2 as the main mobile phase, which allows higher operational flow rates and rapid analysis as compared with LC. Additionally, the application of the CO_2 makes the SFC an environment-friendly method [19–21]. The improvement of SFC instrumentation has been demonstrated with recent studies showcasing the benefits of SFC versus HPLC, as well as their differences in selectivity behaviour [22–24]. In the present study, the selectivity between the two analytical systems the HPLC and the SFC, employing the same C18 column for the separation of tocopherol homologues were compared, and finally, the method was validated on the SFC. The applicability of the new SFC method for qualitative and quantitative identification of tocopherol homologues was evaluated based on analysis of the seeds of nine different fruit species.

2. Materials and Methods

2.1. Reagents. Carbon dioxide (99.8% purity) was purchased from AGA (Riga, Latvia). Methanol, ethanol, 2-propanol, *n*-hexane (MS and HPLC grade), *n*-hexane, ethyl acetate, absolute ethanol, sodium chloride, pyrogallol, and potassium hydroxide (reagent grade) were obtained from Sigma-Aldrich (Steinheim, Germany). Tocopherol homologues (α, β, γ, and δ) (>95% purity) were received from Merck (Darmstadt, Germany).

2.2. Plant Material. Nine different fruit species (fully ripe): red raspberry (*Rubus idaeus* L.), redcurrant (*Ribes rubrum* L.), blackcurrant (*Ribes nigrum* L.), strawberry (*Fragaria* × *ananassa* Duchesne), apple (*Malus domestica* Borkh.), rose hip (*Rosa canina* L.), sea buckthorn (*Hippophae rhamnoides* L.), and Japanese quince (*Chaenomeles japonica* (Thunb.) Lindl. ex Spach) were collected in the Institute of Horticulture, Dobele, Latvia (GPS location: N:56°36′39″ E: 23°17′50″), and the watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) was provided by a local supplier from Ukraine. The seeds were obtained from the fruits during the samples preparation for the analysis of fruit chemical composition. The recovered seeds were oven-dried (3 h) in Orakas 5600 (Marlemi OY, Lemi, Finland) with forced hot air circulation at 55 ± 2°C. The undamaged seeds were selected (~10 g) and milled in with a Knifetec™ 1095 (Foss, Höganäs, Sweden) universal laboratory mill to obtain a powder (mesh size ≤ 0.75 mm). Dry weight basis (dw) of seeds was measured gravimetrically.

2.3. Saponification and Extraction of Tocopherols. The procedure of sample saponification and tocopherols extraction was performed according to Górnaś et al. [25]. In brief, 0.1-0.2 g of powdered seeds, 2.5 mL of absolute ethanol, 0.05 g of pyrogallol, and 0.25 mL of aqueous potassium hydroxide (600 g/L) were placed in a glass tube, sequentially. The tube was closed immediately, mixed (10 sec) before and during the incubation. After 25 min of incubation at 80°C, the sample was rapidly cooled in an ice-water bath for 5 min and then 2.5 mL of sodium chloride (10 g/L) was added and mixed for 5 sec. Then, tocopherols were extracted with 2.5 mL of *n*-hexane:ethyl acetate (9 : 1; v/v) by mixing (15 sec). The organic layer was separated by the centrifugation (1000 × g, at 4°C, 5 min) and transferred to a round-bottom flask, while residues were reextracted twice as described above. The combined extracts were evaporated by a vacuum rotary evaporator till dryness, dissolved in methanol (0.5 mL), and filtrated through a syringe filter (0.22 μm) to a vial, sequentially. The samples were injected directly after preparation into the RP-LC and SFC system.

2.4. SFC and RP-LC Systems. The experiments were performed using Shimadzu Nexera UC system (Kyoto, Japan), which consists of a CBM-20A controller, online DGU-20A5R degasser, an LC-30AD SF CO_2 pump, an LC-30AD pump, an SIL-30AC autosampler (with 20 μL sample loop), a CTO-20AC column oven, an SPD M20A diode-array detector (DAD) (with high pressure cell), and one SFC-30A back pressure regulator (BPR). Additionally, a high-pressure switching six-port valve (FCV-34AH) was installed in the column oven to carry out column switching. All units are connected in the way allowing for using of both systems SFC and RP-LC without configuration changes and using the same column as well as the detection. Data collection and system control were performed using Shimadzu Lab solution DB Ver. 6.70.

2.5. Chromatographic Conditions of Tocopherols Determination by RP-LC. The analysis was performed in the following conditions: mobile phase methanol : water (100 : 0–95 : 5; v/v), flow rate (1.0 mL/min), injection (0.1–10 μL),

temperature of the column oven (25–50°C), and temperature of the room (22°C). The separation of tocopherols was performed on a Kinetex™ C18 column (2.6 μm, 4.6 × 100 mm) (Phenomenex, Torrance, CA, USA). Tocopherol homologues were measured at wavelength $\lambda = 295$ nm by the DAD. Identification was made by comparison of the retention times and UV absorption spectra of individual peaks in the chromatograms of analysed samples with these of the standards.

2.6. Chromatographic Conditions of Tocopherols Determination by SFC. The analysis was performed in the following conditions: mobile phase CO_2 : methanol (100: 0–93 : 7; v/v), flow rate (1.0–4.5 mL/min), temperature of the column oven (25–50°C), temperature of the room (22°C), injection (0.1–10 μL), and back pressure regulator 15 MPa. The detection, identification, and quantification were done as described above in the RP-LC system (Section 2.5.).

