APPLICATION BRIEF

Liquid Chromatography

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Optimizing the Separation of Four Tocopherols and Four Tocotrienols via RP-HPLC by Screening Five Different SPP Phases

Introduction

The knowledge about tocochromanol-related compounds, especially tocotrienols, tocodienols, tocomonoenols, and others, is still limited due to several challenges faced in analytical chemistry. These challenges include separation resolution, co-elution, price and absence of standards, and low analyte concentration in plant material. Application of different column stationary phase chemistries can assist in the challenges faced in compound separation.

Superficially porous particle (SPP) columns can also be used to improve separation of these compounds. SPP particles are made of a solid, non-porous core surrounded by a shell of a porous silica material, resulting in a shorter diffusion path in comparison with fully porous based columns. With a shorter diffusion path within the SPP particle itself, coupled with a uniform packed bed and ultra-inert silica surface, reductions in run times can be observed. Such phases benefit from increased efficiency, with separation resembling that of a UHPLC column. They can be used on standard HPLC instrumentation, without concerns regarding high backpressures, which often compromise column longevity.[1]

In this application brief, five different Quasar™ SPP column phases were screened for the separation of four tocopherol and four tocotrienol homologs (Figure 1), with focus on resolving β and γ isomers.

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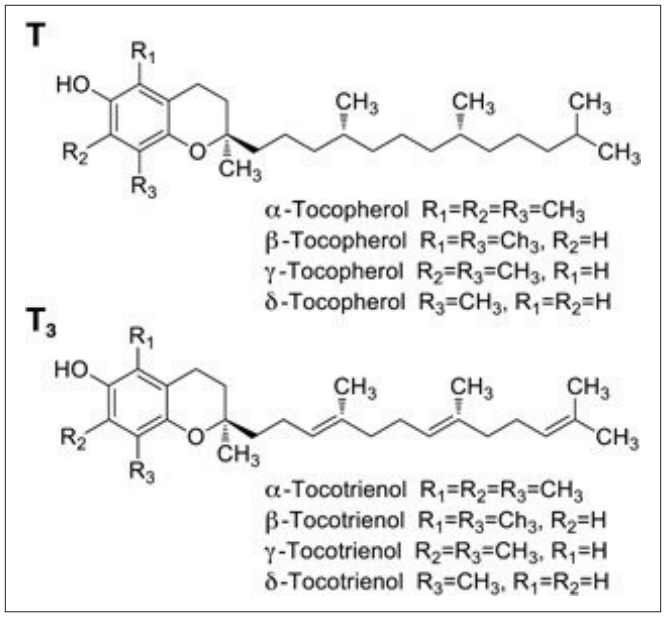

Figure 1. Structure of tocopherols (T) and tocotrienols (T3), with their various homologs (α, β, γ, or δ).[2]

Experimental Conditions

Method Parameters

HPLC method parameters are shown in Table 1.

Table 1. HPLC method parameters for initial screening of five Quasar SPP phases.

Instrument	LC with Fluorescence Detector			
Columns	150 mm	4.6 mm	2.6 µm	Quasar SPP Biphenyl (N9308937)
				Quasar SPP C18 (N9308910)
				Quasar SPP C18/PFP (N9304420)
				Quasar SPP RP-Amide (N9308946)
				Quasar SPP PFP (N9308928)
Mobile Phase	**A:** 1 % Formic Acid in Water* **B:** Methanol 9% **A** 91% **B**			
Flow Rate	1 mL/min			
Temp	40 °C			
Detection	Excitation: 295 nm Emission: 330 nm			
Injection Volume	3 µL			

**Note: For tocopherols and tocotrienols, addition of acid is not required (did not improve separation). Tested mobile phase was also used for other purposes.*

Solvents and Samples

All solvents were of HPLC purity, while tocopherol (α, β, γ, and δ) and tocotrienol (α, β, γ, and δ) standards (purity ≥ 95%) were over 10 years old (kept at -18 °C). Sample diluent was ethanol/methanol.

Results

According to various literature, the separation of tocopherols and tocotrienols by RP-HPLC typically requires methanol with the addition of water from 0 - 15 % v/v as the mobile phase. The addition of water to the mobile phase improves the separation of tocopherols and tocotrienols, especially isomers.[3,4] Therefore, 91 % methanol was used as the initial mobile phase for screening five Quasar SPP columns of various stationary phases chemistries (Biphenyl, C18, C18/PFP, RP-Amide, and PFP).

