Title: Distinction of synthetic DL-α-tocopherol from natural vitamin E (D-α-tocopherol) by reversed-phase liquid chromatography. Enhanced selectivity of a polymeric C18 stationary phase at low temperature and/or at high pressure

Author: Yuko Yui Shota Miyazaki Yan Ma Masayoshi Ohira Oliver Fiehn Tohru Ikegami David V. McCalley Nobuo Tanaka

PII: S0021-9673(16)30532-5
DOI: http://dx.doi.org/doi:10.1016/j.chroma.2016.04.076
Reference: CHROMA 357520

To appear in: *Journal of Chromatography A*

Received date: 20-1-2016
Revised date: 6-4-2016
Accepted date: 27-4-2016

Please cite this article as: Yuko Yui, Shota Miyazaki, Yan Ma, Masayoshi Ohira, Oliver Fiehn, Tohru Ikegami, David V.McCalley, Nobuo Tanaka, Distinction of synthetic DL-*rmalpha*-tocopherol from natural vitamin E (D-*rmalpha*-tocopherol) by reversed-phase liquid chromatography.Enhanced selectivity of a polymeric C18 stationary phase at low temperature and/or at high pressure, Journal of Chromatography A http://dx.doi.org/10.1016/j.chroma.2016.04.076

Distinction of synthetic DL-α-tocopherol from natural vitamin E (D-α-tocopherol) by reversed-phase liquid chromatography. Enhanced selectivity of a polymeric C18 stationary phase at low temperature and/or at high pressure.

Yuko Yui (1), Shota Miyazaki (1), Yan Ma (2), Masayoshi Ohira (1), Oliver Fiehn (2,3), Tohru Ikegami (4), David V. McCalley (5), and Nobuo Tanaka (1)*

(1) GL Sciences Inc., Iruma, Saitama 358-0032, Japan
(2) University of California, Davis, 451 Health Sciences Drive, Davis, CA 95616, USA
(3) University of Jeddah, Biochemistry Department, Saudi Arabia
(4) Kyoto Institute of Technology, Kyoto 606-8585, Japan
(5) University of the West of England, Bristol, BS16 1QY, U.K.

Corresponding author:
Nobuo Tanaka, n-tanaka@gls.co.jp

Highlights

Distinction of synthetic DL-α-tocopherol from natural vitamin E
Separation of DL-α-tocopherol diastereomers by reversed-phase LC
Enhanced selectivity at low temperature and/or at high pressure for this system

Abstract

Separation of diastereomers of DL-α-tocopherol was studied by reversed-phase liquid chromatography using three types of stationary phases, polymeric ODS, polymeric C30, and monomeric ODS. Polymeric ODS stationary phase (Inertsil ODS-P, 3 mmID, 20 cm) was effective for the separation of the isomers created by the presence of three chiral centers on the alkyl chain of synthetic DL-α-tocopherol. Considerable improvement of the separation of isomers was observed on ODS-P phase at high pressure and at low temperature. Complete separation of four pairs of diastereomers was achieved at 12.0 °C, 536 bar, while three peaks were observed when the separation was carried out either at 12.0 °C at low pressure or at 20 °C at 488 bar. Higher temperature (30.0 °C) with the ODS-P phase resulted in only partial separation of the diastereomers even at high pressure. Only slight resolution was observed for the mixture of diastereomers with the C30 stationary phase (Inertsil C30) at 12.0 °C and 441 bar, although the stationary phase afforded greater resolution for β- and γ-tocopherol than ODS-P. A monomeric C18 stationary phase did not show any separation at 12.0 °C and 463 bar. The results suggest that the binding site of the polymeric ODS-P phase is selective for flexible alkyl chains that provided the longest retention for the natural form, (*R,R,R*) form, and the enantiomer, (*S,S,S*) form, of DL-α-tocopherol.

Keywords: DL-α-tocopherol, polymeric ODS, high pressure, low temperature, diastereomer, reversed-phase LC

1. Introduction

Natural and synthetic forms of vitamin E, including tocopherols and tocotrienols, are used as additives in food and food supplements, because of their beneficial effects, namely antioxidant activity. Natural tocopherol has four fundamental forms: α–, β–, γ–, and δ-tocopherol. The structure of tocopherol contains a chromanol head with one to three methyl groups at different positions, and a phytyl tail with three chiral centers at 2, 4', 8'-carbon atoms (Figure 1). Natural tocopherols have 2(*R)*,4'(*R)*,8'(*R)* configuration (abbreviated here as (*R,R,R)*) in the three chiral centers, resulting in D-α-, D-β-, D-γ-, and D-δ-tocopherol. A synthetic tocopherol, DL-α-tocopherol, consists of eight diastereomeric isomers created by the three chiral centers as shown in Figure 1. Distinction of vitamin E isomers is important for quality control analysis of food, supplement, and tablets, because each isomer provides different biological activity, among which natural D-α-tocopherol (*R,R,R*) is most effective [1]. Chromatographic analysis of tocopherol and related substances has been reviewed by Rupérez, *et al* [2].

Separation of D-α–, D-β–, D-γ–, and D-δ-tocopherol by HPLC has been reported with normal-phase HPLC using silica, amino, and diol stationary phases [3,4], with reversed-phase HPLC using silica-C30, pentafluorophenyl, high-density C18 stationary phase with polymeric bonding [5,6,7,8], and with monolithic columns possessing a polymer stationary phase [9,10]. Normal phase LC separates all natural tocopherol isomers, and is well suited for direct injection of oils, but is not used for complicated biological samples in practice.

Some special stationary phases have been employed for the separation of β- and γ-tocopherols in reversed-phase liquid chromatography [5-8]. Two stationary phases, polymeric silica C18 (Inertsil ODS-P) and polymeric C30 (Inertsil C30 S-Select) showed opposite elution orders for β– and γ–isomers. The separation with C30 and ODS-P was improved at lower temperature and/or at higher pressure, but was not possible with monomeric C18 phases [8]. C30 stationary phase was generally more effective for β–, and γ–tocopherol separation than ODS-P phase.

