HALO®

TECHNICAL REPORT

TITLE: SEPARATION OF NINE TOCOCHROMANOLS IN COLD-PRESSED OILS BY SUPERCRITICAL FLUID CHROMATOGRAPHY USING DIFFERENT HALO® STATIONARY PHASES

MARKET SEGMENT: FOOD & BEVERAGE

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ABSTRACT:

Tocopherols, tocotrienols, and plastochromanol-8, commonly named tocochromanols/tocols/vitamin E, are fat-soluble antioxidants that play an important role in human health. The primary dietary sources of tocochromanols are nuts, seeds, and plant oils. Tocochromanols are also used as an ingredient in various food, cosmetic, and pharmaceutical products. The chromatographic analysis of tocochromanols is crucial in research aimed at investigating the bioactive compounds profile of plant material. To meet this need, a fast, reliable, and environmentally friendly alternative to normal-phase chromatography is required. The developed supercritical fluid chromatography method with photodiode array detection presented here allows for simultaneous separation of nine tocochromanols in cold-pressed plant oils in 14.5 min.

INTRODUCTION:

Tocopherols (Ts), tocotrienols (T3s), and plastochromanol-8 (PC-8), commonly named tocochromanols/tocols/vitamin E, are fat-soluble antioxidants that play an important role in human health. The richest sources of tocochromanols are plant oils, including cold-pressed, which are becoming more popular due to the high content of bioactive compounds contained (chlorophylls, carotenoids, phytosterols, phenolic compounds, tocochromanols, and other bioactive compounds). The chromatographic separation and quantification of tocochromanols can be challenging due to the complexity of the oil matrix. Often the saponification protocol and liquid-liquid extraction are utilized to purify the sample, however, it requires large amounts of nonpolar solvents (hexane and ethyl acetate), time, and workload [1].

KEY WORDS:

Tocochromanols, Tocopherols, Tocotrienols, Vitamin E, Biphenyl, Supercritical Fluid Chromatography, Plant Oils Analysis, SPP columns.

Simultaneous separation of nine tocochromanols, four Ts and T3s, and PC-8, is not often reported in the literature because of several challenges: lack of commercial PC-8 standard, difficulties in chromatographical separation of ß- and γ- isomers, high retention of PC-8 in comparison with Ts and T3s in RPLC. The most common method used for the separation of all nine tocochromanols is normal phase chromatography (NPLC). The biggest disadvantage of the NPLC method is high consumption of toxic solvents such as hexane or heptane. Simultaneous separation of four Ts, four T3s and PC-8 in RPLC mode was not reported [1]. SFC is a environmentally friendlier mode of chromatography since the main component of the mobile phase is carbon dioxide (CO_2), which is both non-toxic and readily available. Oil sample dilution

in 2-propanol as a sample preparation method is compatible with SFC. Additionally, it is possible to use SFC with NPLC, RPLC and HILIC stationary phases without adjusting the mobile phase.

HALO 90 Å Biphenyl phase provided high selectivity towards the analytes resulting in an environmentally friendly, robust, fast, and low-cost method for routine screening of various cold-pressed seed oils [2].

EXPERIMENTAL:

The Shimadzu Nexera UC system (Kyoto, Japan), with a CBM-20A controller, two LC-30 CE pumps, an LC30 CE SF CO_2 pump, a DGU-20A5R degasser, a SIL-30AC autosampler, a CTO-20AC column oven, an SPD M20A diode-array detector (DAD) and SFC-30A back pressure regulator (BPR) was used for method development, validation and analysis.

2-propanol, methanol were HPLC grade and the purity of CO_2 was 99.8%. Standards of α, ß, γ, and δ tocopherols and tocotrienols (>95%, HPLC) were obtained from LGC Standards and Merck, respectively. Plastochromanol-8 was isolated from flax oil (Linum usitatissimum L.) in-lab scale and its identification was achieved by LCMS-IT-TOF.

A sample of cold-pressed oils was prepared as follows: oil sample (0.50 g) was added to a 5 mL volumetric flask and dissolved in 2-propanol. Before the injection into an SFC system, samples were centrifuged for 5 minutes at 17500 rpm (Eppendorf 5430 R, Hamburg, Germany), transferred to 2 mL vials, and injected.

