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Journal of Chromatography A, 679 (1994) 329–334

JOURNAL OF CHROMATOGRAPHY A

Supercritical fluid chromatographic determination of tocopherols on an ODS-silica gel column

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First received 8 March 1994; revised manuscript received 26 May 1994

Abstract

A method for the supercritical fluid chromatographic (SFC) determination of tocopherols in vegetable oils was investigated using an ODS-silica gel column with carbon dioxide as the mobile phase. The retention of tocopherols was affected by the density of the mobile phase and the addition of methanol as a modifier. The addition of small concentrations of methanol produced a satisfactory separation of tocopherol homologues, including the positional isomers β- and γ-tocopherol. The results of the determination of tocopherols in vegetable oils by SFC were in satisfactory agreement with those obtained by normal-phase HPLC.

1. Introduction

Tocopherols are well known as components of vitamin E. Four compounds, 5,7,8-trimethyltocol (α-tocopherol), 5,8-dimethyltocol (β-tocopherol), 7,8-dimethyltocol (γ-tocopherol) and 8-methyltocol (δ-tocopherol), are widely distributed in the natural products and their biological activities as vitamin E are different from one another.

HPLC using fluorescence or UV detection is a popular technique for determining tocopherols because of the simplicity and precision [1,2]. There have been many reports on the determination of tocopherols by HPLC under normal- and reversed-phase conditions. However, the separation of the positional isomers β- and γ-tocopherol under reversed-phase conditions has not been reported.

In recent years, supercritical fluid chromatography (SFC) has been developed as separation technique that is a bridge between GC and LC. Carbon dioxide is usually used as the mobile phase in SFC because of its critical properties and safety. However, the addition of a modifier to the mobile phase is often required in order to improve the retention and the peak shapes [3–9]. In packed-column SFC, a UV detector is widely used when an organic solvent is added to the mobile phase. A fluorescence detector can be expected to have higher sensitivity and greater selectivity than a UV detector for the detection of fluorescent species. However, the use of fluorescence detectors in SFC is not convenient owing to the pressure resistivity of the cell.

SFC has been applied to the determination of

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0021-9673/94/$07.00
SSDI 0021-9673(94)00527-G

tocopherols. Upnmoor and Brunner [10] described the retention behaviour of α-tocopherol on various stationary phases. The retention of α-tocopherol decreased with increasing methanol concentration in the mobile phase. Perrin and Prevot [11] reported the separation of tocopherols by SFC on a silica gel column. The elution order of tocopherols in this study was similar to that in normal-phase HPLC. Saito and co-workers [12,13] demonstrated the enrichment of α- and β-tocopherol from wheat germ powder by semi-preparative SFC and supercritical fluid extraction. They also demonstrated the isolation of α- and β-tocopherol using a recycle SFC system [14].

In this paper, we report the determination of tocopherols by SFC on an ODS-silica gel column. The effects of methanol used as a modifier on the retention and the separation of tocopherols and the application of the method to vegetable oil analysis are described.

2. Experimental

2.1. Equipment

SFC system

A Shimadzu (Kyoto, Japan) LC-6A pump was used to deliver carbon dioxide. The pump head was cooled so as to maintain a stable flow. An Isco (Lincoln, NE, USA) Model 100 DM pump was used to deliver methanol. A DKK (Tokyo, Japan) LSA-M mixer was used for mixing carbon dioxide and methanol. A Rheodyne (Cotati, CA, USA) Model 7125 sample injector with a 20-μl sample loop was used for sample injection. The separation column was kept at 40°C in a column oven from a Shimadzu LC-1 system. A Shimadzu SPD-6A UV detector was used for detection at 290 nm. The flow-rate of the mobile phase was controlled by a restrictor made of a capillary tube (200 mm × 50 μm I.D.).

HPLC system

The HPLC system consisted of a Model 576 pump (GL Sciences, Tokyo, Japan), a Rheodyne Model 7125 sample injector with a 20-μl sample loop and a Shimadzu RF-535 fluorescence detector.

2.2. Materials and chemicals

The separation columns used in SFC and HPLC were an L-column ODS (250 mm × 4.6 mm I.D., particle size 5 μm, pore diameter 120 Å) from the Chemicals Inspection and Testing Institute Japan (Tokyo, Japan) and a Chromatorex-SI (250 mm × 4.6 mm I.D., particle size 5 μm, pore diameter 100 Å) from Fuji Silysia (Kasugai, Aichi, Japan), respectively. α-, β-, γ- and δ-tocopherols used as the standards and tocol used as the internal standard were obtained from Eisai (Tokyo, Japan). Wheat germ, cottonseed and soybean oils were obtained from Sigma (St. Louis, MO, USA). Rice bran oil was donated by Tokyo Oil and Fat (Tokyo, Japan). Carbon dioxide used as the mobile phase in the SFC was of standard grade. Methanol, hexane, 1,4-dioxane and 2-propanol were of HPLC grade. The other reagents used were of analytical-reagent-grade and were used as received.