Effects of higher pressure or lower temperature on selectivity have been observed in reversed-phase liquid chromatography [11,12,13,14]. The effect of pressure on solute retention is strongly affected by the combination of solute structure and stationary phase structure. The effect of pressure was shown to be more pronounced for charged solutes [13,14]. For small, uncharged, hydrophobic solutes, the effects were relatively small. However, greater effects were observed for rigid, planar polynuclear aromatic hydrocarbons than for bulky, flexible ones, particularly on polymeric ODS phase [8,14] Polymeric ODS phase also resulted in very large pressure effects on the retention of alkyl-type solutes [8]. The enhanced selectivity was observed at high pressure and/or low temperature with the opposite elution orders on C30 and ODS-P for β- and γ-tocopherols in a previous study, indicating the attractive interaction of the solute with the stationary phase [8]. The reversed-phase mode does not always separate the four fundamental forms of tocopherol, but has been employed frequently because of short equilibration times and good reproducibility.

Separation of isomers in DL-α-tocopherol based on the three chiral centers in the phytyl tail was reported with HPLC using a chiral stationary phase and subsequent capillary GC on an achiral stationary phase [15,16]. Another report also used a chiral stationary phase [17] to separate DL-α-tocopherol acetate isomers into four peaks (peak area

ratio=4:2:1:1) by HPLC using Chiralpak OP(+), where 2(*R)*-isomers constituted the first peak and 2(*S)* isomers were separated into three peaks (peak area ratio=2:1:1). Differentiation of (*E*)/(*Z*) isomers in synthetic DL-α-tocotrienol were also carried out by employing a chiral stationary phase, which separated the mixture into four geometrical E/Z side chain isomers by using a permethylated β-cyclodextrin phase [18]. Each separated peak was resolved with a cellulose-based chiral stationary phase resulting in complete separation of eight isomers. Although these reports showed that the differentiation between synthetic and natural vitamin E was possible using a chiral stationary phase, the differentiation by reversed-phase HPLC would be useful considering the advantages of reversed-phase mode and the cost of the columns.

In this study, the separation of diastereomers of DL-α-tocopherol was examined by using alkyl modified silica stationary phases with polymeric bonding, Inertsil ODS-P and C30, to prove the possibility of differentiation between natural D-α-tocopherol and synthetic DL-α-tocopherol by reversed-phase LC. The separation was attempted in methanol at 12.0-30.0 ºC and with or without applied back-pressure in a range ca. 40-500 bar to study the effects of temperature and pressure on selectivity of the stationary phase for the isomers in the alkyl tail of DL-α-tocopherol.

2. Experimental

2.1. Instrument and columns

An LC800 liquid chromatograph (GL sciences, Tokyo, Japan) equipped with a column oven with heating and cooling functions, and a UV-VIS detector (MU701, GL Sciences) was used. Three types of columns listed in **Table 1**, including two Inertsil ODS-P columns (4 μm particles, 10 cm x 3 mm I.D.), two Inertsil C30 S-Select columns (5 μm particles, 10 cm x 3 mm I. D.), and two InertSustain C18 columns (3 μm particles, 10 cm x 3 mm I. D), were employed for the study. Inertsil ODS-P and Inertsil C30 possess polymeric bonding chemistry on the silica support with ODS and triacontylsilyl moieties, respectively, while InertSustain C18 has monomeric ODS moieties. The 4000QTRAP MS (AB SCIEX, Foster City, CA, USA) was used as a detector to prove the separation of diastereomeric isomers of DL-α-tocopherol.

2. 2. Chemicals

HPLC-grade methanol was obtained from Kishida Chem (Tokyo, Japan). LC-MS-grade methanol was obtained from Wako Pure Chemical Industries (Osaka, Japan). A Milli-Q system (Millipore, Billerica, MA, USA) was used to prepare HPLC-grade water for the chromatographic measurement. Uracil, D-α–, β–, γ–, and δ–tocopherols were purchased from Wako. DL-α–tocopherol was purchased from Tokyo Chemical Industries Co. (Tokyo, Japan). The structures of tocopherols examined in this study are shown in **Figure 1** (the tocopherol structure was adapted from reference 14)**.**

2. 3. Chromatographic measurement

Chromatographic measurements were made in duplicate. Solutes were measured at 210 nm with the UV-VIS detector. Temperature was controlled by the column oven in a range between 12.0 ºC and 30.0 ºC with 0.1 ºC precision. The instrument can control the oven temperature up to 10 degrees below room temperature. Methanol was used as

mobile phase with flow rate of 0.1 mL/min throughout the study. Column backpressure was increased by adding capillary tubes of 30 μm I.D. of various lengths preceded by PEEK tubing (0.13 mm I. D., 8 cm) between the column outlet and the detector using zero-dead-volume connectors for high pressure operation, as reported earlier [8]. The pressure indicated is the pressure at the pump head. The pressure caused by extra-column parts including the PEEK tube and the detector, excluding the fused-silica capillary tube used as a restrictor, was less than 10 bar with the elution conditions employed. Tocopherol samples were dissolved in acetone at a concentration of 0.3 mg/mL. The injection volume was 0.5 μL, unless noted otherwise. The column dead time, t_0, was obtained by injecting a solution of uracil (U) (0.3 mg/mL). The retention times of solutes were determined from the peak maximum. The LC-MS/MS experiment was carried out in ESI positive mode at a LC column temperature of 12.0 ºC. The eluent was 0.1% formic acid in methanol with flow rate of 0.1 mL/min. Vitamin E standards, each 30 ng/mL, were dissolved in acetone, and 10 μL was injected.

3. Results and discussion

The synthetic DL-α-tocopherol mixture contains four diasteromeric pairs, as shown in Figure 1, which could potentially be separated into four peaks on an achiral stationary phase, with each of the pairs consisting of two enantiomers. In contrast, natural tocopherol consists of only the single (*R,R,R*)-enantiomer of α-tocopherol, together with β-, γ- and δ- forms. In this study, polymeric ODS (Inertsil ODS-P) stationary phase was applied for the separation of phytyl tail isomers of DL-α-tocopherol (Figure 1) at low and high pressure, at 12.0, 20.0, and 30.0 ºC. A polymeric C30 stationary phase (Inertsil C30) and a monomeric C18 stationary phase (InertSustain C18) were examined under the most favorable separation conditions, which were 12.0 ºC at high pressure. Low pressure experiments were carried out below 50 bar, and high-pressure experiments were performed at ca. 500 bar by attaching a 30 μmID fused silica tube of 85-130 cm between the column and the detector.