TEST CONDITIONS:

Column: HALO 90 Å RP-Amide, 2.7 µm, 4.6×250 mm
Part Number: 92814-907
Column: HALO 90 Å Phenyl-Hexyl, 2.7 µm, 4.6×250 mm
Part Number: 92814-906
Column: HALO 90 Å PFP, 2.7 µm, 4.6×250 mm
Part Number: 92814-909
Column: HALO 90 Å HILIC, 2.7 µm, 4.6×250 mm
Part Number: 92814-901
Column: HALO 90 Å ES-CN, 2.7 µm, 4.6×250 mm
Part Number: 92814-904
Column: HALO 90 Å C8, 2.7 µm, 4.6×250 mm
Part Number: 92814-908
Column: HALO 160 Å C30, 2.7 µm, 4.6×250 mm
Part Number: 92114-930
Column: HALO 90 Å C18, 2.7 µm, 4.6×250 mm
Part Number: 92814-902
Column: HALO 90 Å AQ-C18, 2.7 µm, 4.6×250 mm
Part Number: 92814-922

OPTIMIZED CONDITIONS:

Column: HALO 90 Å Biphenyl, 2.7 µm, 4.6 × 250 mm
Part Number: 92814-911
Mobile Phase A: CO_2
Mobile Phase B: MeOH

Gradient:	Time	%B
	0.00	5
	5.00	10
	6.00	25
	12.00	25
	13.00	5
	14.50	5

Flow Rate: 3.0 mL/min
Back-pressure regulator: 10.0 MPa
Back-pressure: 25.0-31.5 MPa
Temperature: 25 °C
Detection: DAD at 295 nm
Injection Volume: 5.0 µL
Sample Solvent: 2-propanol
Sample/Solvent ratio: 1:9-3:7, v/v

RESULTS:

To perform the first screening of the column ability for separation of isomers ß and γ and two other tocopherol homologues (α and δ), the standard solution of four tocopherols was injected into an SFC system equipped with ten different stationary phases (RP-Amide, Phenyl-Hexyl, PFP, HILIC, ES-CN, C8, C30, C18, AQ-C18, and Biphenyl) of HALO® columns (all 90 Å, except C30 which is 160 Å, 2.7 µm, 4.6 × 250 mm). Columns were screened under the same conditions to select the column with high selectivity towards the four tocopherols (Fig.1). Among them RP-Amide, HILIC, C8, AQ-C18, and Biphenyl were chosen for further optimization due to the separation of all four tocopherols. From those five stationary phases, the Biphenyl column showed the highest selectivity towards all nine tocochromanols and was chosen for future optimization.

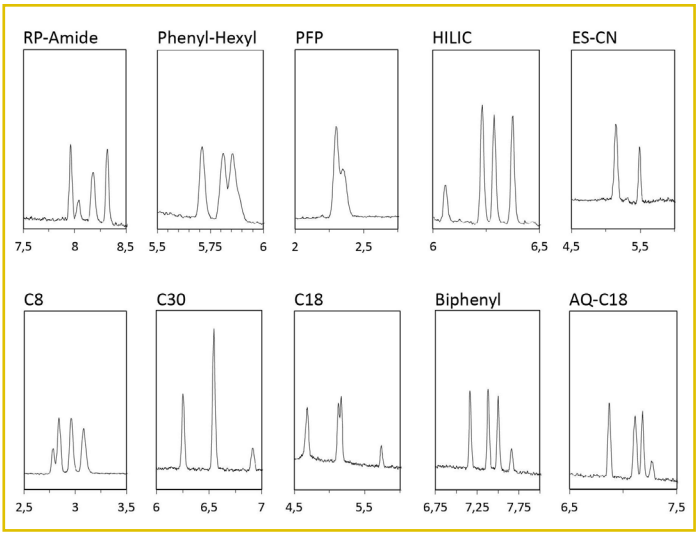

Figure 1. Four tocopherol homologues chromatographic separation using HALO® (250×4.6 mm, 2.7 µm) SPP columns in the SFC mode. Conditions: DAD detection at 295 nm, BPR – 10.0 MPa, temperature 25 °C, flow rate 3.0 mL/min, injection volume 1 µL, mobile phase (A: CO_2; B: MeOH) gradient: 0 min 0.5% B, 3.0 min 0.5% B, 9.0 min 35.0% B, 9.5 min 0.5% B, 10.0 min 0.5%.

Nine tocochromanols were successfully separated by the optimized conditions during 14.5 min using HALO® Biphenyl (Fig.2).

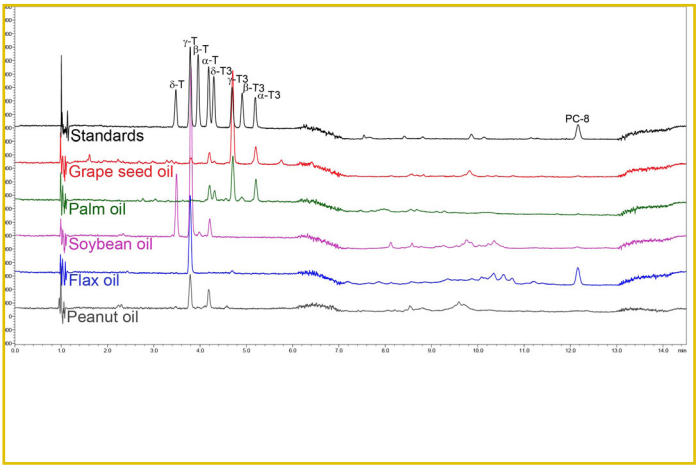

Figure 2. Chromatograms obtained during routine analysis of cold-pressed seed oils. Conditions: see in Experimental.