2.7. SFC Method Validation. The analytical method was validated in terms of selectivity, linearity, limit of detection (LOD), limits of quantification (LOQ), recovery, precision, and accuracy according to the guidelines for bioanalytical method validation of the Center for Drug Evaluation and Research of the U.S. Food and Drug Administration [26]. Spiked samples for method validation (at low, medium, and high concentrations of α-T (2.0, 19.8, and 198.2 μg), β-T (2.4, 24.3, and 243.3 μg), γ-T (2.5, 24.9, and 248.5 μg), and δ-T (2.3, 22.6, and 225.7 μg) were added to the apple seed samples before the saponification procedure.

2.7.1. Selectivity. To confirm the absence of interfering peaks or coeluting, blank and spiked seed samples of nine different fruit species (*R. idaeus*, *R. rubrum*, *R. nigrum*, *F.* × *ananassa*, *M. domestica*, *R. canina*, *H. rhamnoides*, *Ch. japonica*, and *C. lanatus*) were extracted and injected into the SFC system.

2.7.2. Linearity. The linearity of the detector response for standard solutions was tested on five consecutive days by injection of 1 μL of four tocopherol homologues at concentrations 1982.3 ng/μL for α-T, 2432.6 ng/μL for β-T, 2485.4 ng/μL for γ-T, and 2256.6 ng/μL for δ-T and their diluted equivalents in ethanol (75, 50, 25, 10, 7.5, 5, 2.5, and 1% w/w of nominal concentration).

2.7.3. Limits of Detection and Quantification. The limit of detection (LOD) was defined as the amount of the respective analyte injected into the SFC system that could be reliably discerned from the background noise (ca. 3 times the background signal). The limit of quantification (LOQ) was calculated as LOQ = 3LOD.

2.7.4. Recovery. The recovery of the tocopherols was quantified by analysing five independently prepared apple seeds samples and spiked with analytes at low, medium, and high levels (see Section 2.7.) and by comparison of the detector responses with those of standards containing identical concentrations of the tocopherols. The content of tocopherols in the apple seeds was determined at an earlier stage by the proposed method in this study and expressed as a mean value of five independent prepared and quantified samples.

2.7.5. Accuracy and Precision. Intra- and interday accuracy and precision were determined by analysing five independent prepared apple seeds samples that were spiked with low, medium, and high levels of tocopherol standards (Section 2.7.), on the same day and five independent days, respectively. Each of the five samples was running (injected) five times.

2.8. Statistical Analysis. The results were presented as means ± standard deviation ($n = 3$) from three independent replications. The p value ≤0.05 was used to denote significant differences between mean values determined by one-way analysis of variance (ANOVA). The Bonferroni post hoc test was used to denote statistically significant values at $p \leq 0.05$. All statistical analyses were performed with the assistance of Statistica 10.0 (StatSoft, Tulsa, OK, USA) software.

3. Results and Discussion

3.1. RP-LC-DAD vs SFC-DAD

3.1.1. Effect of the Mobile Phase. The advantage of SFC versus RP-LC for the separation of tocopherol homologues employing the Kinetex™ C18 column is illustrated in Figure 1. Four tocopherol homologues cannot be separated on the C18 column by the RP-LC, due to the lack of separation of isomers β and γ, and can by the SFC using CO_2 : methanol (99.8 : 0.2, v/v) as a mobile phase. Based on the peaks intensity, it is clear that the RP-LC method is more sensitive (about three-, four-, and six-fold for α-T, β-T + γ-T, and δ-T, respectively) compared with the SFC method (Figure 1). The lambda max (λ_{max}) of the UV spectra of tocopherol homologues obtained by the RP-LC with the methanol as a mobile phase was the lowest for α-T (292 nm) and the highest for δ-T (297 nm), whereas β-T and γ-T had the same value (296 nm). The application of the SFC with the CO_2 : MeOH (99.8 : 0.2, v/v) as the mobile phase resulted in noticeable changes in the lambda max (λ_{max}) of the UV spectra of tocopherol homologues (295 nm for α-T and β-T and 294 nm for γ-T and δ-T) (Figure 1). Increasing the concentration of the methanol in the mobile phase till 1% (CO_2 : MeOH (99.0 : 1.0, v/v)) resulted in unification of the wavelength $\lambda_{max} = 295$ nm. While at the levels above 7% of the methanol in the mobile phase of the SFC the λ_{max} wavelength of tocopherol homologues was as in 100% methanol. It must be highlighted that the tocopherols separation via SFC decreased methanol consumption by 125-fold compared with the RP-LC approach (Figure 1). Hence, SFC is significantly advantageous for developing environmentally friendlier chromatographic methods and decreasing costs associated to disposal of organic waste.

