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Tocopherol and tocotrienol analysis in raw and cooked vegetables: A validated method with emphasis on sample preparation

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Abstract
Vegetables can be important dietary sources of vitamin E. However, data on vitamin E in raw and cooked vegetables are in part conflicting, indicating analytical pitfalls. The purpose of the study was to develop and validate an HPLC-FLD method for tocochromanol (tocopherols and tocotrienols) analysis equally suitable for raw and cooked vegetables. Significant instability of tocochromanols was observed in raw broccoli and carrot homogenates. Tocochromanols could be stabilized by freeze-drying or ascorbic acid addition prior to homogenization. The optimized protocol for tocochromanol analysis included knife and ball milling of freeze-dried vegetable pieces. Direct acetone extraction of vegetable powders allowed for satisfactory recoveries and precisions. A significant decrease of tocochromanols in baked compared to raw vegetables was shown, the extent of which varied largely between vegetables. For some raw vegetables, such as spinach or broccoli, underestimation of vitamin E in nutrient databases cannot be ruled out and should be examined.

Keywords: Tocopherol, Tocotrienol, Vitamin E, Vegetable, Tocopherol oxidase, Sample preparation, Enzyme activity, Nutrient databases

1. Introduction

Tocopherols and tocotrienols, collectively known as tocochromanols, are a group of naturally occurring antioxidants commonly referred to as vitamin E. α-, β-, γ- and δ-Tocochromanols differ in the number and/or positions of methyl groups at the chromanol ring (Supplementary Fig. 1). There is some controversy about vitamin E activities exhibited by the different forms of tocopherols and tocotrienols. According to the Food and Nutrition Board of the U.S. Institute of Medicine, α-tocopherol alone should be used for estimating vitamin E requirements and vitamin E intake recommendations, since the β-, γ-, and δ-tocochromanols are not converted to α-tocopherol in humans and are poorly recognized by the α-tocopherol transfer protein in the liver (Institute of Medicine, 2000). However, standard methods for vitamin E analysis such as the DIN EN 12822 still refer to the traditional concept of α-tocopherol equivalents calculated from α-, β-, γ- and δ-tocopherol contents multiplied with individual factors accounting for differences in vitamin activities (Deutsches Institut für Normung, 2012) and some nutrient databases still express vitamin E as α-tocopherol equivalents.

Tocopherols and tocotrienols are most commonly analyzed by HPLC. A large number of methods for the analysis of fruits, vegetables and other plant materials has been published in the past, varying in chromatographic and sample preparation procedures. Sample preparation protocols vary in homogenization and/or extraction steps. Most methods include saponification (Chun, Lee, Ye, Exler, & Eitenmiller, 2006; Konings, Roomans, & Beljaars, 1996; Ouchikh et al., 2011; Piironen, Syväoja, Varo, Salminen, & Koivistoinen, 1986), which is also part of DIN EN 12822. However, others use direct extraction protocols varying in the solvents and/or the extraction procedures (Barros, Carvalho, Morais, & Ferreira, 2010; Gómez-Coronado, Ibanez, Ruperez, & Barbas, 2004; Tangolar, Özogul, Tangolar, & Yağmur, 2011).

Own analyses indicated higher vitamin levels in certain baked vegetables than in the corresponding raw samples. This was especially pronounced for vitamin E in broccoli (unpublished data). Other authors also reported higher tocopherol levels in some heat-treated vegetables such as spinach or broccoli as compared to the respective raw vegetables (Bernhardt & Schlich, 2006; Chun et al., 2006). Similar observations have been made for the pro-vitamin β-carotene in various vegetables (Bernhardt & Schlich, 2006; Hart & Scott, 1995; Howard, Wong, Perry, & Klein, 1999; Lessin, Catigani, & Schwartz, 1997; Sungpuag, Tangchitpianvit, Chittchang, & Wasantwisut, 1999). These results were often attributed to increased extractability of the vitamins from heat-treated vegetables due to softening of the tissue by cell disruption (Bernhardt & Schlich, 2006; Hart & Scott, 1995).

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However, it is known that plant tissue damage releases enzymes such as ascorbate oxidase resulting in a loss of vitamin C in raw fruits and vegetables (Takamura, Terao, & Matoba, 2002). Similarly, enzymatic oxidation of vitamin E or b-carotene in raw vegetable homogenates might lead to their underestimation. In fact, some authors have suggested oxidizing enzymes being involved in the loss of vitamin E or b-carotene during food processing (Lessin et al., 1997; Murillo, Plumpton, & Gaunt, 1976).

Due to the conflicting data on vitamin E in some raw and heat-treated vegetables, the aim of the present study was to develop and validate a method equally suitable for the analysis of tocopherols and tocotrienols in raw and heat-treated vegetables. The stability of vitamin E during the homogenization and the extraction procedure of raw and baked vegetables were in the focus of this method development.

2. Materials and methods

2.1. Standards and reagents

a-, b-, c- and d-Tocopherols (P95% by HPLC) and a-, b-, c- and d-tocotrienols (P97% by HPLC) were from Merck KGaA (Calbiochem®, Darmstadt, Germany) and Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany), respectively. Acetone SupraSolv® (used for extractions) and LiChrosolv® gradient grade solvents (used for HPLC) were purchased from VWR International GmbH (Bruchsal, Germany). All other chemicals were analysis grade and obtained from either Sigma–Aldrich or VWR.

2.2. Vegetable samples

The vegetable samples used in this study (carrot, broccoli, red pepper, green pepper, spinach, green beans, kohlrabi, tomato, celery) were purchased in local supermarkets in the region of Karlsruhe, Germany, from September 2012 through March 2013.