Sample diluent optimization was the next step in the method development. Methanol showed the lowest tendency to affect the peak shapes when higher injection volumes were used (Fig.3),

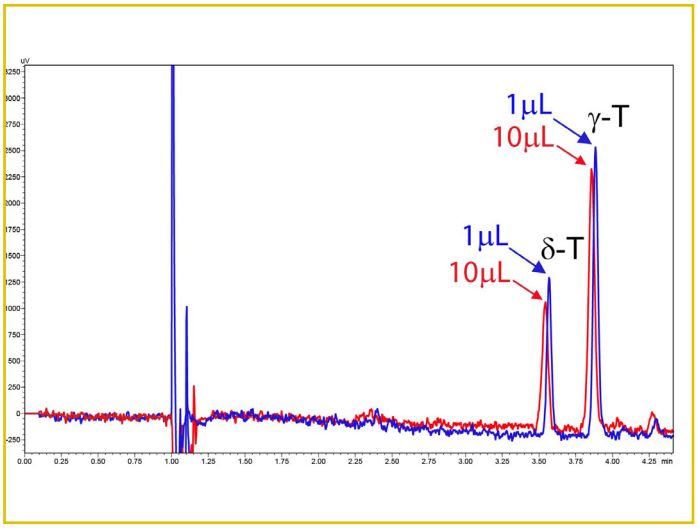

Figure 3. Impact of the injection volume (1 µL of methanol extract compared to a 10 µL aliquot of the same solution diluted 10-times) on peak shape obtained by the HALO® Biphenyl column and SFC-DAD system.

however, methanol is not miscible with oil. Suitable solvents for oil dilution were hexane, ethyl acetate, however 2-propanol was found to be the most suitable solvent. Peak shapes obtained by using 2-propanol as the sample solvent were acceptable with injection volumes up to 7-8 µL. The ratio of 2-propanol to sample was optimized to increase the sample loading and ratio range from 1:9 to 3:7 (w/v) and was found to be suitable for direct injection to the SFC system equipped with the HALO® Biphenyl SPP column (Fig.4).

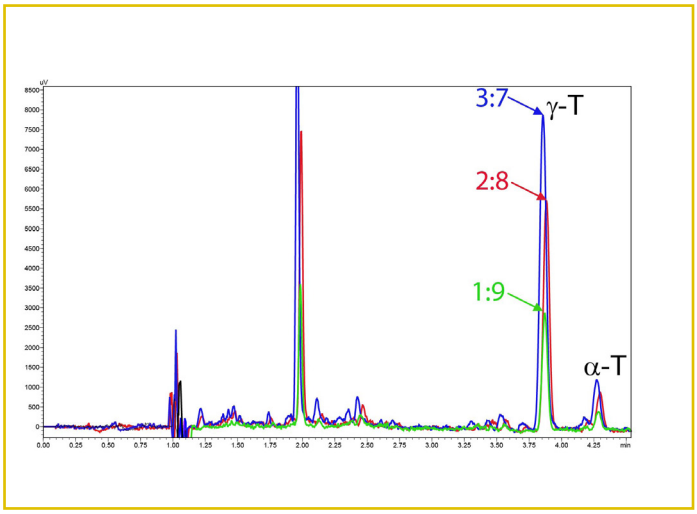

Figure 4. Impact of the sample oil (rapeseed oil) and solvent (2-propanol) ratio on the peak shape via the HALO® Biphenyl column and SFC-DAD system. Ratios of 1:9, 2:8, and 3:7 (w/v) with a 5 µL injection volume.

Instability of retention times and resolution has been an issue for the SFC methods, thus, it is important that the stationary phase utilized is compatible with the SFC. Intraday and interday validation experiments on the reproducibility of the retention time showed that the method is repeatable (0.1–0.31 % and 0.59–0.79 %, respectively). Furthermore, the experiments showed that the injection volume between 1–8 µL and an increase in sample capacity from 1:9 to 3:7, w/v, oil:2-propanol are possible without peak distortion due to volume and sample overload, respectively.

CONCLUSION:

Supercritical fluid chromatography is a greener alternative to NPLC applications with higher environmental sustainability and should be applied if possible. The attentive screening for the right stationary phase chemistry can provide a solution with the highest selectivity towards the analytes and support the quality of the obtained results, especially if the investigated matrix is less known. Moreover, with the HALO® SPP technology, it is possible to use higher injection volumes without sample or volume overload effects.

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