Figure 2 demonstrates the results obtained from the screening of these phases. The Quasar SPP Biphenyl column offered poor separation of the eight compounds, with only three peaks.

Analysis using the Quasar SPP C18 phase provided good separation, excluding the tocopherol and tocotrienol isomers β and γ. In comparison, the Quasar SPP C18/PFP phase, with its alternative selectivity and introduction of a secondary mechanism, provided better separation than the Quasar SPP C18 phase, especially for tocotrienols. It showed partial separation of the β and γ isomers of tocopherol and tocotrienol.

Analysis using the Quasar SPP RP-Amide provided good separation of all eight compounds. However, the analysis time was over 25 minutes, therefore it was not selected for further optimization as resulting run times would be too long. Resolution between the β and γ isomers was greater than the Quasar SPP C18/PFP phase and could be enhanced further by increasing the aqueous content of the mobile phase. However, this would also result in an increased analysis time.

The Quasar SPP PFP phase provided separation of the investigated compounds in a relatively short period of time (8 minutes) compared with the other phases screened, and hence was selected for further method optimisation. Figure 3 demonstrates the addition of more aqueous content to the mobile phase composition, with 85 % methanol being used to successfully resolve all eight peaks on the Quasar SPP PFP phase in 25 minutes.

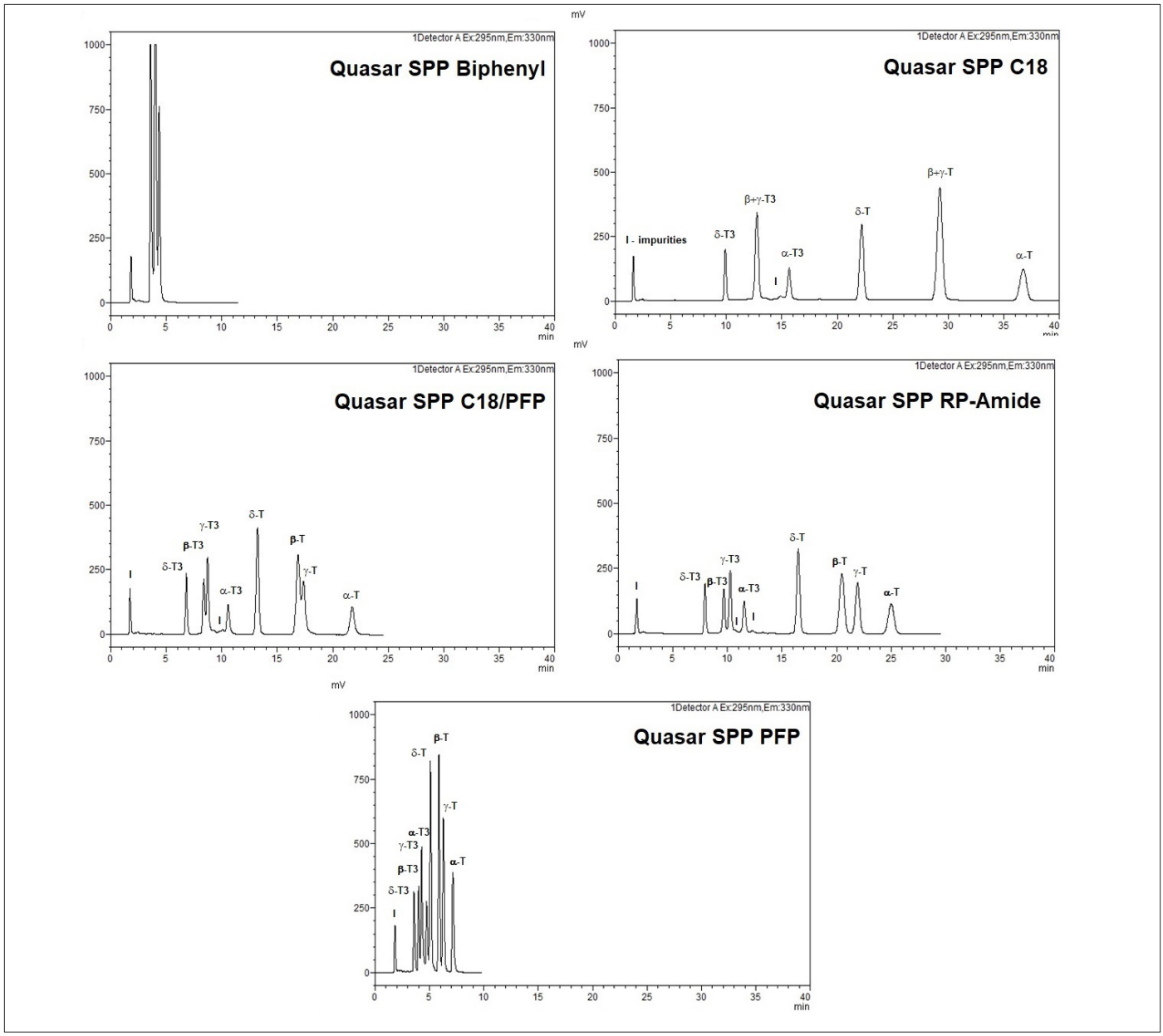

Figure 2. Screening of the five Quasar SPP phases (Biphenyl, C18, C18/PFP, RP-Amide, and PFP) using the experimental conditions in Table 1.

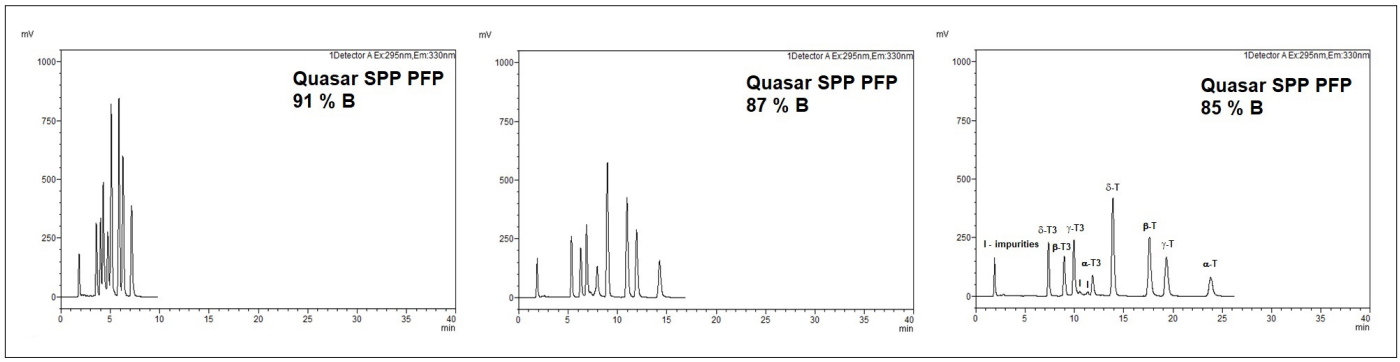

Figure 3. Analysis of tocopherols and tocotrienols using a Quasar SPP PFP using 91 %, 87 % and 85 % methanol in the mobile phase.