3.1. Separation of phytyl-tail isomers of synthetic DL-α-tocopherol on the polymeric C18 phase at 12.0 ºC at low pressure

Upper chromatograms in Figure 2a (chromatogram 2A-L) and 2b (chromatogram 2B-L) shown in red indicate separations carried out at low pressure (48 bar), and bottom chromatograms in Figure 2a and 2b shown in black (chromatograms 2A-H and 2B-H) indicate separations carried out at high pressure (536 bar) with a fused silica tube (30 μmID, 95 cm) attached between the column and the detector. Four kinds of tocopherols, D-α-, D-β-, D-γ-, and D-δ-tocopherol, were separated with ODS-P at 12.0 ºC at low pressure, as shown in Figure 2a (chromatogram 2A-L), while diastereomers of DL-α-tocopherol were separated into three peaks with an intensity ratio of roughly 1:2:1 for peaks 1, 2+3, and 4, respectively, as shown in Figure 2b (chromatogram 2B-L). The last peak of DL-α-tocopherol coeluted with D-α-tocopherol (Figure 2a). (The distinction of synthetic and natural forms of α-tocopherol can be shown in the absence of β–, γ– and δ-forms. The latter three forms of tocopherol were included in the figures in order to show the separation of all possible forms of tocopherol present as vitamin E.),

3. 2. Separation of phytyl-tail isomers of synthetic DL-α-tocopherol on polymeric C18 phase at 12.0 ºC at high pressure

D-α-, D-β-, D-γ-, and D-δ-tocopherol (each possessing (*R,R,R)* configuration on the phytyl tail) were retained 60-70% longer on ODS-P phase with the increase of pressure by ca. 490 bar, as shown in Figure 2a (chromatogram 2A-H) compared to elution at low pressure (chromatogram 2A-L of Figure 2a). Diastereomers of DL-α-tocopherol were separated into four peaks with the intensity ratio of roughly 1:1:1:1 (Figure 2b, chromatogram 2B-H). The last peak of DL-α-tocopherol coeluted with D-α-tocopherol. The last peak of Figure 2b was thus identified as the (*R,R,R*) and (*S,S,S*) forms of DL-α-tocopherol. The retention of the other three diastereomer pairs of DL-α-tocopherol did not increase as much as that of D-α-tocopherol. The elution order was presumably the same as at low pressure except the two middle peaks, peak 2 and peak 3, separated at high pressure. The chromatograms in Figure 2 show the difference in selectivity and the different response of the stationary phase to pressure increase for different isomeric structures. The results obtained by LC-UV were confirmed by LC-MS (see Figure S1 and S2 in supplementary materials). The conditions employed in Figure 2 can be used for the differentiation between synthetic DL-α-tocopherol and natural D-α-tocopherol. The ODS-P column showed an increase in separation factor among the diastereomeric isomers at high pressure, indicating that the binding sites of this stationary phase is selective for the flexible moieties in the phytyl tail of tocopherol.

The four peaks of diastereomers of DL-α-tocopherol were tentatively assigned as (*R,S,R*)+(*S,R,S*), (*R,S,S*)+(*S,R,R*), (*R,R,S*)+(*S,S,R*), and (*R,R,R*)+(*S,S,S*), in the order of elution in Figure 2b (chromatogram 2B-H), assuming that the reversal of configuration at the neighboring chiral carbons increases the bulkiness of the molecule. The less bulky isomer was retained longer presumably, as proposed for the selectivity of polymeric C18 phase for polyaromatic hydrocarbons [19]. In Figure 3, a Newman projection presentation was employed to show the anti-periplanar conformation of the phytyl tail of tocopherol having (*R,R,R*) configuration at the chiral centers. The (*R,R,R*) configuration is least sterically demanding, because configuration reversal at the neighboring chiral centers would bring in the increase in bulkiness, because each methyl group at a chiral center will stick out from the main chain to the opposite directions. The increase in bulkiness was assumed to be greater with two configuration reversals than one reversal, and with a reversal closer to the chromanol head than that at further away [17].

3. 3. Effect of stationary phase structure on selectivity for diastereomeric isomers of synthetic DL-α-tocopherol at high pressure and at 12 °C

Figure 4 shows the chromatograms of tocopherols on the monomeric ODS phase. InertSustain C18 (3 mm ID, 20 cm, 2 columns of 10 cm) did not separate either diastereomeric isomers of DL-α-tocopherol or chromanol head isomers, D-β-tocopherol and D-γ-tocopherol, at 12 °C and at 463 bar in the presence of restriction capillary to increase the system pressure. The conditions were similar to the most favorable separation conditions for ODS-P phase. D-α-tocopherol coeluted with DL-α-tocopherol in Figure 4a, while the latter did not show significant peak broadening (no sign of separation of the isomers) in Figure 4b.

Figure 5 shows the separation of tocopherols on the polymeric C30 phase, Inertsil C30 (3 mm ID, 20 cm, two 10 cm columns connected in series), which barely separated the diastereomeric isomers of DL-α-tocopherol into two peaks of the intensity ratio of ca. 3:1 (Figure 5b) without a clear valley between them under increased pressure at 12.0 °C,

although the stationary phase provided easy separation for the chromanol head isomers (Figure 5a), D-β-tocopherol and D-γ-tocopherol, with the opposite elution order from ODS-P phase shown in Figure 2, as reported previously [8]. The elution order of DL-α-tocopherol diastereomers seemed to be similar to that on ODS-P phase, with the last peak coeluting with D-α-tocopherol. The stationary phase did not provide appreciable separation of diastereomers at low pressure without using restriction capillary at the end of the column (results not shown).

3. 4. Effect of temperature on selectivity of polymeric ODS phase

At 12.0 ºC, the ODS-P stationary phase separated DL-α-tocopherol diastereomers into three peaks at low pressure and into four peaks at high pressure, as shown in Figure 2. The results can be interpreted as follows. The more bulky the structure of an isomer is, the smaller the retention, as proposed with respect to the selectivity of polymeric ODS phase [19]. Presumably, at high pressure or at low temperature, the retention of the first peak of DL-α-tocopherol, or the (*R,S,R*) and (*S,R,S*) isomers, seemed to be hindered with the alkyl chains of the stationary phase having rigidity. (*R,R,S)* and (*S,R,R)* forms and (*R,S,S*) and (*S,R,R*) forms with single configuration inversion presumably coeluted at low pressure, but differentiated at high pressure.