2.3. Vegetable sample preparation

Raw vegetable samples were cleaned and inedible parts were removed. Carrots and kohlrabi were peeled. Subsequently, each vegetable was cut into pieces of about 2 cm in diameter or length. To determine vitamin E stability during sample preparation, different homogenization and/or stabilization procedures were tested using either fresh or freeze-dried vegetables.

In the first experiment, broccoli was homogenized either fresh or after freeze-drying as follows: three out of four broccoli aliquots (125–150 g each) were homogenized fresh in a B-400 mixer (BÜCHI Labortechnik AG, Flawil, Switzerland) for 10 s after addition of about 15 mL of water, about 15 mL of aqueous ascorbic acid solution (11%, w/w), or about 15 mL of aqueous acetic acid solution (11%, w/w), respectively. The final concentration of added ascorbic or acetic acid in the acidified broccoli homogenates was 1.2% (w/w) and the final pH values were 4.24 and 4.28, respectively. The added water content in the three fresh homogenates was 10–11% (w/w). The fourth broccoli aliquot (170 g) was freeze-dried and the lyophilized pieces were homogenized using a knife mill (GRINDOMIX GM 200, Retsch Technology GmbH, Haan, Germany; 25 s, 6000 U) and a ball mill (Mixer Mill MM 200, Retsch Technology GmbH; 1 min, 25 Hz) consecutively. To allow for equal extraction conditions, the fresh broccoli homogenates were also freeze-dried, knife milled and ball milled prior to extraction. Direct extraction and HPLC analysis (pentafluorophenyl (PFP) column) were carried out as detailed in Sections 2.4 and 2.6, respectively.

In the second experiment, broccoli, red pepper, and carrots were each divided into three aliquots (100–150 g each). One aliquot was directly homogenized after addition of aqueous ascorbic acid solution and mixing in a Büchi B-400 mixer as detailed above. The remaining two aliquots were homogenized after freeze-drying using a knife mill only (25 s, 6000 U) or a combination of a knife mill (25 s, 6000 U) and a ball mill (1 min, 25 Hz), respectively. Different from the former experiment, the samples that were homogenized fresh (without prior freeze-drying) were also not freeze-dried prior to extraction. This allowed for a direct comparison of the three sample preparation protocols, including the effect of extracting fresh (i.e. none freeze-dried) vs. freeze-dried material. The samples were further processed using the direct extraction protocol as detailed in Section 2.4 and analyzed by HPLC (PFP column) as described in Section 2.6.

In a third experiment, raw and baked vegetables (carrots, broccoli, and red pepper) were analyzed with and without stabilization prior to homogenization. Baking was carried out in a common household oven at 180 °C for 31 min (broccoli and carrots) or 21 min (red pepper). For analyses "without stabilization" the vegetable samples were homogenized freshly in a Büchi B-400 mixer for 10 s. Analyses "with stabilization" were conducted using the sample preparation protocol including freeze-drying of vegetable pieces and subsequent knife and ball milling as detailed above (first experiment). Direct extraction and HPLC analysis (pentafluorophenyl (PFP) column) were carried out as detailed in Sections 2.4 and 2.6, respectively.

2.4. Direct extraction of tocopherols and tocotrienols

Freeze-dried vegetable powders (100 mg) or fresh vegetable homogenates (1 g) obtained as described in Section 2.3 were weighed into 50 mL-centrifuge tubes (PP, Corning Inc., New York, USA). Following the addition of 10 mL acetone (containing 0.025% butylhydroxytoluene (BHT)), the sample was ultra-sonicated for 2 min and further extracted for 1 min using a Vortex mixer (Corning Inc.) at maximum speed. The sample was then centrifuged (3600×*g*, 6 °C, 2 min) and the acetone extract was collected in a 50 mL-volumetric flask. The extraction was repeated three more times, without the initial ultrasonic treatment. The volume of the combined extracts was made up to 50 mL with acetone (containing 0.025% BHT) and a 10 mL-aliquot was transferred into a 12 mL-glass vial and dried under a stream of nitrogen. The dried extract was re-solubilized in 500]L of a methanol/acetone/water mixture (54:40:6; v/v). Following filtration of the extract (PTFE, 1.2]m, Phenomenex, Aschaffenburg, Germany), HPLC-FLD analysis was carried out as described in Section 2.6 on a PFP column.

2.5. Extraction of tocopherols and tocotrienols following saponification

The saponification was carried out according to DIN EN 12822:2000 (Deutsches Institut für Normung, 2000). In brief, freeze-dried vegetable powders (100 mg) were weighed into 50 mL-screw-top Erlenmeyer flasks and saponified under nitrogen atmosphere after addition of ethanol (25 mL; 96%), sodium sulphide (10 mg), ascorbic acid (250 mg) and potassium hydroxide solution (5 mL; 60%, w/v) for 35 min at 85 °C in a water bath. The suspension was cooled on ice for 30 min prior to extraction. The saponified sample was transferred into a 50 mL-centrifuge tube and extracted four times with 10 mL of n-hexane (1 min, Vortex at maximum speed). The combined n-hexane extracts were washed neutral with water. Centrifugation (3600×*g*, 6 °C, 1 min) had to be carried out after neutralization with water to improve phase separation. The neutralized n-hexane extract was transferred into a 50 mL-volumetric flask and the volume was made up with n-hexane. A 10 mL-aliquot of the extract was transferred into a 12 mL-glass vial and further treated as described in Section 2.4. HPLC-FLD analysis was carried out using a PFP column as detailed in Section 2.6.