Conclusion

- Analysis of tocopherols and tocotrienols using a biphenyl column was shown to be unsuitable due to co-elution and minimal retention. A C18 phase provides much better separation in comparison with a biphenyl phase but it cannot resolve the β and γ isomers.
- Quasar PFP, RP-Amide and C18/PFP phases showed good separation of all eight compounds in the initial screen. With an increase in the aqueous content of the mobile phase, the PFP phase gave the optimal separation of all eight compounds and complete separation between the β and γ tocopherol and tocotrienol isomers.
- Excellent peak shape was obtained due to Quasar's ultra-high purity silica and optimized ligand bonding technology.

References

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3. P. Górnaś, A. Siger, J. Czubinski, K. Dwiecki, D. Seglina, M. Nogala-Kalucka, An alternative RP-HPLC Method for the Separation and Determination of Tocopherol and Tocotrienol Homologues as Butter Authenticity Markers: A Comparative Study Between Two European Countries, European Journal of Lipid Science and Technology, 2014, 116, 895-903.
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Consumables Used

Component	Description	Part Number
Column	Quasar SPP Biphenyl (150 x 4.6 mm, 2.6 µm)	N9308937
	Quasar SPP C18 (150 x 4.6 mm, 2.6 µm)	N9308910
	Quasar SPP C18/PFP (150 x 4.6 mm, 2.6 µm)	N9304420
	Quasar SPP RP-Amide (150 x 4.6 mm, 2.6 µm)	N9308946
	Quasar SPP PFP (150 x 4.6 mm, 2.6 µm)	N9308928
HPLC Vials	2 mL Amber 9 mm Screw Top Vial with Write-on Patch and Fill Lines (100/pack)	N9307802
HPLC Vial Caps	9 mm Screw Top Blue (polypropylene) Cap with PTFE/Silicone pre-slit Septa (100/pack)	N9306203

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