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# Optimization and Validation of the Reversed-Phase High-Performance Liquid Chromatography with Fluorescence Detection Method for the Separation of Tocopherol and Tocotrienol Isomers in Cereals, Employing a Novel Sorbent Material

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**ABSTRACT:** The separation and determination of tocopherols (Ts) and tocotrienols (T3s) by reversed-phase high-performance liquid chromatography with fluorescence detection has been developed and validated after optimization of various chromatographic conditions and other experimental parameters. Analytes were separated on a PerfectSil Target ODS-3 (250 × 4.6 mm, 3 $\mu$m) column filled with a novel sorbent material of ultrapure silica gel. The separation of Ts and T3s was optimized in terms of mobile-phase composition and column temperature on the basis of the best compromise among efficiency, resolution, and analysis time. Using a gradient elution of mobile phase composed of isopropanol/water and 7 °C column temperature, a satisfactory resolution was achieved within 62 min. For the quantitative determination, $\alpha$-T acetate (50 $\mu$g/mL) was used as the internal standard. Detection limits ranged from 0.27 $\mu$g/mL ($\gamma$-T) to 0.76 $\mu$g/mL ($\gamma$-T3). The validation of the method was examined performing intraday ($n$ = 5) and interday ($n$ = 3) assays and was found to be satisfactory, with high accuracy and precision results. Solid-phase extraction provided high relative extraction recoveries from cereal samples: 87.0% for $\gamma$-T3 and 115.5% for $\delta$-T. The method was successfully applied to cereals, such as durum wheat, bread wheat, rice, barley, oat, rye, and corn.

**KEYWORDS:** *reversed-phase HPLC, novel sorbent material, tocopherols, tocotrienols, cereals*

## INTRODUCTION

Vitamin E, an important antioxidant in foods, occurs as eight compounds: $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-tocopherols (Ts) and their corresponding tocotrienols (T3s). The $\alpha$-T has the greatest vitamin E activity; $\alpha$-T3 possesses excellent antioxidant activity and contributes to the nutritive value of cereal grains in the human diet.[1] According to current dietary guidelines, the recommended dietary allowance (RDA) of vitamin E is 15 mg of 2*R*-$\alpha$-T per day (although most tocols are considered to have these vitamin E activities), and the estimated average requirement is 12 mg.[2] As a scavenger of free radicals, vitamin E is believed to protect the body against generative diseases, particularly cancer and cardiovascular disease.[3] Although Ts are considered to be the more biologically active forms of vitamin E, recent evidence suggests that the T3s may have a protective effect by lowering low-density lipoprotein (LDL) cholesterol by inhibiting cholesterol biosynthesis.[4]

Vegetable oils are the main source of tocols, but substantial amounts of these compounds are also reported in most cereal grians, including barley, oats, wheat, rye, and rice.[5,6] Tocols found in various sources can be determined using chromatographic techniques, including normal- and reversed-phase high-performance liquid chromatography (RP-HPLC). When the normal-phase HPLC was used, relatively easy separations of isomeric $\beta$- and $\gamma$-T and -T3 were obtained, with the elution order of Ts and T3s being the following: $\alpha$-T < $\alpha$-T3 < $\beta$-T < $\gamma$-T < $\beta$-T3 < $\gamma$-T3 < $\delta$-T < $\delta$-T3.[5,7]

On the contrary, RP-HPLC using conventional octadecylsilane (ODS) column formats has been widely applied to these compounds because of some practical advantages, such as easy equilibration of stationary phases, reproducible chromatographic peak characteristics, low volatility of solvents, good selectivity, and reduction of the use of hazardous solvents.[8] In RP-HPLC, standard $C_8$ and $C_{18}$ microparticulate stationary phases cannot provide sufficient selectivity for the separation of the problematic $\beta$ and $\gamma$ isomers and the elution of compounds follows the order of increasing analyte hydrophobicity: $\delta$-T3 < ($\beta$ + $\gamma$)-T3 < $\alpha$-T3 < $\delta$-T < ($\beta$ + $\gamma$)-T < $\alpha$-T.[9] $\beta$ and $\gamma$ isomers of Ts and T3s have not been separated on ODS under standard mobile-phase conditions (aqueous acetonitrile or methanol). However, in non-routine applications, with an ODS column, resolution of $\beta$−$\gamma$ pairs of the antioxidant position isomers can be achieved under elution with isopropanol/water eluents.[10] In these systems, the analytes elute from the column at unusually long retention times because of the high column pressure and low eluent flow rates.[8]

However, $\beta$ and $\gamma$ isomers can be separated completely under RP-HPLC conditions using new stationary phases, e.g., long-chain alkyl-bonded $C_{30}$ silica,[11] non-silica-based polyvinyl alcohol,[12] and perfluorinated phenyl silica-based stationary