At 20.0 ºC and at ca. 500 bar, resolution of diastereomers of DL-α-tocopherol was slightly less than at 12.0 ºC at 48 bar on ODS-P, as shown in Figure 6 (chromatogram 6B-H). However, it was not possible to show clear separation at low pressure at 20.0 ºC. At 20 ºC and 43 bar, the diastereomers were recognized only as shoulders for DL-α-tocopherol, as shown in Figure 6 (chromatogram 6B-L).

Similarly, at 30.0 ºC and 535 bar, shoulders were observed, as shown in Figure 7b (chromatogram 7B-H), for DL-α-tocopherol with slightly less resolution than at 20.0 ºC at low pressure. (In these experiment, 30 μmID fused silica capillary tubes of increased length were used to induce high pressure at higher temperature.) At 30.0 ºC at low pressure no appreciable separation was observed for DL-α-tocopherol, although slight peak broadening was observed, as shown in Figure 7a (chromatogram 7A-L) and 7b (chromatogram 7B-L). Therefore differentiation of synthetic and natural vitamin E, that are DL- and D-α-tocopherol, should be carried out using the polymeric ODS stationary phase either at high pressure and/or at low temperature, preferably at ~500 bar and below 20.0 ºC. Experiments at 20.0 ºC or lower may require a column oven with cooling function, but high pressure can be applied to the column simply by attaching a narrow-bore fused silica capillary at the end of the column. At common room temperature, 20-25 ºC for example, chromatograms in-between Figure 6b (chromatogram 6B-H) and Figure 7b (chromatogram 7B-H) can be expected for DL-α-tocopherol if the ODS-P column is utilized at ca. 500 bar or higher.

3. 5. Effect of pressure on solute-stationary phase binding leading to enhanced selectivity

Equation 1 illustrates the effect of pressure on chromatographic parameters, where β and β_0 are phase ratios of the chromatographic system, k and k_0 are retention factors at high and reference pressure, respectively, and R and T are gas constant and absolute temperature. The variation in a retention factor is correlated with a volume change (ΔV) of the solute-mobile phase-stationary phase system associated with the transfer of the

solute from the mobile phase to the stationary phase.

$$\ln\left(\frac{k}{k_0}\right) = -\frac{\Delta V}{RT}P + \ln\left(\frac{\beta}{\beta_0}\right) \qquad (1)$$

$$\alpha = k_2 / k_1 \qquad (2)$$

$$\Delta\Delta V = \Delta V_2 - \Delta V_1 \qquad (3)$$

In Table 2, the retention factors (k), separation factors (α), and volume change (ΔV) upon binding of DL-α-tocopherols to ODS-P stationary phase are listed for 12, 20, and 30 °C. The separation factors were calculated by equation 2 for the first and the last peak of DL-α-tocopherol (peak 1 and peak 4 in Figure 2b and 6b), and the $\Delta\Delta V$ values for the isomer pairs by equation 3, where ΔV_1 and ΔV_2 are the volume changes associated with the solute transfer from the mobile phase to the stationary phase. The ΔV values, however, were calculated by the measurement at two pressures assuming constant phase ratio, thus should be regarded as a rough estimate for comparison purposes in this work. Larger t_0 values by 5-13% were observed at high pressures than at low pressures, as shown in Figures 2, 6, and 7, the difference being in a similar range reported [20], indicating possible reduction of the phase ratio. Therefore the ΔV values calculated by neglecting the phase ratio term of equation 1 may actually include the contribution of the change in the phase ratio of the system. However, the difference in the volume changes for the two isomers ($\Delta\Delta V$, equation 3) should be free from the problem, because the contribution of the phase ratio term is cancelled out.

The results indicate that the stationary phase structure is of prime importance for the selectivity towards isomeric structure in the long alkyl tail. The C30 is more effective than ODS-P stationary phase for the separation based on the rigid moieties (β- and γ-tocopherol) [8], while ODS-P is more selective for the flexible moieties (phytyl tail isomers) compared to C30. Hydrophobic interactions alone, or the solute partition between aqueous mobile phase and monomeric ODS stationary phase (InertSustain C18) which was shown to be not selective for isomers of different steric requirement [8], cannot differentiate the diastereomers of DL-α-tocopherol, as shown in Figure 4.

The magnitude of the effect of pressure on retention, that is the volume change upon solute-stationary phase binding, is dominated by the combination of solutes and the stationary phase. Much smaller effect has been reported for the retention of uncharged solutes compared to charged solutes with the pressure increase in a similar range [13]. Present results showed a large increase in retention for tocopherols that are uncharged solutes with larger size. Major reason for a large increase in retention seems to be the stationary phase properties. Alkyl-type, flexible solutes resulted in a large increase in retention with a pressure increase (corresponding to large negative ΔV) on ODS-P phase, as shown by the previous study [8]. This is the feature of ODS-P stationary phase used in this study. Large ΔV values were observed for ODS-P phase in combination with tocopherol, as was the case with fatty acids and their methyl esters and triacyl glycerols containing long alkyl chains in a previous study [8]. The volume change is smaller with more bulky solutes and at higher temperature that are presumably unfavorable for the binding process. The results also indicate that the effects of pressure and temperature are additive in the present case, and may suggest the similarity in mechanism of the effect of high pressure and low temperature.

The successful separation of DL-α-tocopherol diastereomers with ODS-P stationary phase in the present study indicates that this stationary phase can provide steric interactions with the solutes. For a stationary phase to be selective, there must be some interactions between the stationary phase and the solutes. The selective stationary phase presumably provides dispersion interactions for the differentiation of the diastereomers of DL-α-tocopherol based on the structure of phytyl tail. The interactions were increased at lower temperature or at higher pressure that brought the interacting entities closer to each other with the increase in density of the system. The longest retention and the largest volume reduction upon binding observed for (*R,R,R*) form and (*S,S,S)* form of DL-α-tocopherol of least steric bulkiness among the diastereomers, suggest that steric fit between the solute and the stationary phase contributes to the isomer differentiation. The volume reduction associated with binding of the most bulky (*R,S,R*) and (*S,R,S*) forms with ODS-P stationary phase was smaller than that for the least bulky (*R,R,R*) and (*S,S,S*) forms by up to ca. 4 mL/mol (Table 2), indicating more favorable interactions for the latter at high pressure. The ΔΔV values show the difference in interaction between the stationary phase and the different phytyl tail of tocopherol. The difference was smaller at higher temperature, which was reflected in the smaller separation factor of the diastereomers.