2.3. Preparation of vegetable oils

Vegetable oils were prepared according to Ishikawa et al. [15] as follows. About 10 g of vegetable oil samples were saponified with potassium hydroxide after adding tocol as the internal standard and pyrogallol–ethanol solution. The unsaponifiable matter was extracted with light petroleum (b.p. 30–60°C). The extracted solutions were concentrated to about 5 ml after washing and dehydration with sodium sulfate. The recoveries of tocopherols by this method were 100% [15].

2.4. HPLC procedure

HPLC was applied to the determination of tocopherols in vegetable oils under normal-phase conditions [1]. Hexane–1,4-dioxane–2-propanol (985:10:5, v/v/v) was used as the mobile phase at a flow-rate of 1.0 ml min^{-1}. The silica gel column was used for separation at room temperature. Fluorescence detection was performed

with excitation at 290 nm and emission at 325 nm.

3. Results and discussion

3.1. Retention of tocopherols

The peak shapes of tocopherols were poor when carbon dioxide was used as the mobile phase without a modifier. The retention behaviour of tocopherols and tocol was investigated at carbon dioxide pressures of 12–30 MPa. An increase in carbon dioxide pressure decreased the retention of all the solutes. Approximately liner relationships were obtained from the relationship between log k' and density of carbon dioxide. Chloroform was used to obtain t_0 for a calculating k' with UV detection at 220 nm. A thermodynamic description of this behaviour was reported in detail by Chester and Innis [16]. The elution order remained the same in this pressure range: tocol, δ-, β-, γ and α-tocopherol. Tocopherols having fewer methyl groups in the molecules were eluted faster than those having more methyl groups. Therefore, it seems that the elution order of tocopherols in SFC on the ODS-silica gel column was based on the number of methyl groups in the molecules and was similar to that in reversed-phase LC. On the other hand, the positional isomers β- and γ-tocopherol were separated under these conditions.

The peak shapes of tocopherols improved when methanol was added as a modifier to the mobile phase. The effect of the modifier on the retention of tocopherols at a mobile phase pressure of 15 MPa was investigated by plotting log k' of tocopherols and tocol *vs.* the concentration of methanol in the mobile phase, as shown in Fig. 1. The addition of methanol decreases the retention of all the solutes. The elution order of tocopherols is based on the number of methyl groups in the molecules under these conditions. The elution order of β- and γ-tocopherol reverses depending on the amount of methanol added. In practice, β- and γ-tocopherol eluted as one peak when high concentrations of methanol were added to the mobile phase.

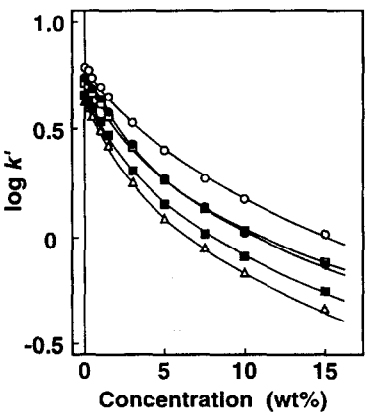


Fig. 1. Effect of the methanol modifier on k'. Samples: ○ = α-tocopherol; ● = γ-tocopherol; □ = β-tocopherol; ■ = δ-tocopherol; △ = tocol.

3.2. Separation of tocopherols

The effect of methanol used as the modifier on the separation of tocopherols at a mobile phase pressure of 15 MPa was investigated. The separation factor (α) between adjacent peaks increased with increasing addition of methanol to the mobile phase, except for the positional isomers β- and γ-tocopherol. The α value between β- and γ-tocopherol approached to 1 according to the amount of methanol added. This suggests that the separation of β- and γ-tocopherol is difficult when high concentrations of methanol are added as the modifier.