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phases.[12,13] Recently, Nùñez et al.[14] and Chaisuwan et al.[15] reported the use of monolith columns for the separation of Ts. The use of reversed-phase conventional particulate ODS columns seem to be the method of choice for the analysis of Ts and T3s in various matrices because monolithic columns are expensive in comparison to particulate columns. As far as the stationary phase is concerned, all researchers who have adopted the RP-HPLC method have used the $C_{18}$ column from different manufacturers, with different lengths, particle sizes, and internal diameters. Examples of the RP-$C_{18}$ column used are NovaPak $C_{18}$, 3.5 $\mu$m;[16] Nucleosil 100 $C_{18}$, 5 $\mu$m;[9,17] Synergi Hydro-RP, 4 $\mu$m;[18] Zorbax SB-$C_{18}$, 5 $\mu$m;[19] Hypersil ODS, 5 $\mu$m;[20] Ultrasphere ODS, 5 $\mu$m;[21] Lichrospher 100 RP-18, 5 $\mu$m;[22] Spherisorb ODS-2, 3 $\mu$m;[23] XTerra MS $C_{18}$, 3.5 $\mu$m;[24] and Luna $C_{18}$, 5 $\mu$m.[25] All of these materials are spherical in shape and end-capped, except for Zorbax SB-$C_{18}$, with a surface area in the range from 150 to 400 $m^2$/g. PerfectSil Target (MZ-Analysntechnik, Mainz, Germany) is an ultrapure silica gel (>99.999%) that is supplied with a novel state of the art bonding and end-capping technology and characterized by excellent chemical and mechanical stability and provides excellent peak symmetry for basic analytes with a surface area of 450 $m^2$/g.[26]

The aim of the present study is to develop and validate an accurate, precise, and sensitive RP-HPLC method for the determination of all four Ts and T3s using a novel sorbent material as the stationary phase. To the best of our knowledge, no conventional RP-HPLC method for the simultaneous determination of all T and T3 isomers in cereals using the above-mentioned sorbent material has been published thus far. The developed method was applied for the determination of $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-T3 and -T in cereal samples. The analytical column was proven to be sufficient for the separation of the problematically resolved pair of $\beta$ and $\gamma$ isomers.

## MATERIALS AND METHODS

**Chemicals and Materials.** All analytical standards used were of high purity from various sources. Standards of D,L-$\alpha$-T, $\gamma$-T, and $\delta$-T were supplied by Supelco (Bellefonte, PA). D-$\beta$-T and $\alpha$-, D-$\beta$-, $\gamma$-, and $\delta$-T3 were supplied by LGC Promochem GmBH (Wesel, Germany). $\alpha$-T acetate was supplied from Sigma (St. Louis, MO). Methanol, 2-propanol, dicloromethane, acetone, ethanol, and water were purchased from Panreac (Barcelona, Spain), all of HPLC grade. All other reagents were of analytical or HPLC grade and were purchased from Panreac (Barcelona, Spain). The solid-phase extraction (SPE) cartridges used were Oasis hydrophilic−lipophilic balance (HLB) (200 mg/6 mL) from Waters (Milford, MA).

The following six cereal varieties and hybrids, in the case of corn, were cultivated in 2009: Athos (*Triticum durum*), Dion (*Triticum aestivum*), Kos (*Hordeum vulgare*), Alexandros (*Oryza sativa*), Pallini (*Avena sativa*), and Aris (*Zea mays*). Rye grain samples were taken from the market. All grain samples (except for rye) were supplied from the Cereal Institute, National Agriculture Research Foundation (NAGREF, Thessaloniki, Greece), and before analysis were stored in the fridge at 4 °C. Then, they were milled using a mill (Retsch, model ZM 1000, Germany) with a 0.25 mm screen, blended, and stored at −20 °C until analysis. Rice grains were milled using a dehusked mill (Taka Yama MTH-35A, Taiwan) and analyzed as brown.

**Standard Preparation.** Because fat-soluble vitamins are light-sensitive, all glassware was covered with aluminum foil. The standard stock solutions of Ts ($\alpha$-, $\beta$-, $\gamma$-, and $\delta$-T) and T3s ($\alpha$-, $\beta$-, $\gamma$-, and $\delta$-T3) were prepared in ethanol. The internal standard (IS) solution was prepared by diluting $\alpha$-T acetate stock solution with methanol to a final concentration of 50 $\mu$g/mL. All of the above solutions were kept deep frozen at −20 °C. Their concentrations were evaluated spectrophotometrically according to their specific absorption coefficients: $\alpha$-T = 75.8 at 292 nm, $\beta$-T = 89.4 at 296 nm, $\gamma$-T = 91.4 at 298 nm, $\delta$-T = 87.3 at 298 nm, $\alpha$-T3 = 91.0 at 292 nm, $\beta$-T3 = 87.5 at 295 nm, $\gamma$-T3 = 103.0 at 298 nm, $\delta$-T3 = 83.0 at 292 nm, and a-T acetate = 42 at 286 nm.[27] Working standard solutions were prepared in methanol by appropriate dilution of the stock standard solutions, spiking with an appropriate amount of the IS.