Structural rigidity and ordered alkyl chains have been discussed previously with polymeric ODS and C30 stationary phases [19,21,22,23]. Lower temperature facilitates ordering and increase in rigidity of the bonded alkyl groups of the stationary phase, resulting in higher steric selectivity [11,12,21,22,23]. Increase in pressure causes an increase in density and ordering of bonded alkyl moieties to result in similar increase in steric selectivity [24,25]. Right stationary phase should be employed in combination with certain structural characteristics of target solutes to utilize the selectivity of the stationary phase and the selective conditions such as high pressure or low temperature.

4. Conclusions

1. DL-α-tocopherol diastereomers were successfully separated with the polymeric ODS phase (Inertsil ODS-P) using methanol as mobile phase at 12.0-20.0 ºC. By increasing pressure, the selectivity for a flexible moiety, the phytyl tail, was enhanced on ODS-P.

2. The separations were improved at lower temperature or at higher pressure, resulting in baseline separation of all the isomers at 12.0 ºC and at ca. 500 bar with ODS-P.

3. Inertsil ODS-P can be used either at low temperature or under high pressure for the differentiation of natural (D-α-) and synthetic (DL-α-) form of tocopherol (vitamin E) by reversed-phase LC.

4. The binding sites of ODS-P phase provided the most favorable interactions for the least bulky (*R,R,R*) and (*S,S,S*) forms of DL-α-tocopherol compared to other diastereomers, as indicated by the largest volume reduction with pressure.

Acknowledgment
The authors thank Masahiro Furuno and Hideyuki Uzu of GL Sciences for their kind support.

References

[1] H. Scherf, L.J. Machlin, T.M. Frye, B.A. Krautmann, S.N. Williams, Vitamin E biopotency: Comparison of various 'natural-derived' and chemically synthesized α-tocopherols, Animal Feed Sci. Tech. 59 (1996) 115-126.

[2] F.J Rupérez, D Martín, E Herrera, C Barbas, Chromatographic analysis of α-tocopherol and related compounds in various matrices, J. Chromatogr. A 935 (2001) 45-69.

[3] A. Kamal-Eldin, R. Andersson, A multivariate study of the correlation between tocopherol content and fatty acid composition in vegetable oils, J. Am. Oil Chem. Soc. 74 (1997) 375-380.

[4] S. L. Abidi, T.L. Mounts, Normal phase high-performance liquid chromatography of tocopherols on polar phases, J. Liq. Chromatogr. Rel. Technol. 19 (1996) 509-520.

[5] S. Strohschein, M. Pursch, D. Lubda, K. Albert, Shape Selectivity of C30 phases for RP-HPLC Separation of tocopherol isomers and correlation with MAS NMR data from suspended stationary phases, Anal. Chem. 70 (1998) 13–18.

[6] A. A. Franke, C. M. Morrison, L. J. Custer, X. Li, J. F. Lai, Simultaneous analysis of circulating 25-hydroxy-vitamin D3, 25-hydroxy-vitamin D2, retinol, tocopherols, carotenoids, and oxidized and reduced coenzyme Q10 by high performance liquid chromatography with photo diode-array detection using C18 and C30 columns alone or in combination, J. Chromatogr. A 1301 (2013) 1-9.

[7] N. Grebenstein, J. Frank, Rapid baseline-separation of all eight tocopherols and tocotrienols by reversed-phase liquid-chromatography with a solid-core pentafluorophenyl column and their sensitive quantification in plasma and liver, J. Chromatogr. A 1243 (2012) 39-46.

[8] K. Okusa, Y. Iwasaki, I. Kuroda, S. Miwa, M. Ohira, T. Nagai, H. Mizobe, N. Gotoh, T. Ikegami, D. V. McCalley, N. Tanaka, Effect of pressure on the selectivity of polymeric C18 and C30 stationary phases in reversed-phase liquid chromatography. Increased separation of isomeric fatty acid methyl esters, triacylglycerols, and tocopherols at high pressure, J. Chromatogr. A 1339 (2014) 86-95.

[9] O. Núñez, T. Ikegami, K. Miyamoto, N. Tanaka, Study of a monolithic silica capillary column coated with poly(octadecyl methacrylate) for the reversed-phase liquid chromatographic separation of some polar and non-polar compounds, J. Chromatogr. A 1175 (2007) 7-15.

[10] Q. Duan, C. Liu, Z. Liu, Z. Zhou, W. Chen, Q. Wang, J. Crommen, Z. Jiang, Preparation and evaluation of a novel monolithic column containing double octadecyl chains for reverse-phase micro high performance liquid chromatography, J. Chromatogr. A 1345 (2014) 174–181.

[11] K. A. Lippa, L. C. Sander, R. D. Mountain, Molecular dynamics simulations of alkylsilane stationary-phase order and disorder. 2. Effects of temperature and chain length, Anal. Chem., 77 (2005) 7862-7871.

[12] M. Pursch, S. Strohschein, H. Händel, K. Albert, Temperature-dependent behavior of C30 interphases. A solid-state NMR and LC−NMR study, Anal. Chem. 68 (1996)

386–393.

[13] D. Åsberg, J. Samuelsson, M. Lesko, A. Cavazzini, K. Kaczmarski, T. Fornstedt, Method transfer from high-pressure liquid chromatography to ultra-high-pressure liquid chromatography. II. Temperature and pressure effect. J. Chromatogr. A, 1401 (2015) 52–59.

[14] D. V. McCalley, The impact of pressure and frictional heating on retention, selectivity, and efficiency in ultra-high-pressure liquid chromatography. Trends in Anal. Chem. 63 (2014) 31–43.

[15] M. Vecchi, W. Walther, E. Glinz, T. Netscher, R. Schmid, M. Lalonde, W. Vetter, Separation and Quantitation of All Eight Stereoisomers of α-Tocopherol by Chromatography. Helv. Chim. Acta 73 (1990) 782-789.

[16] G. Riss, A. W. Kormann, E. Glinz, W. Walther, U. B. Ranalder, Separation of the eight stereoisomers of all-rac-α-tocopherol from tissues and plasma: Chiral phase high-performance liquid chromatography and capillary gas chromatography, Methods Enzymol. 234 (1994) 302-310.

[17] T. Ueda, H. Ichikawa, O. Igarashi, Determination of α-tocopherol stereoisomers in biological specimens using chiral phase high-performance liquid chromatography, J. Nutr. Sci. Vitaminol. Tokyo 39 (1993) 207-219.