2.6. HPLC-FLD analyses

HPLC analyses were carried out on a Shimadzu high pressure gradient system consisting of a DGU-20A$_5$ degasser, two LC-30AD pumps, an SIL-30AC autosampler, a CTO-20AC column thermostat, an SPD-M20A diode array detector (DAD), and an RF-511 fluorescence detector (FLD).

Chromatographic analyses of tocopherols and tocotrienols were generally performed on a PFP stationary phase (Kinetex PFP, 150 × 3 mm, 2.6 lm equipped with a KrudKatcher ULTRA HPLC In-Line Filter, 0,5 lm depth filter, Phenomenex, Aschaffenburg, Germany) with a column temperature of 24 °C and a flow rate of 1.3 mL/min. Elution was carried out using the following gradient of methanol/water (85:15, v/v; eluent A) and *tert*-methylbutylether/methanol/water (80:18:2, v/v/v; eluent B): 0–15 min 0% B, 15–25 min 0–15% B, 25–28 min 15–80% B, 28–31 min 80% B, 31–33 min 80–0% B, and 33–45 min 0% B. The autosampler temperature was set at 4 °C and the injection volume was 10 lL. Tocopherols and tocotrienols were detected and quantified at 295 nm excitation and 330 nm emission wavelengths, respectively. A standard chromatogram is shown in Fig. 1A. In addition, the UV/Vis absorbance was monitored (DAD, 190–600 nm).

A second chromatographic method was developed using a C30 phase (Develosil RP Aqueous C30, 150 × 3 mm, 3 lm, Phenomenex, Aschaffenburg, Germany) to confirm selectivity of the PFP separation delineated above. The C30-HPLC parameters were as follows: column temperature 18 °C; flow rate 0.5 mL/min; gradient elution with methanol/water (91:9, v/v; eluent A) and *tert*-methylbutylether/methanol/water (80:18:2; v/v/v; eluent B): 0–20.5 min 0% B, 20.5–25 min 0–10% B, 25–36 min 10% B, 36–46 min 10–55% B, 46–48 min 55–80% B, 48–51 min 80% B, 51–53 min 80–0% B, 53–63 min 0% B (Fig. 1B).

2.7. Calculation of α-tocopherol equivalents and statistical analyses

a-Tocopherol equivalents were calculated according to McLaughlin and Weihrauch (1979). Statistical comparisons of results were made by the two-tailed *t*-test at the 0.05 level of significance using the program SigmaPlot 12.0.

2.8. Method validation

2.8.1. Linearity

External calibration curves were used for quantitation in the working ranges of 0.5–40.5 lg/mL, 0.5–8.1 lg/mL, and 0.1–8.1 lg/mL for a-tocopherol, a-tocotrienol, and b-, c- and d-tocopherols and -tocotrienols, respectively. Weighted linear regression (1/y) was performed using the program SigmaPlot 12.0. Visual inspection of residual plots and correlation coefficients, which typically ranged from 0.9996 to 1.0000, indicated a good fit of the regression model.

2.8.2. Accuracy and precision

The sample preparation and extraction protocol that showed the best results (freeze-drying of vegetable pieces and subsequent homogenization by knife and ball milling as detailed in Section 2.3; direct extraction method as described in Section 2.4; PFP column) was subjected to further method validation. Validation was carried out using carrots, broccoli and red pepper as representatives for the matrix "vegetables".

Accuracy was tested by recovery studies, spiking the vegetable samples with known amounts of a-, b-, c- and d-tocopherols as well as a- and b-tocotrienols. For each analyte the recovery was determined at three concentration levels (low, medium and high) analyzing six replicates per concentration level. The levels chosen covered the respective working ranges and slightly differed between vegetables due to naturally occurring tocopherol and/or tocotrienol levels in unspiked vegetables. Low levels: 0.5–1.7 lg/mL of extract for b-tocopherol, c-tocopherol, d-tocopherol, a-tocotrienol and b-tocotrienol or 3.5 lg/mL of extract for a-tocopherol. Medium levels: 3.0–3.4 lg/mL of extract for b-tocopherol, c-tocopherol, d-tocopherol, a-tocotrienol and b-tocotrienol or 14.5 lg/mL of extract for a-tocopherol. High levels: 5.2–6.6 lg/mL of extract for b-tocopherol, c-tocopherol, d-tocopherol, a-tocotrienol and b-tocotrienol or 29.5–30.6 lg/mL of extract for a-tocopherol.

The within-run (intra-batch) precision was calculated from the results of the recovery studies detailed above (three concentration levels per analyte). The coefficients of variation (CV) were calculated for each concentration level from the standard deviation of six replicates.

The between-run (inter-batch) precision of the method was assessed by triplicate analysis of freeze-dried baked red pepper at three different days within the time frame of four months.