**Instrumentation.** A 1200 series liquid chromatograph provided with a quaternary pump, a membrane degasser, and a fluorescent detector (Agilent Technology, Inc., Urdorf, Switzerland) was used. The column was coupled to a Rheodyne injection valve with a 20 $\mu$L loop. Chromatograms were recorded and evaluated by Agilent Chemstation software, version B.04.01 (Agilent Technologies). The analytical column PerfectSil Target ODS-3, 3 $\mu$m, 250 × 4.6 mm, was kindly donated by MZ-Analysentechnik.

A Stuart (Bibby Sterlin, Ltd., U.K.) small vortexer, a Griffin Christ centrifuge (Griffin and George, Ltd., U.K.), and a Fisher Scientific ultrasonic bath (Elma-Ultrasonic, Germany) were employed for sample pretreatment. SPE was carried out on a 12-port vacuum manifold from Supelco (Bellefonte, PA).

**Chromatographic Conditions.** The chromatographic separation of the examined analytes was achieved by RP-HPLC using a 250 × 4.6 mm inner diameter , 3 $\mu$m particle size, PerfectSil Target ODS-3 column (MZ-Analysentechnik, Mainz, Germany) thermostatted at 7 °C. The mobile phase consisted of isopropanol (IsOH) and water and was delivered to the analytical column according to the gradient program of Table 1. The HPLC column was allowed to re-equilibrate for another 10 min prior the next injection. Ts and T3s were detected using fluorescence at 290 nm excitation and 320 nm emission, while the $\alpha$-T acetate (IS) was detected at 285 nm excitation and 330 nm emission.

**Table 1. Gradient Timetable**

| time (min) | isopropanol (%) | water (%) | flow rate (%) |
|---|---|---|---|
| 0 | 75 | 25 | 0.24 |
| 30 | 80 | 20 | 0.25 |
| 45 | 90 | 10 | 0.28 |
| 60 | 90 | 10 | 0.31 |
| 65 | 75 | 25 | 0.24 |

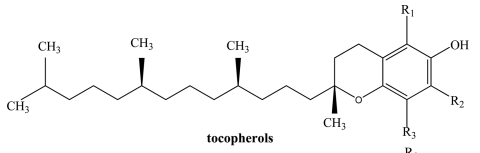
**Sample Preparation.** Tocols were extracted according to the method previously reported by the same authors[17] as follows: 0.1 g of well-homogenized pooled cereal flour was placed in a 15 mL screw-capped test tube with 2 mL of absolute ethanol, to remove proteins, and 200 $\mu$L of IS (50 $\mu$g/mL). After vortex mixing for 1 min, the test tube was placed in a sonication bath for 20 min. After removal from the sonication bath, the test tube was centrifuged at 3000 rpm for 5 min. The above procedure was repeated 1 more time. An aliquot of 2 mL of water was added to the combined extracts to decrease the percentage content of ethanol of supernatants and passed through a solid-phase cartridge (Oasis HLB), which was first conditioned with 3 mL of methanol and 3 mL of water. After washing with 2 mL of water, retained constituents were eluted with 2 mL of dichloromethane, followed by the evaporation to dryness under a gentle flow of nitrogen at 30 °C. The residue was reconstituted with 200 $\mu$L of methanol, and aliquots of 20 $\mu$L were injected into the HPLC column.

**Method Validation.** The proposed analytical method was fully validated in terms of linearity, inter- and intraday precision, accuracy, sensitivity, and selectivity. Linearity was checked by constructing the calibration curves using standard solutions and the spiked cereal sample at eight concentration levels, i.e., 0.1−40 $\mu$g/mL. Calibration curves were obtained by graphing the ratio of the peak area of the analyte to the peak area of the IS versus the nominal cereal concentration. Linear regression analysis was used to calculate the slope, intercept, and correlation coefficient of each calibration line. The calculations for the limit of detection (LOD) and the limit of quantification (LOQ) were based on the standard deviation (SD) of $y$ intercepts of regression analysis ($r$) and the slope ($S$), using the equations LOD = 3.3$r$/$S$ and LOQ = 10$r$/$S$. The assay precision

[relative standard deviation (RSD)] was studied by analyzing spiked samples at three concentration levels (2.5, 5, and 10 $\mu$g/mL), by expressing the SD of repeated measurements as a percentage of the mean value. Interday precision was estimated by three replicate measurements at the same concentration levels, while intraday precision was conducted during routine operation of the system over the period of 5 days. Accuracy was studied by analyzing three concentration levels (2.5, 5, and 10 $\mu$g/mL) and was expressed as a percentage recovery of spiked samples at the same concentrations. Selectivity was demonstrated by the analysis of blank matrices and was assessed by the absence of interference in the same chromatographic analysis as examined analytes in cereal samples.