[18] A. M. Drotleff, W. Ternes, Determination of RS,E/Z-tocotrienols by HPLC, J. Chromatogr. A 909 (2001) 215–223.

[19] L. C.Sander, S. A. Wise, Influence of stationary phase chemistry on shape recognition in liquid chromatography, Anal. Chem., 67 (1995) 3284–3292.

[20] F. Gritti, G. Guiochon, Influence of the pressure on the properties of chromatographic columns I. Measurement of the compressibility of methanol–water mixtures on a mesoporous silica adsorbent, J. Chromatogr. A, 1070 (2005) 1-12.

[21] K. Jinno, T. Ibuki, N. Tanaka, M. Okamoto, J. C. Fetzer, W. R. Biggs, P. R. Griffiths, J. M. Olinger, Retention behaviour of large polycyclic aromatic hydrocarbons in reversed-phase liquid chromatography on a polymeric octadecylsilica stationary phase, J. Chromatogr. A 461 (1989) 209-227.

[22] G. Srinivasan, C. Meyer, N. Welsch, K. Albert, K. Müller, Influence of synthetic routes on the conformational order and mobility of C18 and C30 stationary phases, J. Chromatogr. A 1113 (2006) 45-54.

[23] K. A. Lippa, L. C. Sander, R. D. Mountain, Molecular dynamics simulations of alkylsilane stationary-phase order and disorder. 1. Effects of surface coverage and bonding chemistry, Anal. Chem. 77 (2005) 7852-7861.

[24] V. L. McGuffin, S. -H. Chen, Theoretical and experimental studies of the effect of pressure on solute retention in liquid chromatography, Anal. Chem. 69 (1997) 930-943.

[25] V. L. McGuffin, S. -H. Chen, Molar enthalpy and molar volume of methylene and benzene homologues in reversed-phase liquid chromatography, J. Chromatogr. A 762 (1997) 35-46.

Figure Captions

Figure 1. Structures of tocopherol isomers. The asterisks indicate the position of chiral centers, while the numbers indicate the carbon number on the phytyl tail.

Figure 2. (a) Separation of synthetic DL-α-tocopherol and four kinds of natural tocopherols, D-α-, D-β-, D-γ-, and D-δ-tocopherol, and (b) separation of DL-α-tocopherol. Column: Inertsil ODS-P (3 μm), 3 mm I.D. 10 cm column x 2. Mobile phase: CH_3OH. Temperature: 12.0 °C. Flow rate: 0.1 mL/min. Detection wavelength: 210 nm. Overlaid chromatograms in red refer to low pressure operation (48 bar, chromatograms 2A-L and 2B-L), whereas chromatograms in black high pressure operation (536 bar, chromatograms 2A-H and 2B-H), respectively, the latter with 95 cm, 30 μm I. D. fused silica tubing between the column and the detector. Peak identification is indicated in (a), while tentative assignment will be given for the peaks of phytyl tail isomers in (b) in section 3.2. Sample: 0.5 μL of an acetone solution at 0.3 mg/mL of each tocopherol.

Figure 3. Structure of D-α-tocopherol, having 2*R*,4'*R*,8'*R* configuration(*R,R,R*) at three chiral centers. Newman projection presentation is used. The numbers indicate the carbon number on the alkyl chain.

Figure 4. (a) Separation of DL-α-tocopherol and four kinds of tocopherols, D-α-, D-β-, D-γ-, and D-δ-tocopherol, (b) separation of DL-α-tocopherol, (c) elution of D-α-tocopherol. Column: InertSustain C18 (3 μm), 3 mm I.D. 10 cm column x 2. Temperature: 12.0 °C. Pressure: 463 bar with 85 cm, 30 μm I. D. fused silica tubing between the column and the detector. See Figure 2 for other experimental conditions.

Figure 5. (a) Separation of DL-α-tocopherol and four kinds of tocopherols, D-α-, D-β-, D-γ-, and D-δ-tocopherol, (b) Separation of DL-α-tocopherol, (c) elution of D-α-tocopherol. Column: Inertsil C30 (3 μm), 3 mm I.D. 10 cm column x 2. Temperature: 12.0 °C. Pressure: 441 bar with 85 cm, 30 μm I. D. fused silica tubing between the column and the detector. See Figure 2 for other experimental conditions.

Figure 6. (a) Separation of DL-α-tocopherol and four kinds of tocopherols, D-α-, D-β-, D-γ-, and D-δ-tocopherol, (b) separation of DL-α-tocopherol, and (c) chromatogram of D-α-tocopherol. Column: Inertsil ODS-P (3 μm), 3 mm I.D. 10 cm column x 2. Temperature: 20.0 °C. Overlaid chromatograms in red refer to low pressure operation (43 bar, chromatograms 6A-L, 6B-L, and 6C-L), whereas chromatograms in black high pressure operation (488 bar, chromatograms 6A-H, 6B-H and 6C-H)), respectively, the latter with 100 cm, 30 μm I. D. fused silic a tubing between the column and the detector. See Figure 2 for other experimental conditions.

Figure 7. (a) Separation of DL-α-tocopherol and four kinds of tocopherols, D-α-, D-β-, D-γ-, and D-δ-tocopherol, (b) separation of DL-α-tocopherol, and (c) chromatogram of D-α-tocopherol. Column: Inertsil ODS-P (3 μm), 2.1 mm I.D. 10 cm column x 2. Temperature: 30.0 °C. Overlaid chromatograms in red refer to low pressure operation (38 bar, chromatograms 7A-L, 7B-L, and 7C-L), whereas chromatograms in black high pressure operation (535 bar, chromatograms 7A-H, 7B-H and 7C-H)), respectively, the latter with 130 cm, 30 μm I. D. fused silica tubing between the column and the detector. See Figure 2 for other experimental conditions.