Fig. 2 shows the relationship between the methanol concentration in the mobile phase and resolution (R_s) between adjacent peaks. When a small amount of methanol is added to pure carbon dioxide, the R_s values increase considerably, probably owing to the improvement of the peak shapes. At higher concentrations of methanol, on the other hand, all the R_s values decrease with increasing methanol concentration, presumably because the theoretical plate number concerning the separation of tocopherols on this separation column decreases. The theoretical plate number for α-tocopherol at methanol concentrations of 0.5 and 15% (w/w) were about 12 500 and 3200, respectively. When the methanol concentration in the mobile phase is above

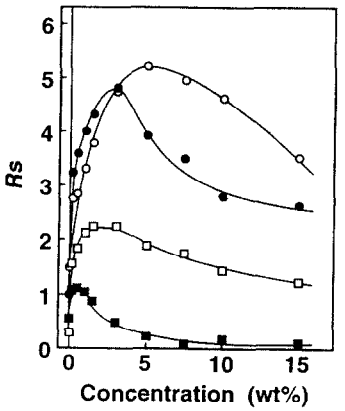
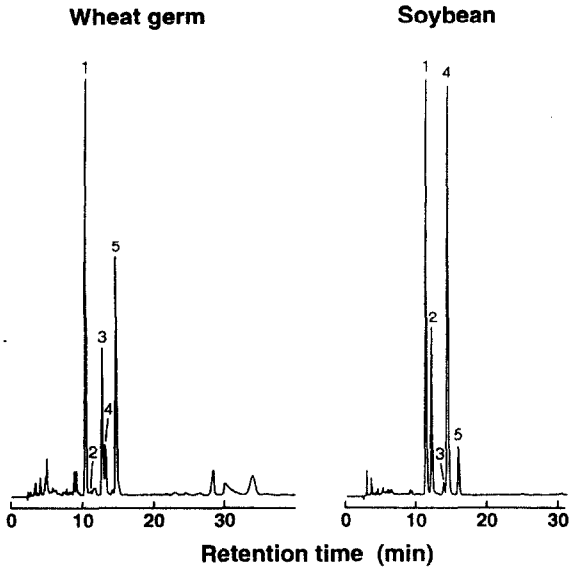


Fig. 2. Effect of the methanol modifier on R_s. The R_s values were obtained for (○) α- and γ-tocopherol, (●) β- and γ-tocopherol, (□) δ-tocopherol and tocol and (■) β- and γ-tocopherol.

3% (w/w), the R_s value between β- and γ-tocopherol is below 0.5 and suggests overlapping of their chromatographic peaks. The best separation of β- and γ-tocopherol is expected when 0.5% (w/w) of methanol is added to the mobile phase.

3.3. Application to vegetable oils

The determination of tocopherols in several kinds of vegetable oils was performed by SFC on the ODS-silica gel column, as shown in Fig. 3. The pressure of mobile phase was kept at 15 MPa and 0.5% (w/w) of methanol was added as the modifier. Some peaks were detected after 18 min and were, probably due to sterols included in the vegetable oils. The results for the determination of tocopherols in vegetable oils are given in Table 1. It is found that the compositions of tocopherols in four vegetable oils differ. The reproducibilities ($n = 5$) are good except for the components present in small amounts. The detection limit and detector linear dynamic range for α-tocopherol under these conditions were 16 ng (signal-to-noise ratio = 3) and four orders of magnitude, respectively. The detection limit is sufficient for vegetable oil analysis. However, fluorescence detection is needed for the determination of tocopherols if greater sensitivity is required, such as in plasma analysis.



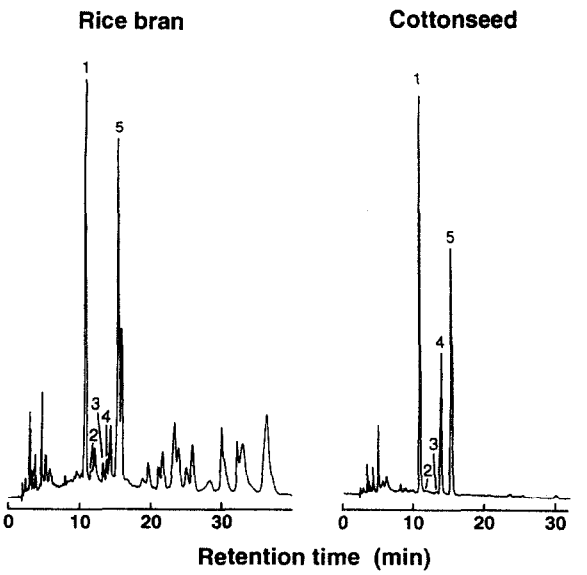


Fig. 3. SFC of tocopherols in vegetable oils. Peaks: 1 = tocol (internal standard); 2 = δ-tocopherol; 3 = β-tocopherol; 4 = γ-tocopherol; 5 = α-tocopherol.