3. Results and discussion

3.1. Development of the chromatographic procedure

Separation of tocopherols and tocotrienols can be carried out by using normal phase (Stöggl, Huck, Scherz, Popp, & Bonn, 2001) or reversed phase (RP) columns. However, the use of standard C18 phases does not allow for a separation of the b- and c-isomers under routine conditions. Recently, a satisfactory separation of b- and c-tocopherols as well as b- and c-tocotrienols was achieved under RP-HPLC conditions using a PerfectSil Target ODS-3 column as the stationary phase (Irakli, Samanidou, & Papadoyannis, 2012). However, this method requires low column temperatures (7 °C) – a prerequisite which cannot always be met due to equipment limitations, i.e. column oven cooling capacity – and also resulted in long run times (75 min including column cleaning and equilibration). In addition, b- and c-tocotrienols were not fully resolved. Alternative stationary phases which have been applied for tocopherol and tocotrienol separation in RP-HPLC mode are for example C30 (Stöggl, Huck, Wongyai, Scherz, & Bonn, 2005), PFP (Grebenstein & Frank, 2012), or monolithic silica coated with poly(octadecyl methacrylate) (Núñez, Ikegami, Miyamoto, & Tanaka, 2007). In this study, chromatographic conditions were optimized on both, a PFP and a C30 column. The standard chromatograms obtained are shown in Fig. 1A and B, respectively. By using *tert*-methylbutylether besides methanol as organic modifier in the eluent, strongly retained sample matrix components can be rinsed off the column during the cleaning step. Due to different selectivity, the retention behaviour of the analytes varied between the chromatographic methods: on the PFP column, the elution order was d–b–c–a, whereas on the C30 column it was d–c–b–a. Better resolution and a shorter run time (45 min including column cleaning and equilibration) were realized using the PFP column, making it the preferred method. In addition, a rather low column oven temperature (18 °C) was required for c- and b-isomer resolution on the C30 column. Therefore, this method can only be applied when a column oven with cooling function is available. However, using the C30 column at higher temperatures, satisfying resolution of c- and b-isomers may be achieved by mobile phase adjustments, but this will lead to longer run times. Fig. 1C–J show sample chromatograms obtained from the analyses of various vegetables using the PFP column.

Although more time consuming, the method developed on the C30 phase is useful as a secondary method to confirm peak identity in unknown matrices.

3.2. Development of the sample preparation procedure

During early method development different solvents or solvent mixtures (hexane, acetone, hexane/ethyl acetate (85:15, v/v) and

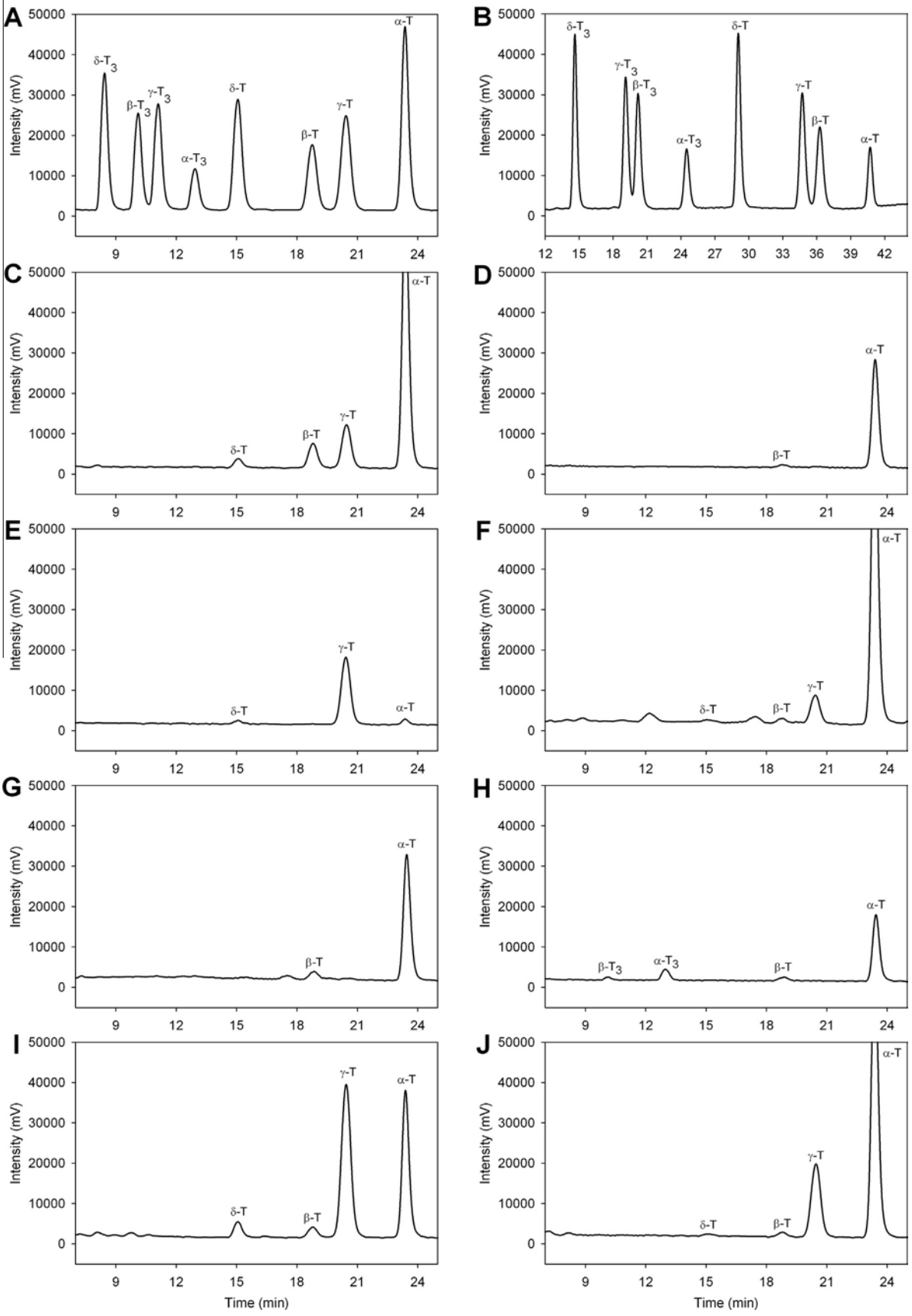

Fig. 1. Sample HPLC-FLD chromatograms (excitation at 295 nm, emission at 330 nm); (A) tocopherol and tocotrienol standard mixture analyzed on a Kinetex PFP column; (B) tocopherol and tocotrienol standard mixture analyzed on a Develosil RP-Aqueous C30 column; (C–J) vegetable extracts analyzed on the Kinetex PFP column; (C) red pepper; (D) green pepper; (E) princess beans; (F) spinach; (G) celery; (H) carrot; (I) tomato; (J) broccoli. (T3) tocotrienols; (T) tocopherols.

ethanol/hexane (50:50, v/v) were tested for their extraction efficiencies for tocopherols and tocotrienols. The best results were obtained with acetone, which therefore was chosen for all further analyses. However, when adopting this method, blank extractions should be performed to test the purity of the acetone used. In our laboratory, some batches of analysis grade acetone contained trace amounts of an unknown fluorescent compound that co-eluted with c-tocotrienol, on both the PFP column and on the C30 column. This impurity was not observed when using acetone of a higher grade (see Section 2.1).