## RESULTS AND DISCUSSION

**Optimization of T and T3 Separation.** Ts and T3s are closely related homologues and isomers depending upon the number and position of methyl groups on the aromatic ring of the tocol backbone in Ts, respectively: (1) $\delta$-T with one methyl group, (2) $\beta$- and $\gamma$-Ts with two methyl groups, and (3) $\alpha$-T with three methyl groups (Figure 1). The unsaturated




| form | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| α-T or α-$T_3$ | $CH_3$ | $CH_3$ | $CH_3$ |
| β-T or β-$T_3$ | $CH_3$ | H | $CH_3$ |
| γ-T or γ-$T_3$ | H | $CH_3$ | $CH_3$ |
| δ-T or δ-$T_3$ | H | H | $CH_3$ |

**Figure 1.** Chemical structures of Ts and T3s examined in this study.

analogues of Ts are T3s. The two dimethylated compounds in class 2 differ in structures merely by the position of methyl groups. In the RP-HPLC system, resolution of $\beta$- and $\gamma$-T has often been reported to be either poor or co-eluted.[6,9,16−25] The searching of new stationary phases capable of resolving the above problem may cause a challenge for the analysts. In the present study, the PerfectSil Target ODS-3 (3 $\mu$m, 250 × 4.6 mm inner diameter) stationary phase was used for the separation of T and T3 isomers. This novel sorbent was proven to be of excellent performance for the determination of quinolones[26] or drugs[28] in pharmaceutical formulations. Previously, Abidi and Mounts[12] demonstrated that RP-HPLC with mobile phases containing alkanols with carbon atoms greater than 2 favored the separation of the $\beta$−$\gamma$ pair on ODS columns. Thus, in the present study, isopropanol/water mixtures in various proportions were first tested as mobile phases in isocratic elution mode of 0.3 mL/min at 25 °C. As observed in Figure 2, the studied mobile phases at the above conditions were enable to resolve the $\beta$−$\gamma$ pair of Ts and T3s ($R_s$ < 1.5). Moreover, increasing the water content in the mobile phase resulted in a higher resolution factor and longer run times. To improve this separation, the column temperature was progressively reduced from 25 to 5 °C, keeping the mobile phase constant. As shown in Figure 3, an increase of selectivity

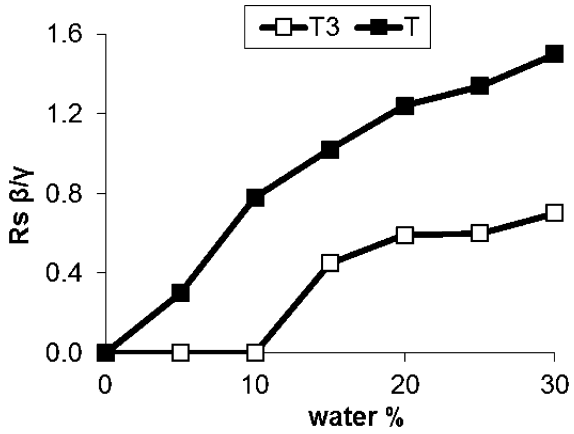



**Figure 2.** Influence of the mobile-phase composition on the separation of $\beta/\gamma$ isomers on the PerfectSil Target column. *T*, 25 °C; flow rate, 0.3 mL/min.

was observed when the column temperature decreased but the retention times were quite high. At 10 °C, the peaks of $\beta$- and $\gamma$-T were baseline-separated, in contrast with $\beta$- and $\gamma$-T3 that required a lower column temperature to increase selectivity. With respect to the duration of run analysis and the selectivity of the $\beta$−$\gamma$ pair of Ts and T3s, the column temperature was adjusted to 7 °C.

Because of the long run time analysis, a gradient elution system was applied, starting with 25% water content followed by a gradient decrease up to 10% to reduce the duration of analysis (<62 min). On the basis of these results, a gradient program as described in Table 1 at 7 °C column temperature was selected as the best compromise between efficiency, resolution, and analysis time. Under the described experimental conditions, the T and T3 isomers were well-resolved, with resolution factors ranged from 1.1 to 9.4, as shown in Figure 4A.