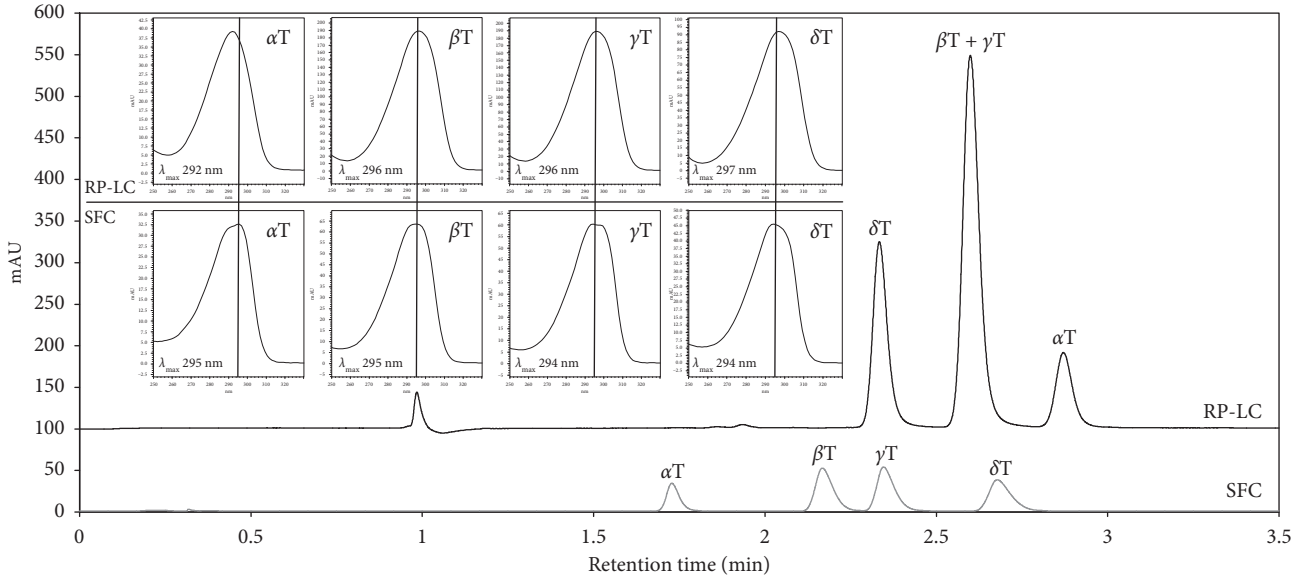

Figure 1: Chromatographic separation of four tocopherol homologues on the Kinetex™ C18 column by RP-LC and SFC. The concentration of tocopherol homologues injected in both systems was equal. The chromatographic conditions of the RP-LC method: mobile phase: MeOH; isocratic flow rate: 1 mL/min; column oven temperature: 25°C; room temperature: 22°C; injection volume: 1 μL. The chromatographic conditions of the SFC method—mobile phase: CO_2 : MeOH (99.8 : 0.2, v/v); isocratic flow rate: 4 mL/min; column oven temperature: 25°C; room temperature: 22°C; injection volume: 1 μL; back pressure regulator: 15 MPa.

3.1.2. Impact of the Mobile Phase Organic Modifier and the Column Temperature. The RP-LC approach employing 100% methanol was much more sensitive than the SFC method which contained only 0.2% methanol (Figure 1). The reduction of methanol content in the mobile phase from 100% to 95%, in the RP-LC, resulted in a significant decrease in sensitivity and increase in analysis time. Despite the increased resolution between the three peaks, the 5% decrease in the organic modifier failed to separate the coeluting peak containing both β and γ isomers. The sensitivity of the RP-LC method, for all tocopherol homologues, was negatively correlated with the increase content of water in the mobile phase as a methanol replacement (Figure 2).

In the case of the SFC system, the impact of methanol concentration in the mobile phase, especially on selectivity, was much more complex. With increased levels of methanol in the mobile phase, a higher sensitivity for all analytes was observed. The sensitivity of the SFC method, for all tocopherol homologues, was positively correlated with the increase content of methanol in the mobile phase. The rate of increasing sensitivity of individual tocopherol homologues, along with the increasing concentration of methanol in the mobile phase, was the highest for β-T and γ-T and the lowest for α-T. Therefore, changing the concentration of methanol in the mobile phase from 0.1 to 0.7% resulted in a nearly 2-fold higher sensitivity of forms β and γ in relation to α, while at the lowest concentration of the methanol (0.1%) the values of all homologues are comparable (Figure 3).

The selectivity of the SFC separation of the tocopherol homologues was extremely sensitive to minimal organic modifier changes to the mobile phase. The elution of homologue δ and α, especially δ, were effected the most by the small changes of the organic modifier in the mobile phase, while the β and γ were quite stable in the concentration range 0.1–1.0 of the methanol (Figure 3). Based on Figure 3, it can be stated that up until 2% organic modifier composition of methanol in the mobile phase, of the SFC system, the elution order is typical of NP, while at 3%, the elution order typical of RP-LC conditions.

For both systems RP-LC and SFC, the increase in temperature of the column oven decreased the analysis time and the increased sensitivity of the method. The resolution of the peaks decreased with increased temperature (data shown only for the SFC method, Figure 4).

3.1.3. Effect of the Injection Volume and Solvent Environment. Changing the sample's solvent environment between methanol, ethanol, 2-propanol, and *n*-hexane, in the injection volume range of 0.1–1 μL into the SFC system with CO_2:methanol (99.8 : 0.2, v/v) as mobile phase did not have a significant impact on the tocopherols separation nor the peaks shape (data not shown). An increased injection volume from 3 μL and greater experienced significant changes to the peak shape, decreased retention time, and fronting behaviour of the peaks, which is clearly illustrated by the 10 μL injection volume chromatogram in Figure 5.

The injection volume for the samples diluted in different solvent environments where peak distortion was quite significant are as follows and ranked in the order of worst peak shape: *n*-hexane 3 μL > 2-propanol 5 μL > ethanol 7 μL > methanol 10 μL (data shown only for methanol, Figure 5).