Preliminary experiments spiking broccoli with b-tocopherol (which is only found in negligible amounts in broccoli) indicated a higher instability of tocopherols in raw broccoli homogenate than in heat-treated broccoli homogenate (data not shown). Since the samples were immediately extracted following homogenization, the degradation of tocopherols occurs very rapidly. Thus, stabilization is required not only during extraction, but already during the initial homogenization step. In this study, different methods of stabilization were tested for the homogenization of raw broccoli samples (Fig. 2). Both, ascorbic acid addition and freeze-drying resulted in a significant increase of tocopherols in the sample extracts as compared to the non-stabilized sample, whereas addition of acetic acid only led to a small increase, which may be explained by decreased enzyme activity due to the reduced pH. The results indicate the involvement of oxidizing enzymes in tocopherol degradation in the raw broccoli homogenate. When vegetable cells are damaged, for example by cutting or mixing, oxidizing enzymes such as polyphenol oxidases or ascorbate oxidases are activated due to the loss of cell compartments (Takamura et al., 2002). Tocopherols and tocotrienols are not known as substrates for polyphenol oxidases. In a-tocopherol, which is the dominant isomer in broccoli and most other vegetables, both *ortho* positions of the hydroxyl function are occupied by methyl groups, making *ortho* hydroxylation by polyphenol oxidase impossible. Broccoli was also shown to contain only trace amounts of polyphenol oxidases but rather high amounts of ascorbate oxidase (Yamaguchi et al., 2003). Ascorbic acid is a plant cell antioxidant protecting susceptible compounds such as tocopherols or carotenoids from oxidative damage. It is able to regenerate tocopherols from their oxidized forms. The loss of vitamin E during homogenization of raw broccoli may partly be a result of ascorbate oxidation and reduced tocopherol protection and/or regeneration. However, although tocopherol degradation in plant material is most commonly ascribed to non-enzymatic oxidation by reactive oxygen species, several studies demonstrated the presence of specific tocopherol oxidases in plant tissues (Barlow & Gaunt, 1972; Murillo et al., 1976; Szymańska & Kruk, 2013). a-Tocopherol oxidase was first identified in peas (*Pisum sativum* L.) (Barlow & Gaunt, 1972). The authors showed, that tocopherol oxidase from pea shoots requires molecular oxygen and phospholipids for its activity. Tocopherol oxidase has been found in a variety of food plants, including legumes, plants of the genus *Brassica*, and many fruits (Murillo et al., 1976). It is therefore most likely the main driver of the pronounced tocopherol degradation observed in raw broccoli, which belongs to the genus *Brassica*. Tocopherol oxidase may be present in all parts of the plant (Murillo et al., 1976). In *Phaseolus coccineus* seedlings, the highest tocopherol oxidase activities were measured in the leaves (Szymańska & Kruk, 2013). Moreover, this enzyme was shown to have a high substrate specificity (a » b > c > d) and most likely belongs to the multicopper oxidases, a family which also comprises polyphenol oxidases or laccases (Szymańska & Kruk, 2013). The authors identified tocopherolquinones as reaction products, which appeared to be more dominant for a-tocopherol as compared to b-, c- or d-tocopherol. However, the results indicated the presence of other, yet unidentified products. Barlow and Gaunt (1972) also described three unidentified reaction products when a-tocopherol was used as a substrate. The mechanism of the enzymatic oxidation by tocopherol oxidase therefore remains to be elucidated.

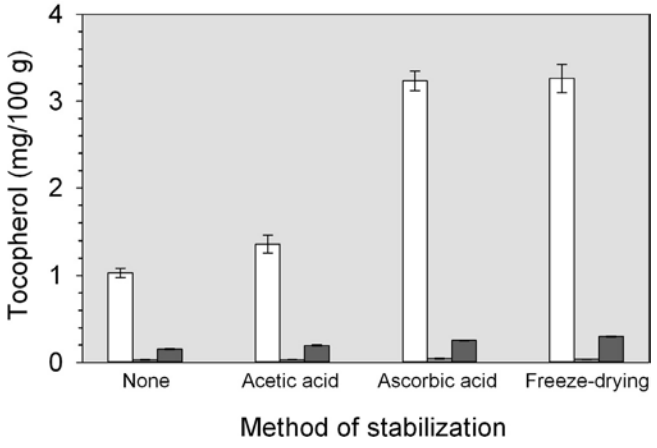

Fig. 2. a, b- and c-Tocopherol contents in raw broccoli obtained by different homogenization/stabilization methods. None: addition of water and mixing in a Büchi GM 200; acetic acid: addition of acetic acid and mixing in a Büchi GM 200 (final acetic acid concentration 1.2% by weight, pH 4.28); ascorbic acid: addition of ascorbic acid and mixing in a Büchi GM 200 (final ascorbic acid concentration 1.2% by weight, pH 4.24); freeze-drying: broccoli pieces were freeze-dried and milled (subsequent knife and ball milling). To allow for equal extraction conditions the fresh broccoli homogenates were also freeze-dried and milled prior to analysis. Extraction was carried out with acetone containing 0.025% BHT. White bars, a-tocopherol, light grey bars, b-tocopherol, and dark grey bars, c-tocopherol. Tocopherol contents were calculated on a fresh weight basis. Each sample was analyzed in duplicate.