*Validation of the Method. Linearity and Sensitivity.* Calibration curves of eight points using a mixture of standard solutions were constructed covering a concentration range from 0.1 to 40 $\mu$g/mL. Equations were obtained by least-squares linear regression analysis of the peak area ratio of analyte/IS versus the analyte concentration. All calibration data as well as LOD and LOQ are presented in Table 2. The analytical method was linear up to 40 $\mu$g/mL for $\gamma$-T3, $\alpha$-T3, and $\alpha$-T, 30 $\mu$g/mL for $\beta$-T3, 20 $\mu$g/mL for $\delta$-T3, $\delta$-T, and $\beta$-T, and 15 $\mu$g/mL for $\gamma$-T.

Calibration curves constructed for the spiked cereal sample with standard solution, prepared as described in the Materials and Methods, are also presented in the same table. The analytical procedure was linear as mentioned above for standard solutions. Correlation coefficients were found to be more than 0.9980 for all analytes. All calibration data as well as LOD and LOQ are presented in Table 2. LOD values were 0.47−0.75 and 0.27−0.76 $\mu$g/mL for standard and spiked cereal samples, and corresponding LOQ values were 1.42−2.26 and 0.82−2.32 $\mu$g/mL, respectively.

*Selectivity.* With regard to selectivity, this has been proven to be satisfactory, as shown in chromatograms B and C of Figure 4 that present typical high-performance liquid chromatograms of Ts and T3s extracted from the spiked cereal sample at 5 $\mu$g/mL and the corresponding blank cereal sample. All T and T3 isomers were determined without any interference peaks

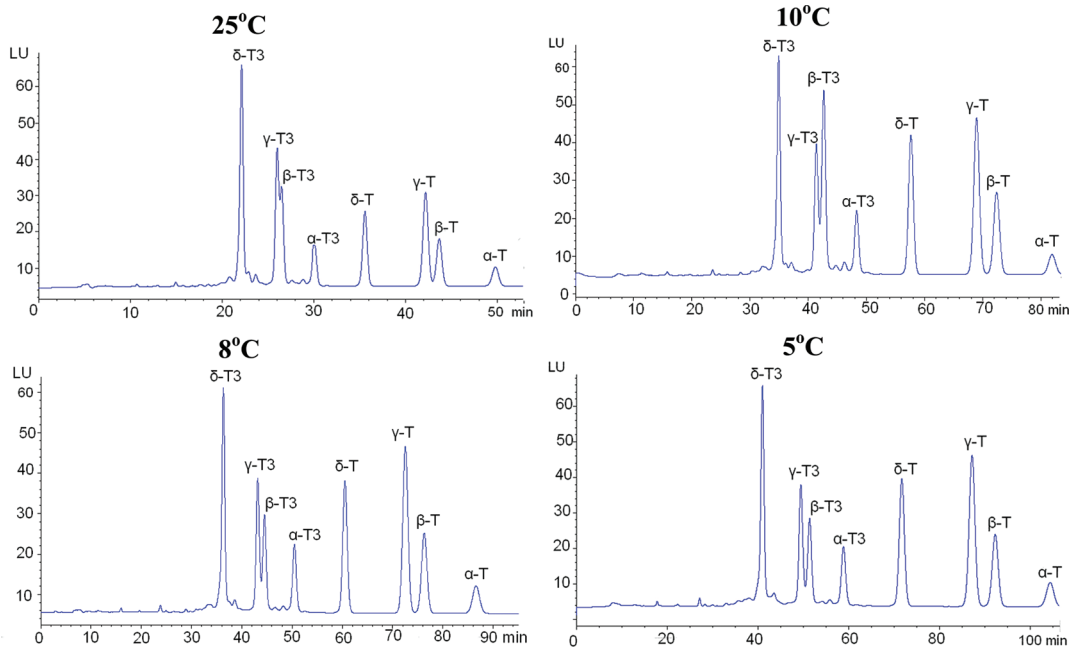



**Figure 3.** HPLC separation of T and T3 isomers at different temperatures with the PerfectSil Target stationary phase. Mobile phase, isopropanol/water (75:25, v/v); flow rate, 0.3 mL/min.