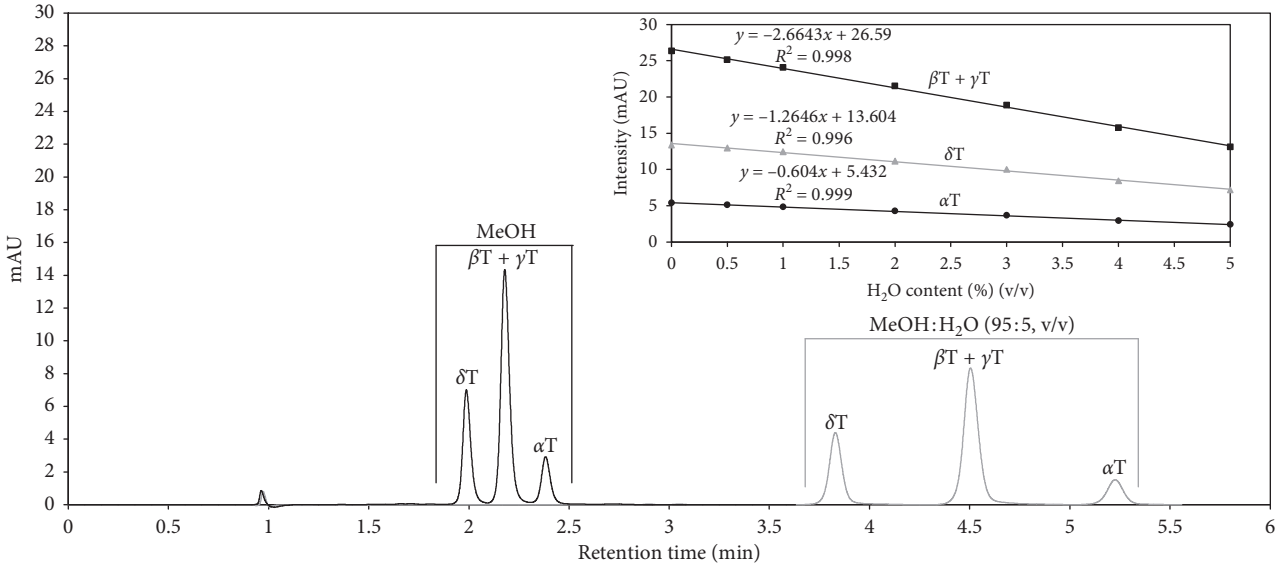

Figure 2: Impact of the methanol concentration in the mobile phase on the tocopherol homologues selectivity and sensitivity of the RP-LC-DAD method. The concentration of tocopherol homologues in each injection was equal. The chromatographic conditions: mobile phase: MeOH : H_2O (100 : 0–95 : 5, v/v); isocratic flow rate: 1 mL/min; column oven temperature: 40°C; room temperature: 22°C; injection volume: 1 μL.