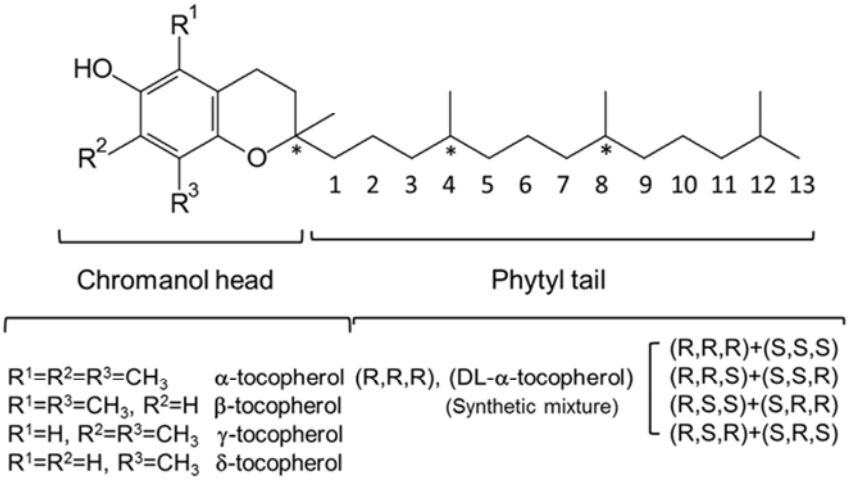

Figure 1

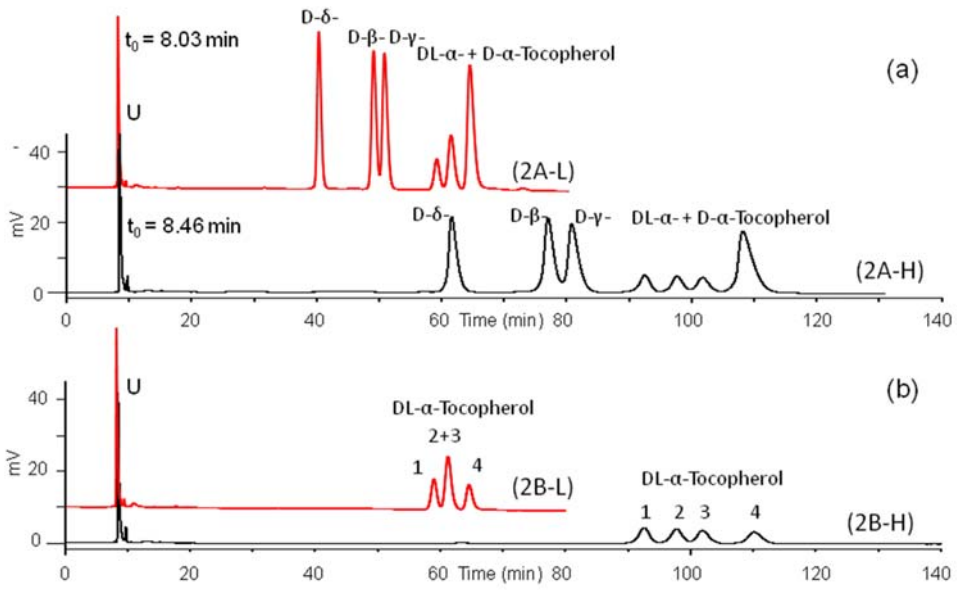

Figure 2

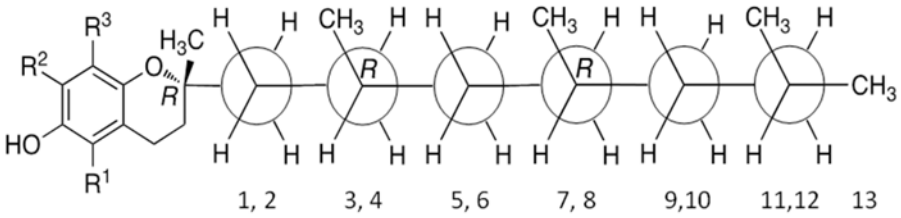

Figure 3

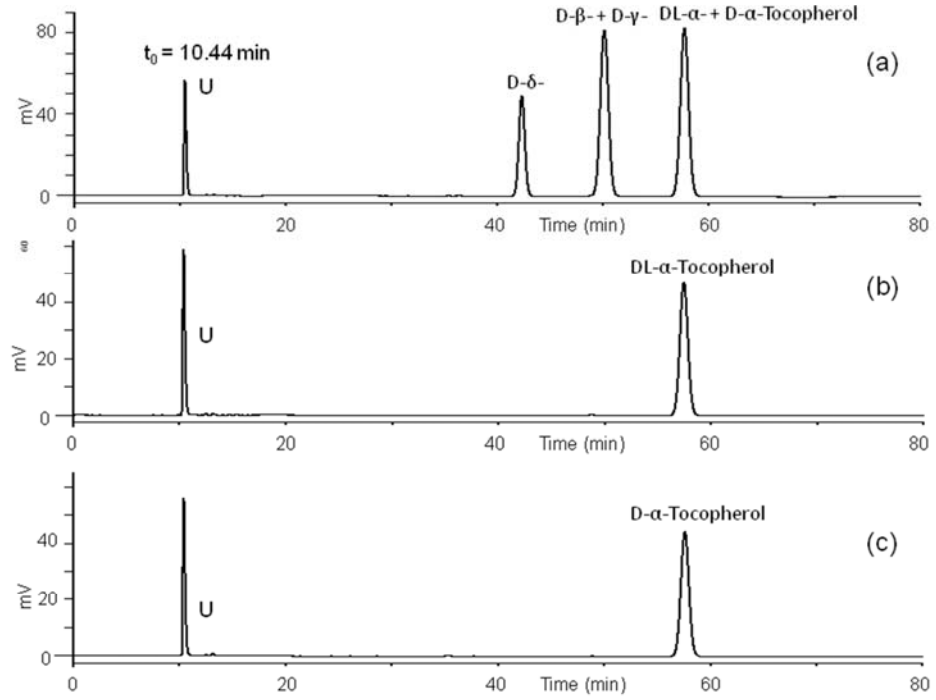

Figure 4

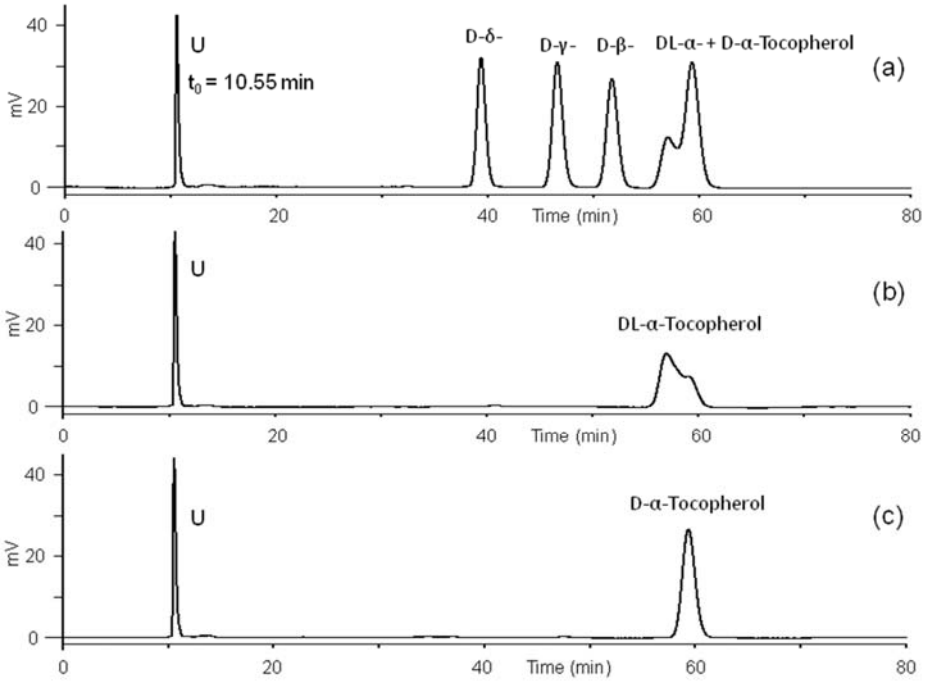

Figure 5

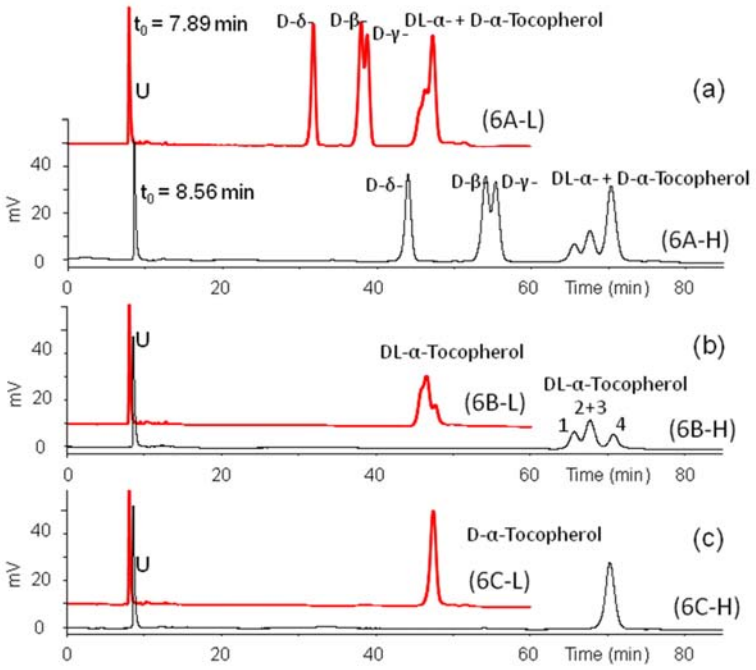

Figure 6

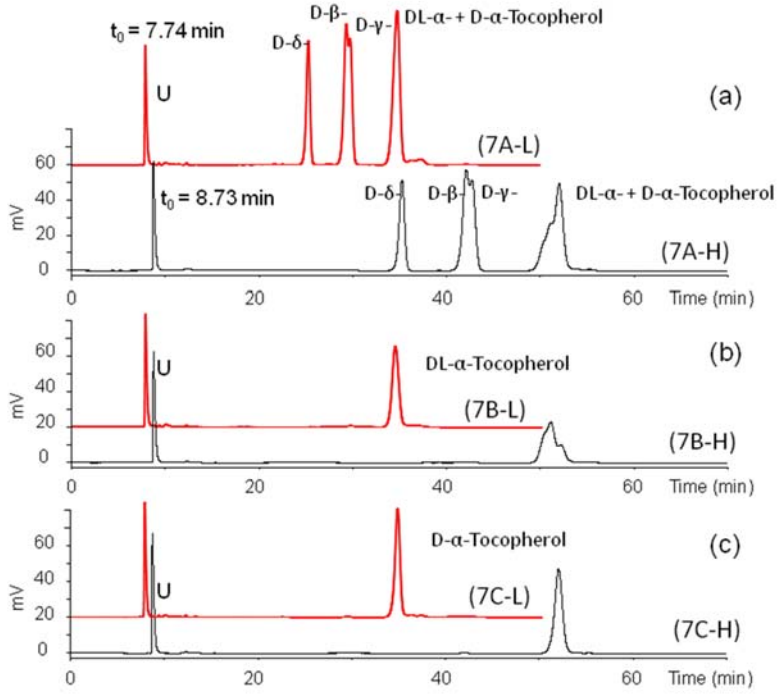

Figure 7

Table 1. Columns employed for the study on separation of DL-α-tocopherol diastereomers.

Column	Stationary phase	Carbon content (%)[a]	Surface area (m2/g)[a,b]	Pore size (nm) [a,b]	Pore volume (mL/g)[a,b]
Inertsil ODS-P	polymeric C_{18}	29	450	10	1.05
Inertsil C30	polymeric C_{30}	16	450	10	1.05
InertSustain C18	monomeric C_{18}	14	350	10	0.85

[a] Specification of a commercial product
[b] Properties of the corresponding silica support without surface modification

Table 2. Volume change associated with binding of tocopherols with ODS-P stationary phase

T (°C)	*P* (bar)	*k*1 (DL-1) [a]	*k*2 (DL-4) [a]	α (DL-4 /DL-1)	ΔV(DL-1) [b] (mL/mol)	ΔV(DL-4) [b] (mL/mol)	$\Delta\Delta V$ (ΔV(DL-4)-ΔV(DL-1)) (mL/mol)
12.0	48	6.34	7.04	1.13	-21.8	-25.9	-4.1
	536	9.94	12.01	1.20			
20.0	43	4.83	5.02	1.04	-17.4	-20.0	-2.6
	488	6.64	7.23	1.09			
30.0	38	ND	3.47	ND	ND	-18.1	ND
	535	ND	4.96	ND			

[a] DL-1 and DL-4 stand for the first and the forth peak of DL-α-tocopherol, respectively, separated at high pressure with ODS-P column in Figure 2b and Figure 6b.
[b] The ΔV value may contain the contribution of the change in phase ratio (see text).