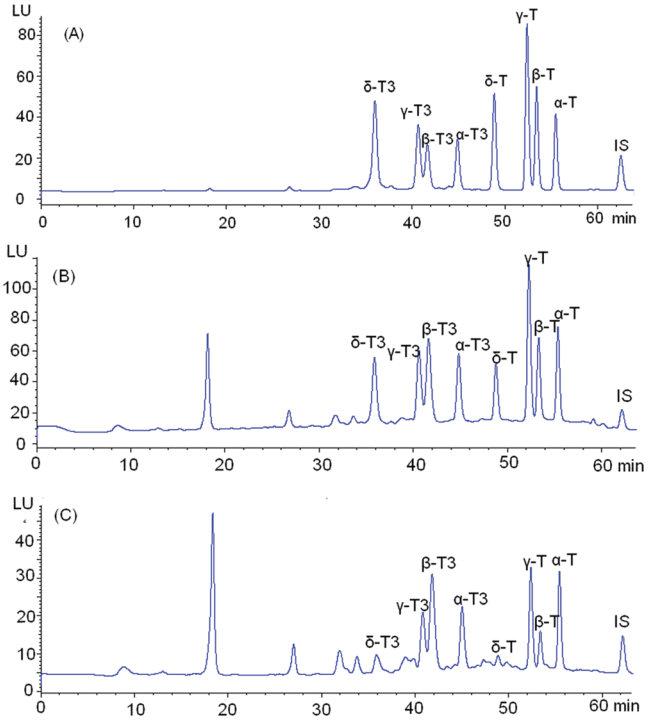



**Figure 4.** Chromatograms of the (A) mixture of standard solutions, (B) cereal sample spiked with a mixture of standard solutions at 5 μg/mL, and (C) blank cereal sample.

around the retention time of the analytes in any of the samples analyzed.

*Precision and Accuracy.* Intra- and interday precision and accuracy results for the spiked cereal sample at 50, 100, and 200 ng are summarized in Table 3. Excellent repeatability and satisfactory interday precision were noticed with RSD values lower than 6.2 and 8.9%, respectively, while mean recoveries were between 87.0 and 115.5% for the selected levels.

**Table 2. Linearity and Sensitivity Data of Ts and T3s in Standard Solutions and Spiked Cereal Sample (with *x* Values in Nanograms)**

| analyte | regression equation | $r^2$ | LOD (μg/mL) | LOQ (μg/mL) |
|---|---|---|---|---|
| | Standard Solutions | | | |
| α-T | $y = (0.0126 \pm 0.0001)x + (0.4576 \pm 0.0571)$ | 0.9993 | 0.75 | 2.26 |
| β-T | $y = (0.0226 \pm 0.0003)x + (0.0415 \pm 0.0664)$ | 0.9990 | 0.49 | 1.47 |
| γ-T | $y = (0.0335 \pm 0.0006)x + (0.2511 \pm 0.1065)$ | 0.9985 | 0.53 | 1.59 |
| δ-T | $y = (0.0230 \pm 0.0004)x + (0.0614 \pm 0.0701)$ | 0.9990 | 0.50 | 1.52 |
| α-T3 | $y = (0.0134 \pm 0.0002)x + (0.1565 \pm 0.0552)$ | 0.9992 | 0.68 | 2.06 |
| β-T3 | $y = (0.0177 \pm 0.0002)x -$ $(0.0146 \pm 0.0503)$ | 0.9995 | 0.47 | 1.42 |
| γ- T3 | $y = (0.0183 \pm 0.0002)x + (0.2045 \pm 0.0678)$ | 0.9995 | 0.61 | 1.85 |
| δ-T3 | $y = (0.0300 \pm 0.0005)x + (0.1171 \pm 0.1164)$ | 0.9988 | 0.64 | 1.94 |
| | Cereal Sample | | | |
| α-T | $y = (0.0135 \pm 0.0001)x + (0.0796 \pm 0.0374)$ | 0.9995 | 0.46 | 1.38 |
| β-T | $y = (0.0213 \pm 0.0004)x + (0.3952 \pm 0.0778)$ | 0.9986 | 0.60 | 1.82 |
| γ-T | $y = (0.0382 \pm 0.0006)x + (0.6233 \pm 0.0628)$ | 0.9991 | 0.27 | 0.82 |
| δ-T | $y = (0.0202 \pm 0.0002)x + (0.2001 \pm 0.0508)$ | 0.9995 | 0.41 | 1.26 |
| α-T3 | $y = (0.0169 \pm 0.0002)x + (0.1131 \pm 0.0559)$ | 0.9994 | 0.55 | 1.65 |
| β-T3 | $y = (0.0181 \pm 0.0002)x + (0.2713 \pm 0.0481)$ | 0.9996 | 0.44 | 1.33 |
| γ- T3 | $y = (0.0222 \pm 0.0003)x + (0.6368 \pm 0.1030)$ | 0.9988 | 0.76 | 2.32 |
| δ-T3 | $y = (0.0301 \pm 0.0006)x + (0.3721 \pm 0.1355)$ | 0.9980 | 0.74 | 2.25 |

**Column Validation.** To establish the applicability of the PerfectSil Target sorbent to T and T3 analyses, column validation