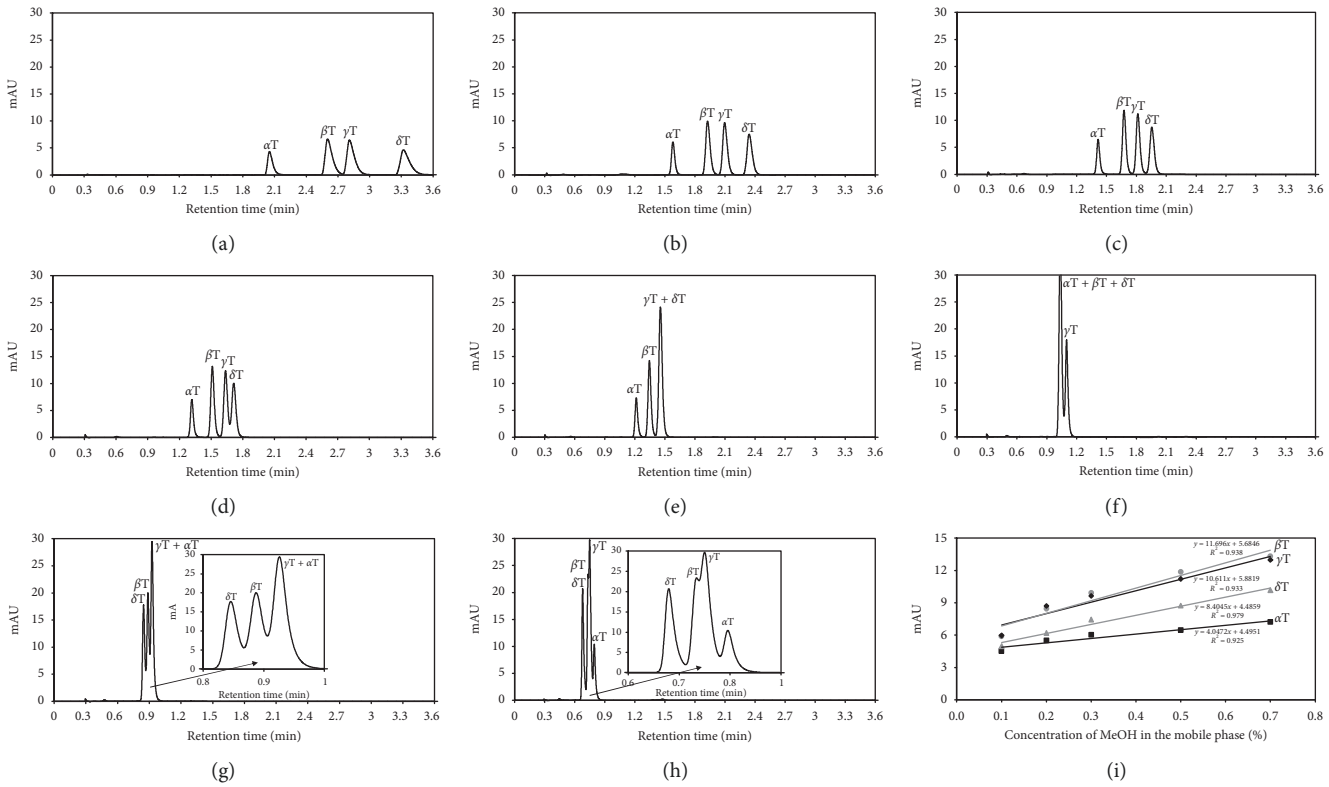

Figure 3: Impact of the methanol concentration in the mobile phase on the tocopherol homologues selectivity and sensitivity of the SFC-DAD method. The concentration of tocopherol homologues injected in each case was equal. The chromatographic conditions of the SFC method: isocratic flow rate: 4 mL/min; column oven temperature: 25°C; room temperature: 22°C; injection volume: 1 μL; back pressure regulator: 15 MPa; with different concentrations of methanol in the mobile phase CO_2 : MeOH (v/v): 99.9 : 0.1 (a), 99.7 : 0.3 (b), 99.5 : 0.5 (c), 99.3 : 0.7 (d), 99.0 : 1.0 (e), 98.0 : 2.0 (f), 97.0 : 3.0 (g), 95.0 : 5.0 (h), and the correlations between the sensitivity (mAU) of the tocopherol homologues and the content of methanol in the mobile phase (%) (i).

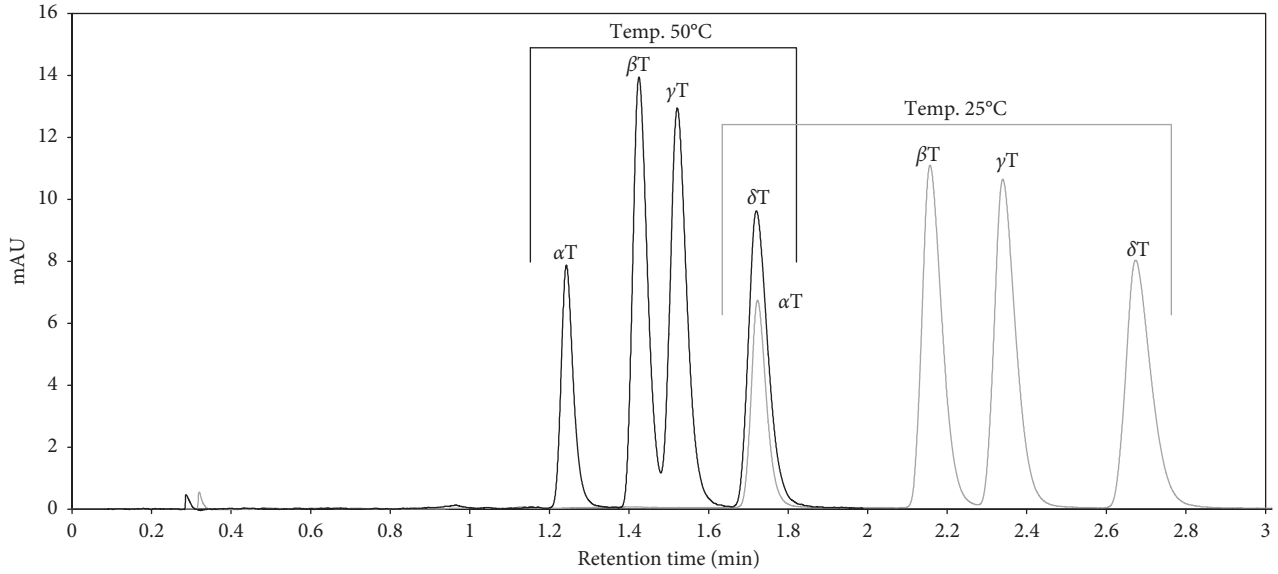

Figure 4: Impact of the column oven temperature (25 vs 50°C) on the tocopherol homologues selectivity and sensitivity of the SFC-DAD method. The concentration of tocopherol homologues injected in each case was equal. The chromatographic conditions of the SFC method: mobile phase: CO_2 : MeOH (99.8 : 0.2, v/v); isocratic flow rate: 4 mL/min; room temperature: 22°C; injection volume: 1 μL; back pressure regulator: 15 MPa.