Vegetable oils contain not only tocopherols but also structurally related compounds, such as tocotrienols and tocopherol esters. Determina-

Table 1
Comparison of SFC and HPLC determinations of tocopherols in vegetable oils

Oil	Method	Content of tocopherol (mg per 100 g)[a]			
		α-	β-	γ-	δ-
Wheat germ	SFC	259 ± 2	119 ± 1	41.7 ± 1.2	3.1 ± 0.5
	HPLC	267 ± 5	105 ± 1	35.5 ± 3.2	1.6 ± 0.4
Soybean	SFC	19.9 ± 0.4	3.4 ± 0.2	136 ± 2	47.0 ± 0.5
	HPLC	19.9 ± 0.6	6.4 ± 0.2	132 ± 1	46.9 ± 0.4
Rice bran	SFC	28.2 ± 0.4	1.0 ± 0.1	1.2 ± 0.1	Trace
	HPLC	27.6 ± 0.2	1.4 ± 0.2	1.3 ± 0.3	Trace
Cottonseed	SFC	133 ± 1	1.2 ± 0.2	61.4 ± 0.5	Trace
	HPLC	130 ± 1	8.6 ± 0.2	58.4 ± 0.4	Trace

[a] Mean ± S.D. ($n = 5$).

tion of tocopherols in vegetable oils by normal-phase HPLC using a fluorescence detector was performed in order to compare it with SFC, as shown in Fig. 4. Tocopherols having more methyl groups in the molecules are eluted faster than those having fewer methyl groups under these conditions. The results for the determination of tocopherols in vegetable oils by HPLC are also given in Table 1. The results obtained by SFC and HPLC show good agreement. A small difference between the SFC and HPLC results occurred for some tocopherols present in small

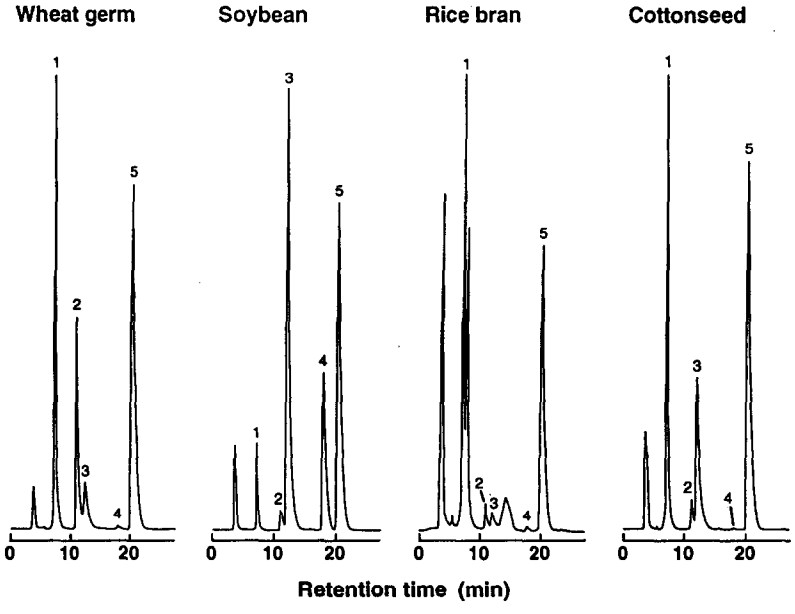


Fig. 4. HPLC of tocopherols in vegetable oils. Peaks: 1 = α-tocopherol; 2 = β-tocopherol; 3 = γ-tocopherol; 4 = δ-tocopherol; 5 = tocol (internal standard).

amounts, such as β-tocopherol in cottonseed oil. This is probably due to the peak overlap with other components in vegetable oils.

4. Conclusions

The determination of tocopherols by SFC using an ODS-silica gel column was reported. The retention behaviour of tocopherols under these conditions was similar to that in reversed-phase HPLC. However, SFC could separate tocopherols, including positional isomers. The addition of small concentrations of methanol as modifier produced satisfactory separations.

Both SFC and normal-phase HPLC were applied to the determination of tocopherols in vegetable oils. It is significant that SFC could effect separates in a different chromatographic mode to that in normal-phase HPLC because vegetable oils contain tocopherols as well as their structurally related compound. We conclude that SFC is suitable as a quantitative method for the determination of tocopherols.

Acknowledgement

The authors thank Mr. Iimura of Tokyo Oil and Fat for discussions concerning vegetable oils.

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