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**Table 3. Precision and Accuracy Data in Spiked Cereal Samples**

| | | intraday ($n$ = 3) | | | interday ($n$ = 5 × 3) | | |
|---|---|---|---|---|---|---|---|
| analyte | added (ng) | found ± SD (ng) | RSD (%) | recovery (%) | found ± SD (ng) | RSD (%) | recovery (%) |
| | 50 | 48.14 ± 1.02 | 2.1 | 96.3 | 49.74 ± 1.37 | 2.7 | 99.5 |
| α-T | 100 | 95.42 ± 4.41 | 4.6 | 95.4 | 102.10 ± 5.17 | 5.1 | 102.1 |
| | 200 | 226.39 ± 4.90 | 2.2 | 113.2 | 220.61 ± 7.29 | 3.3 | 110.3 |
| | 50 | 45.15 ± 1.42 | 3.1 | 90.3 | 45.43 ± 1.54 | 3.4 | 90.9 |
| β-T | 100 | 94.95 ± 3.73 | 3.9 | 95.0 | 96.08 ± 8.23 | 8.6 | 96.1 |
| | 200 | 219.09 ± 11.05 | 5.0 | 109.5 | 205.56 ± 7.59 | 3.7 | 102.8 |
| | 50 | 45.00 ± 0.82 | 1.8 | 90.0 | 44.77 ± 3.59 | 8.0 | 89.5 |
| γ-T | 100 | 95.64 ± 4.14 | 4.3 | 95.6 | 96.19 ± 6.32 | 6.6 | 96.2 |
| | 200 | 194.59 ± 11.99 | 6.2 | 97.3 | 182.86 ± 15.18 | 8.3 | 91.4 |
| | 50 | 57.74 ± 2.86 | 5.0 | 115.5 | 46.22 ± 1.46 | 3.2 | 92.4 |
| δ-T | 100 | 94.13 ± 2.69 | 2.9 | 94.1 | 99.07 ± 6.58 | 6.6 | 99.1 |
| | 200 | 224.00 ± 5.86 | 2.6 | 112.0 | 230.20 ± 19.04 | 8.3 | 115.1 |
| | 50 | 50.02 ± 0.71 | 1.4 | 100.0 | 50.55 ± 1.40 | 2.8 | 101.1 |
| α-T3 | 100 | 90.04 ± 3.92 | 4.4 | 90.0 | 97.30 ± 6.64 | 6.8 | 97.3 |
| | 200 | 199.61 ± 6.95 | 3.5 | 99.8 | 193.57 ± 4.99 | 2.6 | 96.8 |
| | 50 | 50.43 ± 0.83 | 1.6 | 100.9 | 50.88 ± 0.86 | 1.7 | 101.8 |
| β-T3 | 100 | 90.13 ± 2.19 | 2.4 | 90.1 | 93.65 ± 8.29 | 8.9 | 93.6 |
| | 200 | 199.53 ± 6.31 | 3.2 | 99.8 | 190.40 ± 15.86 | 8.3 | 95.2 |
| | 50 | 45.07 ± 0.64 | 1.4 | 90.1 | 48.18 ± 3.46 | 7.2 | 96.4 |
| γ-T3 | 100 | 87.00 ± 2.64 | 3.0 | 87.0 | 88.40 ± 1.60 | 1.8 | 88.4 |
| | 200 | 209.85 ± 11.40 | 5.4 | 104.9 | 198.99 ± 14.45 | 7.3 | 99.5 |
| | 50 | 50.92 ± 0.88 | 1.7 | 101.8 | 51.73 ± 1.11 | 2.2 | 103.5 |
| δ-T3 | 100 | 92.09 ± 5.37 | 5.8 | 92.1 | 98.10 ± 8.01 | 8.6 | 98.1 |
| | 200 | 212.18 ± 4.39 | 2.1 | 106.1 | 206.96 ± 7.57 | 3.7 | 103.5 |

**Table 4. PerfectSil Target Analytical Column Performance Characteristics**

| analyte | retention time ($t$) | relative retention time (RRT) | theoretical plate ($N$) | retention factor ($k$) | selectivity factor ($a$) | resolution ($R_s$) |
|---|---|---|---|---|---|---|
| δ-T3 | 34.1 | 0.56 | 23947 | 2.53 | | |
| γ-T3 | 38.7 | 0.64 | 29638 | 3.00 | 4.6 | 5.19 |
| β-T3 | 39.7 | 0.65 | 28042 | 3.11 | 1.0 | 1.07 |
| α-T3 | 43.2 | 0.71 | 40999 | 3.47 | 3.5 | 3.72 |
| δ-T | 47.3 | 0.78 | 63084 | 3.89 | 4.1 | 5.24 |
| γ-T | 50.9 | 0.84 | 88519 | 4.27 | 3.6 | 5.16 |
| β-T | 52.1 | 0.86 | 93992 | 4.39 | 1.2 | 1.60 |
| α-T | 54.2 | 0.89 | 103841 | 4.61 | 2.1 | 3.20 |
| IS | 60.8 | | 90575 | 5.29 | 6.6 | 9.41 |