Raw vegetable homogenates may be stabilized by ascorbic acid addition or by reducing the water activity prior to homogenization, for example by freeze-drying. In some studies, vegetable samples were homogenized prior to freeze-drying (Chun et al., 2006; Konings et al., 1996). However, this may result in a significant loss of tocopherols/tocotrienols and possibly other analytes that are prone to enzymatic oxidation. Therefore, when freeze-drying is part of the sample preparation protocol for vitamin E analyses in raw vegetables, it is essential to lyophilize the samples in pieces allowing for a significant reduction in water activity *prior* to homogenization. Ascorbic acid addition and freeze-drying are equally efficient stabilizing methods (Fig. 2). However, in the experiment shown in Fig. 2, the homogenate stabilized with ascorbic acid was also freeze-dried and ball milled prior to extraction (after homogenization) allowing for equal analysis conditions.

In another experiment, tocopherols in carrots, broccoli, and red pepper were analyzed after direct extraction with acetone containing 0.025% BHT (Fig. 3). Extraction yields increased, when using freeze-dried powders (Fig. 3, dark grey bars) as compared to fresh homogenates stabilized with ascorbic acid (Fig. 3, white bars). However, freeze-dried vegetables should be consecutively milled in a knife mill and a ball mill as opposed to using a knife mill only (Fig. 3, light grey bars). Otherwise, the extraction yields of some freeze-dried vegetables may be limited if the particle size of the powder is too large. This was, for example, observed for red pepper, where larger exocarp particles were visible in knife milled samples, which could be broken down by subsequent ball milling resulting in a higher extraction yield (Fig. 3). Overall, ball milling promotes the disruption of cell walls ensuring maximum extraction yields. However, it has to be kept in mind, that the extraction solvent acetone is slightly diluted with water when extracting fresh samples. The direct extraction protocol using freeze-dried vegetable powders and acetone (containing 0.025% BHT) as extraction

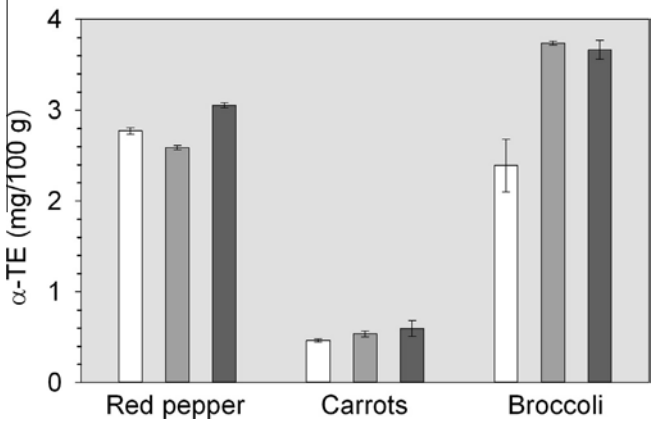

Fig. 3. Vitamin E contents of raw vegetables analyzed after acetone extraction (acetone containing 0.025% BHT) of differently prepared samples. White bars, fresh homogenates (stabilized with 1.2% ascorbic acid); light grey bars, coarse powders (vegetable pieces were freeze-dried and knife milled); dark grey bars, fine powders (vegetable pieces were freeze-dried, knife milled and subsequently ball milled). Samples were analyzed by HPLC-FLD using a Kinetex PFP column. Vitamin E contents were calculated as a-tocopherol equivalents (a-TE) on a fresh weight basis. Each sample was analyzed in triplicate.

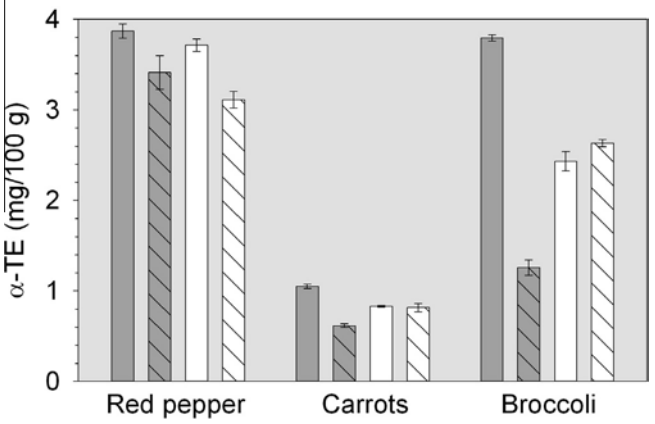

Fig. 4. Vitamin E contents of raw and baked vegetables with and without stabilization during homogenization. Grey bars, raw vegetable; white bars, baked vegetable; solid bars, stabilized homogenate (freeze-dried in pieces and homogenized by knife milling and ball milling); striped bars, non-stabilized homogenate (homogenized prior to freeze-drying). To allow for equal extraction conditions the non-stabilized homogenates were also freeze-dried and milled prior to analysis. Extraction was carried out with acetone containing 0.025% BHT. Samples were analyzed by HPLC-FLD using a Kinetex PFP column. Vitamin E contents were calculated as a-tocopherol equivalents (a-TE) on a raw, fresh weight basis. Each sample was analyzed in triplicate.

solvent also led to higher yields than the extraction following saponification (DIN EN 12822:2000) (data not shown).