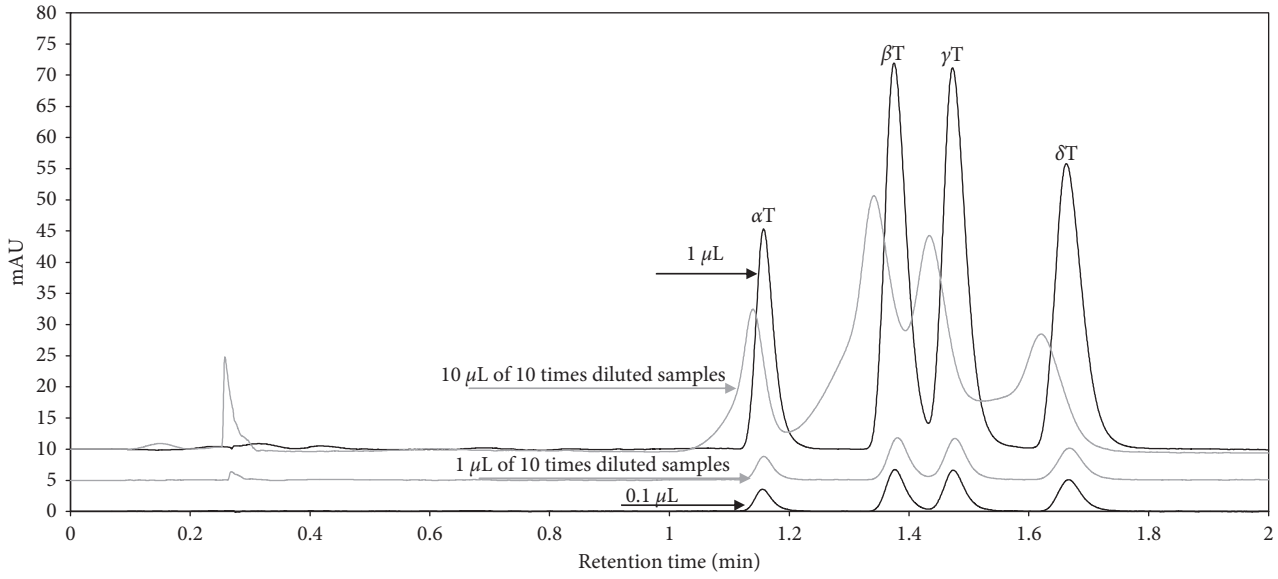

Figure 5: Impact of the injection volume of standards diluted in methanol on the tocopherol homologues peak shape via SFC-DAD. The concentration of tocopherol homologues in the sample was equal with the exception of grey chromatograms where the sample was diluted 10 times in methanol. The chromatographic conditions of the SFC method: mobile phase: CO_2 : MeOH (99.8 : 0.2, v/v); isocratic flow rate: 4.5 mL/min; column oven temperature: 40°C; room temperature: 22°C; back pressure regulator: 15 MPa.

The maximum injection volume of samples diluted in methanol and/or ethanol, that did not induce significant peak distortion for the SFC analysis was 2 μL (Figure 6). The concentration of tocopherols did not have a significant effect on the resolution of the peaks and peak tailing or fronting behaviour (Figure 5). In the case of the RP-LC system, the impact of the solvent environment (with the exception of *n*-hexane, which was not tested) used for the sample dilution and the injection volume (0.1–10 μL) did not have a significant impact on the peak shape tailing, fronting, nor the separation resolution (data not shown). Over 800 injections on the Kinetex™ C18 were throughout this study with no significant loss in column integrity/performance (data not shown).

3.2. SFC Method Validation

3.2.1. Separation Parameters. Table 1 lists the retention times obtained with the optimized SFC separation conditions of the four tocopherol homologues standards and calculated

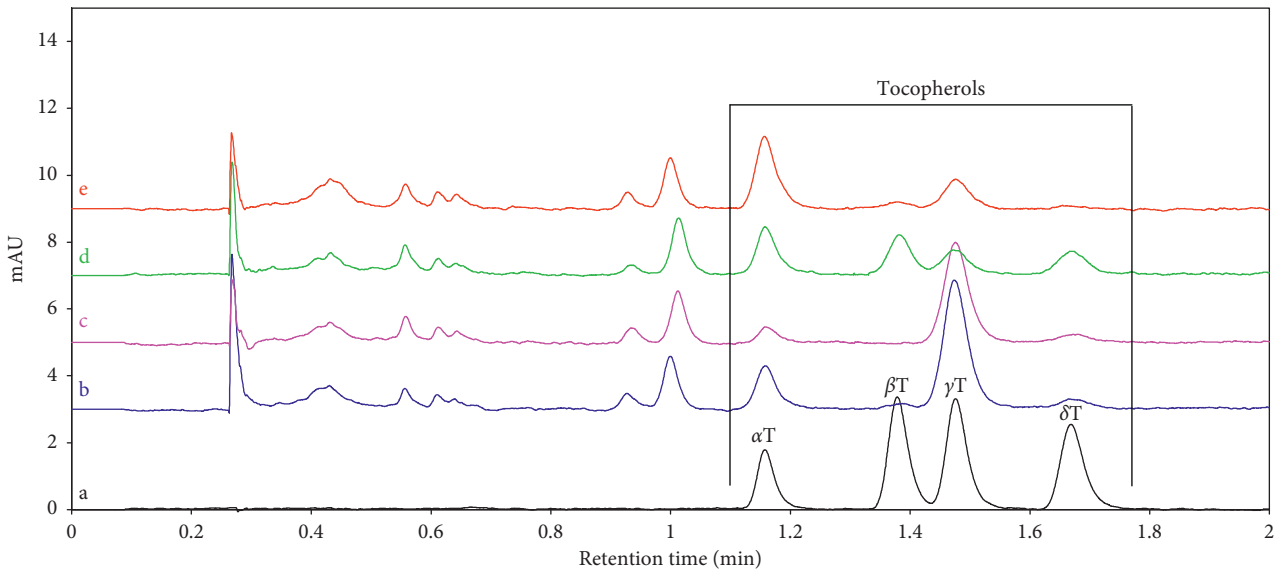

Figure 6: Chromatograms of the tocopherol homologues separation in four selected fruit seed samples via SFC-DAD. The 0.1 g of each fruit seed sample was used for tocopherols extraction and finally diluted in 0.5 mL of methanol. The chromatographic conditions of the SFC method: mobile phase: CO_2 : MeOH (99.8 : 0.2, v/v); isocratic flow rate: 4.5 mL/min; column oven temperature: 40°C; room temperature: 22°C; injection volume: 2 μL; back pressure regulator: 15 MPa.

Table 1: Separation parameters of the four tocopherol homologues on the Kinetex C18 column with the optimized SFC chromatographic conditions*.

Compounds	Retention time, T_R (min)	Retention factor, k	Selectivity factor, $\alpha_{B/A}$	Resolution, R_s	Number of theoretical plates, N
α-T	1.152	3.28	—	—	5523.8
β-T	1.354	4.03	1.23	3.015	5658.4
γ-T	1.459	4.42	1.10	1.400	5598.1
δ-T	1.646	5.12	1.16	2.267	5727.2

*Details of the SFC separation conditions: flow rate: 4.5 mL/min; column dead time (T_0): 0.269 min; mobile phase: CO_2 : MeOH (99.8 : 0.2, v/v); column oven temperature: 40°C; room temperature: 22°C; back pressure regulator: 15 MPa.

parameters such as the retention factor (k), selectivity factor ($\alpha_{B/A}$), resolution (R_s) and the number of theoretical plates (N). The developed method facilitated high throughput/rapid separation of all tocopherol homologues with adequate resolution completed in a total analysis time below two min (Figure 6).

