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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.

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 β-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 β-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

α-, β-, γ- and δ-Tocopherols (⩾95% by HPLC) and α-, β-, γ- and δ-tocotrienols (⩾97% 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 μL of a methanol/acetone/water mixture (54:40:6; v/v). Following filtration of the extract (PTFE, 0.2 μm, 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.