Fig. 4 shows the vitamin E contents measured in raw and baked red pepper, carrot and broccoli samples, which have been homogenized before or after freeze-drying. The data indicates that the extent of thermally induced vitamin E loss differs between vegetables, in this case being most pronounced for broccoli. Baking led to a significant vitamin E reduction of 21% or 36% for carrots and broccoli, respectively. This loss may be due to thermally catalyzed non-enzymatic processes and/or tocopherol oxidase activity in the initial phase of baking – when tissue damage already occurs and enzymes are not yet fully heat-inactivated. These data refute former results for vitamin E in raw and cooked broccoli (Bernhardt & Schlich, 2006). The authors reported that the a-tocopherol level in fresh broccoli substantially increased (factor of 5) by various cooking methods. They assumed that cooking leads to a better availability of a-tocopherol. The present study, however, showed that the results are most likely due to enzymatic a-tocopherol losses in raw broccoli. The smaller, but also significant ($p = 0.008$), loss of vitamin E in red pepper may be explained by a shorter baking time (21 min vs. 31 min, as required for doneness) and/or a higher natural ascorbic acid level compared to broccoli and carrots.

Table 1
Recovery of tocopherols (T) and tocotrienols (T_3) and within-run precision as determined in recovery experiments (coefficient of variation, CV; $n = 6$).

Analyte	Low level[a]		Medium level[b]		High level[c]	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
Broccoli						
a-T	n. a.[d]	n. a.	n. a.	n. a.	103.6	2.1
b-T	89.1	2.7	94.9	1.2	92.4	2.5
c-T	n. a.	n. a.	93.9	0.4	92.1	1.9
d-T	93.0	2.6	92.4	1.5	95.6	2.5
a-T_3	91.6	2.5	91.9	1.3	91.3	2.7
b-T_3	114.9	4.1	104.5	1.8	102.2	1.9
Carrot						
a-T	105.2	2.2	98.7	4.7	108.3	1.9
b-T	n. a.	n. a.	97.7	4.4	101.1	1.8
c-T	98.4	2.7	85.3	4.3	92.1	1.7
d-T	99.8	1.8	86.4	4.6	95.7	1.4
a-T_3	n. a.	n. a.	84.1	4.5	91.5	1.9
b-T_3	101.7	3.8	100.2	3.1	104.4	0.8
Red pepper						
a-T	n. a.	n. a.	n. a.	n. a.	105.5	1.6
b-T	n. a.	n. a.	112.1	1.3	109.0	2.0
c-T	n. a.	n. a.	89.6	1.6	88.3	1.9
d-T	n. a.	n. a.	88.3	1.8	90.0	2.1
a-T_3	99.6	1.8	102.1	1.7	101.3	2.6
b-T_3	113.0	2.9	99.9	3.2	105.1	1.7

n. a., Not analyzed.

[a] Low levels were 0.5–1.7 lg/mL of extract for b-T, c-T, d-T, a-T_3 and b-T_3 or 3.5 lg/mL of extract for a-T.

[b] Medium levels were 3.0–3.4 lg/mL of extract for b-T, c-T, d-T, a-T_3 or b-T_3 or 14.5 lg/mL of extract for a-T.

[c] High levels were 5.2–6.6 lg/mL of extract for b-T, c-T, d-T, a-T_3 or b-T_3 or 29.5–30.6 lg/mL of extract for a-T.

[d] Recovery was not analyzed at this level due to naturally occurring analyte level.

Table 2
Tocopherol (T), tocotrienol (T_3) and α-T equivalents (α-TE) in raw vegetables analyzed in this study and vitamin E levels listed in nutrient databases.

Vegetable (raw)	α-T (mg/100 g fresh weight)	β-T	γ-T	δ-T	α-T_3	β-T_3	γ-T_3	δ-T_3	α-TE (calculated)	Vitamin E databases[a]
Red sweet pepper A	3.78 ± 0.08[b]	0.19 ± 0.00	0.17 ± 0.00	0.04 ± 0.00	n. d.	n. d.	n. d.	n. d.	3.87 ± 0.08	1.6–3.2
Red sweet pepper B	3.01 ± 0.03	0.11 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	<LOQ[c]	n. d.	n. d.	n. d.	3.06 ± 0.03	
Red sweet pepper C	2.72 ± 0.05	0.10 ± 0.00	0.03 ± 0.00	<LOQ	n. d.	n. d.	n. d.	n. d.	2.77 ± 0.05	
Broccoli A	3.75 ± 0.03	0.03 ± 0.00	0.29 ± 0.00	<LOQ	n. d.	n. d.	n. d.	n. d.	3.80 ± 0.03	0.4–1.3
Broccoli B	3.62 ± 0.12	0.02 ± 0.00	0.40 ± 0.01	0.02 ± 0.00	n. d.	n. d.	n. d.	n. d.	3.67 ± 0.03	
Broccoli C	3.33 ± 0.09	<LOQ	0.38 ± 0.00	<LOQ	n. d.	n. d.	n. d.	n. d.	3.37 ± 0.09	
Carrot A	1.03 ± 0.03	0.06 ± 0.00	n. d.	n. d.	<LOQ	n. d.	n. d.	n. d.	1.05 ± 0.03	0.5–0.7
Carrot B	0.58 ± 0.09	0.02 ± 0.00	n. d.	<LOQ	0.19 ± 0.00	<LOQ	n. d.	n. d.	0.59 ± 0.09	
Carrot C	0.53 ± 0.04	<LOQ	n. d.	n. d.	n. d.	<LOQ	n. d.	n. d.	0.53 ± 0.04	
Spinach	3.97 ± 0.04	<LOQ	0.13 ± 0.00	<LOQ	n. d.	n. d.	n. d.	n. d.	3.98 ± 0.04	1.2–2.9
Tomato	1.00 ± 0.02	0.04 ± 0.00	0.38 ± 0.00	0.03 ± 0.00	n. d.	n. d.	n. d.	<LOQ	1.06 ± 0.02	0.5–1.1
Celery	0.44 ± 0.03	0.01 ± 0.00	<LOQ	n. d.	<LOQ	n. d.	n. d.	n. d.	0.44 ± 0.03	0.2–0.3
Kohlrabi	<LOQ	n. d.	<LOQ	n. d.	n. d.	n. d.	n. d.	n. d.	<LOQ	<0.1–0.5
Green beans	<LOQ	n. d.	0.19 ± 0.01	<LOQ	n. d.	n. d.	n. d.	n. d.	0.02 ± 0.00	0.1–0.4
Green sweet pepper	0.63 ± 0.03	<LOQ	<LOQ	n. d.	<LOQ	n. d.	n. d.	n. d.	0.63 ± 0.01	0.4–2.5