The retention factor ranged from 3.28 to 5.12 for α-T and δ-T, respectively, indicating that all are below the upper limit (20–30) referred as too long elution time [27]. The recorded selectivity factor in each case was over one (>1), indicating a well-performed separation of the analytes. The lowest resolution was calculated between the isomers β and γ (1.4), however this value indicates that the peaks are separated from each other and do not overlap at 0.2%. The lowest number of theoretical plates was recorded for α-T (5523.8) and the highest for δ-T (5727.2).

Injection of samples from nine different fruit species (*R. idaeus*, *R. rubrum*, *R. nigrum*, *F.* × *ananassa*, *M. domestica*, *R. canina*, *H. rhamnoides*, *Ch. japonica*, and *C. lanatus*) revealed no peaks coeluted with the four tocopherols under the optimized chromatographic conditions. All investigated samples contained a number of unidentified peaks that eluted before the tocopherol homologues (Figure 6).

3.2.2. Linearity and Limits of Detection and Quantification. The linear regression equations obtained for the calibration curves of four tocopherol homologues, including determination coefficients (R^2), LOD, and LOQ, are presented in Table 2. The detection responses for tocopherol standard solutions were linear with a $R^2 > 0.99$ for all four tocopherol homologues over a wide range of concentrations (20–2500 ng/μL). The lowest LOD and LOQ were recorded for δ-T, while the highest for β-T. The LOD and LOQ for each isomer were comparable and ranged from 27 to 32 ng/μL and 83–97 ng/μL, respectively. The opposite was reported for tocopherols determined by the RP-LC [9, 10], where the difference between the homologues was two- to threefold.

The developed method was an order of magnitude less sensitive in comparison to RP-LC where fluorescence detection was utilized (ng vs pg) [9, 10]. This finding is not surprising because the LODs for tocopherols determined by the fluorescence detection compared to UV detection are from 150 until over 1000 times more sensitive, depending on the homologue [28]. Unfortunately, fluorescence detection is currently unavailable for any SFC system. The obtained in the present study, the LODs with

Table 2: Linearity, limit of detection (LOD), and limit of quantification (LOQ) of the developed method employing the Kinetex C18 column and the SFC-DAD system[a].

	Tocopherols			
	α-T	β-T	γ-T	δ-T
Standard solutions (R^2)	0.9985 ± 0.0008	0.9988 ± 0.0005	0.9990 ± 0.0006	0.9991 ± 0.0007
Slope and y intercept	$y = 41.458x - 1349.4$	$y = 70.363x - 2807.5$	$y = 72.367x - 3144.6$	$y = 70.056x - 2939.1$
LOD (ng/μL)	29	32	30	27
LOQ (ng/μL)	88	97	90	83

x, concentration (ng); y, peak area (mAU). [a]Tocopherols presented as the absolute amount injected and dissolved in 1 μL of solvent.

the use of SFC-DAD detection was by 60 until over 600 times less sensitive, depending on the tocopherol homologue, in comparison with RP-HPLC-FLD [9, 10]. The difference in the LODs is not only the matter of the used detector, but also used solvents a mobile phase, especially H_2O addition, and temperature of column oven (Figures 1–4) and effect of peak broadening. The cross-check between the SFC-UV detection and HPLC-FLD was reported with the similar observation as in the present study [29]. The LOD and LOQ reported in this study were represented as the amount of analyte required with each injection (ng per injection, where 1 μL was used as a constant injection volume) (Table 2).

3.2.3. Recovery. The intraday and interday recoveries for tocopherols extracted from apple seeds were excellent for all spiked concentrations and within the limits set by the FDA (≤20% deviation from the expected value at low concentrations and ≤15% at medium and high concentrations) [26]. The lowest recovery was noted for samples spiked with the low concentrations of tocopherols (88–96%). When taking into account all concentrations of the spiked analytes (low, medium, and high), as well as the intraday and interday performance, the lowest recovery variation was noted for α-T (95–99%) and the highest for δ-T (88–99%) (Table 3).

3.2.4. Accuracy and Precision. Intraday and interday precision were excellent with no values outside of the FDA limits (±15%). Generally, the precision was below 5%, with the exception of samples spiked with the low concentrations of tocopherols where values were in the range between 5 and 8% (Table 3). Intraday and interday accuracies were excellent and similar as precision with no values outside of the FDA limits (±15%). Generally, the accuracies were much better for samples spiked with the higher concentrations of tocopherols (0.4–1.7%), than for the spiked samples at low level concentrations (4.4–11.8%) (Table 3).

3.3. Tocopherols in Seeds of Nine Fruit Species. The composition of tocopherols in the seeds of nine different fruit species (*R. idaeus*, *R. rubrum*, *R. nigrum*, *F.* × *ananassa*, *M. domestica*, *R. canina*, *H. rhamnoides*, *Ch. japonica*, and *C. lanatus*) have all been presented in Table 4. In four species (*R. idaeus*, *R. nigrum*, *M. domestica*, and *H. rhamnoides*), 44% of the studied samples detected all four tocopherol homologues. This observation highlights the usefulness of isomers β and γ separation to obtain detailed information about the tocopherols composition. The development of chromatographic methods that provide resolution and detection selectivity of all four tocopherols must be utilized instead of methods that use the sum of $\beta + \gamma$ tocopherol.