**Table 5. Contents of Eight Vitamin E Isomers in Different Cereal Species[a]**

| | Ts (μg/g) | | | | T3s (μg/g) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| cereal | α-T | β-T | γ-T | δ-T | α-T3 | β-T3 | γ-T3 | δ-T3 | total tocols |
| durum wheat | 12.1 ± 0.8 | 3.4 ± 0.0 | nd[b] | 1.3 ± 0.1 | 6.1 ± 0.1 | 38.5 ± 1.4 | 2.4 ± 0.5 | nd | 63.8 ± 0.1 |
| bread wheat | 20.0 ± 0.7 | 10.0 ± 0.3 | 1.2 ± 0.2 | 1.2 ± 0.2 | 7.5 ± 0.9 | 40.3 ± 0.1 | nd | nd | 80.2 ± 0.6 |
| barley | 17.9 ± 0.2 | 0.9 ± 0.1 | 2.7 ± 0.4 | 0.4 ± 0.1 | 30.9 ± 1.1 | 16.4 ± 1.3 | 9.6 ± 0.3 | 3.0 ± 0.1 | 81.8 ± 0.6 |
| oat | 11.8 ± 0.3 | 0.4 ± 0.1 | nd | 1.4 ± 0.3 | 10.2 ± 0.1 | 8.3 ± 0.3 | nd | 2.4 ± 0.1 | 34.8 ± 0.3 |
| rye | 18.9 ± 0.3 | 5.2 ± 0.3 | nd | nd | 12.0 ± 0.6 | 20.0 ± 0.6 | 1.7 ± 0.5 | nd | 58.0 ± 1.5 |
| corn | 11.9 ± 0.1 | 0.5 ± 0.1 | 11.7 ± 0.2 | 2.0 ± 0.0 | 5.0 ± 0.6 | nd | 11.4 ± 0.7 | nd | 42.4 ± 1.6 |
| rice | 14.6 ± 0.2 | 0.6 ± 0.2 | 2.2 ± 0.3 | nd | 7.4 ± 0.4 | 5.4 ± 0.4 | 9.5 ± 0.7 | nd | 39.7 ± 1.1 |

[a]Values are the mean of three replicates ± SD. [b]nd = not detected.

was performed in terms of the relative retention time, RRT = $(t_{ri}/t_{rIS})$, the number of theoretical plates, $N = 16(t_r/w)^2$, the retention factor, $k = (t_r - t_0)/t_0$, the separation factor, $a = (t_{r2} - t_0)/(t_{r1} - t_0)$, and the resolution factor $R_s = 2(t_{r2} - t_{r1})/(w_1 + w_2)$.


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Here, $t_0$ refers to the retention time for an unretained compound; $t_{r1}$ and $t_{r2}$ are the retention times; and $w_1$ and $w_2$ are the baseline peak widths of successive peaks. The column performance for the separation of analytes is given in Table 4. A value of 1.5 for resolution implies a complete separation of all studied analytes, whereas $\beta$- and $\gamma$-T3 exhibit a satisfactory resolution ($R_s$ > 1.1).

**Application.** The results from the application of the method for the determination of T and T3 isomers in seven species of cereals (durum wheat, bread wheat, barley, oat, rye, rice, and corn) are presented in Table 5, where Ts and T3s are reported as single isomers and as the sum of single T or T3 (total T + T3). $\alpha$-T was the main isomer found among analyzed Ts, and $\beta$-T was not detected only in barley and oat. Small quantities of $\delta$-T were not found only in rye and rice, whereas $\gamma$-T was present in bread wheat, barley, rice, and corn. The main T3 found in bread wheat, durum wheat, and rye was $\beta$-T3, whereas that found in barley and oat was $\alpha$-T3 and that found in corn and rice was $\gamma$-T3. According to the results, the levels of total T + T3 ranged between 34.8 $\mu$g/g (oat) and 81.8 $\mu$g/g (barley) and were in good agreement with several previous reports.[5,6,29]

Therefore, the herein described method is a successful approach to separate T and T3 isomers in cereals. The developed method justified that retention can be controlled by either the amount of water in the mobile phase or the column temperature. The best compromise among efficiency, resolution, and analysis time (<62 min) was achieved with a 7 °C column temperature and a linear gradient elution system starting with isopropanol/water (75:25, v/v) and ending with isopropanol/water (90:10, v/v). The proposed method was successfully applied to simultaneous determination of T and T3 isomers in cereals.

The optimized RP-HPLC method offers advantages over the normal-phase HPLC method, such as column stability, reproducibility of retention times, and quicker equilibration. In the real world of routine chromatographic applications, the PerfectSil Target column offers no advantages for the separation of T and T3 isomers compared to $C_{30}$ stationary phases and monolithic columns, but their practical use is recommended with respect to the relatively low cost, although longer analysis times were achieved.

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### Notes

The authors declare no competing financial interest.

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