[a] Average α-tocopherol values listed in the nutrient databases of the following institutions: USDA (USA, α-T), MRI (Germany, α-TE), DTU (Denmark, α-TE), NFA (Sweden, α-TE), THL (Finland, α-T).
[b] Values are mean ± SD of each vegetable sample analyzed in triplicate.
[c] Since freeze-dried vegetable powders were weighed for analysis, the limit of quantitation (LOQ) per fresh weight varies slightly depending on moisture loss during freeze-drying: 0.01–0.02 mg/100 g fresh weight for δ-T and δ-T_3; 0.01-0.04 mg/100 g fresh weight for β-T, γ-T, and β-T_3; 0.05-0.2 mg/100 g fresh weight for α-T and α-T_3.

Contrarily, without sufficient stabilization during homogenization (Fig. 4, striped bars), significantly higher vitamin E levels were found in baked than in raw vegetables (factor of 2.1 and 1.3 for broccoli and carrots, respectively). In the case of red pepper no such effect could be observed. This is in accordance with data from Bernhardt and Schlich (2006) who reported a slight decrease in α-tocopherol levels in cooked vs. raw red pepper (factor of 0.9) in contrast to their results for broccoli. The data indicate that there was low or no tocopherol oxidase activity in the red sweet pepper samples used.

The possibility of enzymatic oxidation reactions in raw vegetable samples seems obvious. However, oftentimes it is neglected when it comes to sample homogenization. In a recently published method for the quantitation of vitamin E in green leafy vegetables, fresh vegetable samples were homogenized in a food processor without any stabilization (Cruz & Casal, 2013). Subsequent sample preparation included the addition of ascorbic acid to the homogenate prior to extraction, which may have allowed for regeneration of oxidized tocopherols. However, it should be confirmed if oxidized vitamin E is quantitatively regenerated by this method. Otherwise, the method may lead to underestimation of vitamin E levels.

3.3. Method validation

Since tocopherols and tocotrienols naturally occur in vegetables, no suitable blank matrix is available to fully demonstrate selectivity of the chromatographic method. However, fluorescence detection is generally characterized by a higher selectivity compared to UV detection. Furthermore, the vegetable extracts were also analyzed using a C30 phase (see chapter 2.6), which has an alternative chromatographic selectivity compared to the PFP phase (compare standard chromatograms in Fig. 1A and B). Applying both chromatographic methods, the retention times of all analytes exactly matched those of the respective reference compounds. Also, no additional peaks could be detected in the tocopherol and tocotrienol retention time window when using the C30 method compared to the PFP method. Moreover, the DAD data did not indicate any significant impurities co-eluting with tocopherols and tocotrienols.

Satisfactory recoveries of tocopherols and tocotrienols were found at low, medium and high concentration levels (Table 1). In addition, the developed method was found to be very precise at all three concentration levels, with the CV generally being <5% (within-run precision, Table 1). To determine between-run precisions red pepper was chosen, because it contains all four tocopherol isomers. Between-run precisions were also acceptable (CV < 10%) (Supplementary Table 1). The analytes were also shown to be stable in red pepper extracts for at least 24 h of cool (4 °C) and dark storage in an autosampler (data not shown).

3.4. Analysis of raw vegetables

The validated method was subsequently applied to other vegetables. Sample chromatograms are shown in Fig. 1. The results obtained are summarized in Table 2. Besides carrots – which may contain relatively high amounts of α-tocotrienol – none of the vegetables analyzed in this study contained significant amounts of tocotrienols (Table 2). Green beans were the only vegetable which contained almost no α-tocopherol, but relatively high amounts of γ-tocopherol.

In Table 2, the tocopherol levels analyzed by using the validated method are compared to the values listed in various national nutrient databases. It should be noted that in this study random samples were analyzed and do not reflect a representative range. Nevertheless, Table 2 shows that for most of the analyzed vegetables there is a good agreement between the results obtained here, and the values found in nutrient databases, whereas for other raw vegetables, such as spinach or broccoli, underestimation of vitamin E in the databases cannot be ruled out. Therefore, vitamin E data for raw plant foods should be revisited and updated if necessary.

In conclusion, the results of the present study demonstrate the importance of sample stabilization for vitamin E analysis in raw plant materials. Standard protocols, such as the DIN EN 12822 for the analysis of vitamin E in foodstuffs, should provide corresponding notes or instructions for raw fruits and vegetables to avoid any pitfalls in sample preparation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.07.099.

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