The composition of tocopherols in the seeds of nine species was characterised, and the lowest and highest levels for each homologue is as follows: *C. lanatus* vs. *H. rhamnoides* for α-T (1.1 vs. 39.0 mg/100 g dw), *Ch. japonica* vs. *M. domestica* for β-T (trace amount <0.1 mg (tr) vs. 14.0 mg/100 g dw), *Ch. japonica* vs. *R. idaeus* for γ-T (tr vs. 47.3 mg/100 g dw), *F.* × *ananassa* vs. *R. rubrum* for δ-T (tr vs. 12.2 mg/100 g dw), and *Ch. japonica* vs. *R. idaeus* for total tocopherols (13.9 vs. 79.2 mg/100 g dw). Because of the dominance of one of the homologues, the studied samples can be divided into three groups of seeds dominated by α-T (*M. domestica*, *H. rhamnoides*, and *Ch. japonica*), γ-T (*R. idaeus*, *R. rubrum*, *R. nigrum*, and *C. lanatus*) and both forms α-T and γ-T in similar levels (*F.* × *ananassa* and *R. canina*). The profile and concentration of tocopherols in the studied seed samples of nine different fruit species was similar to previous reports [6, 30].

4. Conclusion

In the past, the concentrations of tocopherol isomers β and γ, could only be reported as their respective sums due to lack of resolving power/selectivity provided by the C18 column employed for RP-LC. The present study shows, that the tocopherol isomers β and γ can be separated by employing the same C18 column via SFC. The developed SFC method with UV detection, despite an order of magnitude lower sensitivity relative to RP-LC with fluorescence detection, did not require different procedures of the sample preparation than those used in RP-LC method with fluorescence detection. With the development of this rapid, precise, accurate, and most importantly environmentally-friendlier chromatographic method, it is now possible to characterize all four tocopherol homologues in plant material <2 minutes. The present study sheds a new light on the use of the C18 column, and new technology using supercritical CO_2 as the main mobile phase.

Table 3: Intra- ($n = 5 \times 5$) and interday ($n = 25 \times 5$) recoveries, precisions, and accuracies of tocopherols extracted from apple seeds spiked at low, medium, and high levels and detected by the SFC-DAD system.

Recovery, precision, and accuracy	Levels of tocopherol standards	Tocopherols			
		α-T	β-T	γ-T	δ-T
Intraday recovery (%)	Low	95	94	89	88
	Medium	98	95	97	94
	High	99	99	98	99
Interday recovery (%)	Low	96	94	91	91
	Medium	98	97	98	96
	High	99	100	99	98
Intraday precision (CV%)	Low	5.0	6.0	6.6	5.7
	Medium	2.6	2.4	2.8	2.9
	High	2.3	2.9	2.3	3.5
Interday precision (CV%)	Low	5.7	6.7	7.0	8.0
	Medium	2.7	3.2	3.3	4.0
	High	2.0	3.2	3.0	3.4
Intraday accuracy (bias%)	Low	4.6	6.5	10.9	11.8
	Medium	2.4	4.9	2.7	5.9
	High	1.2	0.8	1.7	0.6
Interday accuracy (bias%)	Low	4.4	6.0	9.3	9.4
	Medium	2.3	3.0	2.5	4.2
	High	0.7	0.4	1.3	1.6

Table 4: The composition of tocopherols (mg/100 g dw) in the seeds of nine different fruit species determined by the SFC-DAD system.

Species	Tocopherols				
	α-T	β-T	γ-T	δ-T	Total Ts
R. idaeus	25.3 ± 1.5^{e}	2.3 ± 0.1^{a}	47.3 ± 2.5^{e}	4.3 ± 0.2^{b}	79.2 ± 4.3^{e}
F. × ananassa	13.7 ± 0.7^{d}	nd	11.6 ± 0.5^{a}	tr	25.3 ± 1.3^{bc}
R. rubrum	5.9 ± 0.5^{b}	1.5 ± 0.2^{a}	32.8 ± 1.5^{c}	12.2 ± 0.7^{c}	52.4 ± 3.0^{d}
R. nigrum	11.0 ± 0.9^{c}	nd	37.0 ± 2.5^{d}	4.2 ± 0.2^{b}	52.2 ± 3.7^{d}
M. domestica	22.4 ± 2.2^{e}	14.0 ± 1.0^{b}	9.9 ± 0.5^{a}	10.4 ± 0.4^{c}	56.7 ± 4.1^{d}
H. rhamnoides	39.0 ± 2.5^{f}	2.4 ± 0.2^{a}	11.1 ± 0.7^{a}	1.0 ± 0.1^{a}	53.5 ± 3.5^{d}
Ch. japonica	13.9 ± 0.8^{d}	tr	tr	nd	13.9 ± 0.8^{a}
R. canina	11.9 ± 0.6^{c}	nd	11.5 ± 0.5^{a}	nd	23.4 ± 1.2^{b}
C. lanatus	1.1 ± 0.1^{a}	nd	25.3 ± 1.5^{b}	0.5 ± 0.1^{a}	26.9 ± 1.7^{c}

Values are expressed as the mean ± standard deviation ($n = 3$). Different letters in the same column indicate statistically significant differences at $p \leq 0.05$. T, tocopherol; nd, not detected; tr, traces (<0.1 mg/100 g dw).

Abbreviations

T: Tocopherol
SFC: Supercritical fluid chromatography
RP-LC: Reversed-phase liquid chromatography
NP-LC: Normal-phase liquid chromatography
CO_2: Carbon dioxide.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this article. A.S. is a recipient of an ANII PD scholarship and a researcher from CSIC and SNI, Uruguay.

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