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Original Research Article

Comparing extraction methods for the determination of tocopherols and tocotrienols in seeds and germinating seeds of soybean transformed with *OsHGGT*

Yu Young Lee [a,1], Hyang Mi Park [a,1], Choon Ki Lee [a], Sun Lim Kim [a], Tae-Young Hwang [a], Man Soo Choi [a], Young-Up Kwon [a], Wook Han Kim [a], Si Ju Kim [a], Sang Chul Lee [b], Yul Ho Kim [a,*]

[a] National Institute of Crop Science, RDA, Suwon 441-857, Republic of Korea
[b] School of Applied Bioscience, College of Agriculture and Life Sciences, Kyungpook National University, Daegu 702-701, Republic of Korea

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ABSTRACT

Previously, transgenic soybeans were generated and reported to produce tocotrienols (α-, γ- and δ-tocotrienols), compounds not normally found in soybean. Three procedures were evaluated to optimize the extraction method for transgenic soybean seeds and germinating seeds. Significant differences were observed among the extraction methods in seeds and germinating seeds. In seeds, the highest analytical values (tocopherols, 37.11 mg 100 g^{-1}; and tocotrienols, 1.54 mg 100 g^{-1}) were observed by using rapid Soxhlet extraction. In germinating seeds, the content of transgenic soybean (B20 and C5) total vitamin E (tocopherols, 18.04, 20.73 mg 100 g^{-1}; and tocotrienols, 0.82 and 0.84 mg 100 g^{-1}) by direct extraction was approximately 16% and 9% greater than the amount obtained by saponification. In addition, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) tests demonstrated a direct correlation between the radical-scavenging capacity and the total content of natural tocopherols and tocotrienols. Therefore, these results suggest that an optimal extraction method will provide a fast, simple, reproducible procedure for analyzing tocotrienols and tocopherols. Furthermore, this method may be used to determine novel minor functional compounds such as tocotrienols for the evaluation of biological activity.

1. Introduction

Tocopherols and tocotrienols (vitamin E), crucial lipid-soluble antioxidants, are essential to human health. These molecules play important roles in scavenging free radicals and inhibiting lipid peroxidation in biological membranes (Folk and Munne-Bosch, 2010). In recent years, tocotrienols have received greater attention than tocopherols. Apart from vitamin E activity, tocotrienols have shown promise in numerous treatment areas. These include reducing blood cholesterol levels and preventing stroke-induced brain damage, as well as exhibiting anti-inflammatory and anti-angiogenesis properties (Aggarwal et al., 2010; Lee et al., 2008; Schaffer et al., 2005; Sen et al., 2006; Serbinova et al., 1991; Theriault et al., 1999). Tocotrienols also exhibit potent anti-cancer activity in various human cancer cells from tissues including the prostate, breast and colon (Constantinou et al., 2008; Miyazawa et al., 2009). These biological properties have resulted in the inclusion of tocotrienols in a broad spectrum of dietary supplements and functional foods (as well as nutra- and cosmeceutical applications).

The extraction procedure for vitamin E is traditionally performed by enzymatic hydrolysis, saponification and Soxhlet extraction (Ruperez et al., 2001; Slover et al., 1969; Wrolstad, 2002). Among these methods, saponification has been widely used for the quantification of vitamin E in cereals (Chun et al., 2006; Lerma-Garcia et al., 2009; Piironen et al., 1986; Ruperez et al., 2001). Following saponification, the unsaponifiable components, including vitamin E, are extracted into an organic solvent, while fatty acid salts, glycerols and other potentially interfering substances remain in the alkaline aqueous phase. Nevertheless, saponification is time-consuming, complex and not always necessary. In previous studies, the saponification results did not vary from the traditional extraction methods (Ruperez et al., 2001).

Residue oil from crude palm oil has been processed classically using Soxhlet extraction by screw press (Ollanketo et al., 2002). Supercritical carbon dioxide (SC-CO_2) (Lau et al., 2006, 2008) and pressurized liquid extraction (PLE) (Sanagi et al., 2005) are currently used to extract from palm mesocarp. In rice bran, saponification is normally used for the determination of vitamin E (Diack and Saska, 1994), and the effects of solvent, extraction temperature and time have been studied (Chen and Bergman,

* Corresponding author. Tel.: +82 31 290 6751; fax: +82 32 290 6742.
E-mail address: Kimyuh77@korea.kr (Y.H. Kim).
[1] These authors contributed equally to this work.

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2005; Duvernay et al., 2005; Devi and Arumughan, 2007; Hu et al., 1996; Proctor et al., 1994; Proctor and Bowen, 1996). One-step equilibrium direct solvent extraction and SC-CO_2 are extraction methods used for the determination of tocopherols and tocotrienols from rice bran (Imsanguan et al., 2008; Lerma-Garcia et al., 2009; Sarmento et al., 2006).

Soybean represents a good source of protein, polyunsaturated fats and fat-soluble vitamin E, especially tocopherol. Recently, tocopherols from deodorizer distillate of soybean oil (DDSO) were concentrated using SC-CO_2, thus increasing the tocopherol value (Clark and Synder, 1989; Mendes et al., 2005). Soxhlet extraction, in comparison to saponification and direct solvent extraction, has been the best method for quantifying tocopherols in soybeans (Lim et al., 2007). The quantity of vitamin E in cereals, as well as vegetables, is influenced by the species, variety, maturity and growing conditions (Tsochatzis et al., 2012). Moreover, variations in vitamin E values result from many factors, including sample preparation, processing procedures and the conditions of the analytical methods (Clark and Synder, 1989; Lee et al., 2006; Peres et al., 2006; Tasai et al., 2007).

Previous studies have demonstrated the production of transgenic soybeans producing tocotrienols, compounds not normally found in soybean, by metabolic engineering of the biosynthesis pathways. Transgenic soybean over-expressed rice *HGGT* (*OsHGGT*) using two different promoters produced four tocopherol isomers, two new γ- and δ-tocotrienols. Moreover, in transgenic plants, significantly higher antioxidant activities were detected in germinated than in non-germinated seeds. In particular, after 3 days of germination, the DPPH and ABTS radical-scavenging activities of seed extracts from transgenic plants were up to 17% and 35.3% higher, respectively, than extracts from un-germinated wild-type seeds. In addition, germinating seeds from transgenic lines exhibited dramatically lower MDA contents than intact and germinating wild-type seeds. Increased tocotrienol levels correlated with significant improvements in antioxidant activity (Kim et al., 2008, 2011).

An optimum extraction method is desirable for various crop materials such as seeds and roots. To numerous plant-derived extracts and phytochemicals a variety of potentially health-promoting biological activities have been ascribed (Ollanketo et al., 2002). Therefore, the purpose of this study was to optimize the extraction procedures for the quantitative determination of vitamin E, especially novel minor functional compounds such as tocotrienols, in transgenic soybean. In addition, an evaluation of the health-promoting biological activity of the extracted compounds should be included when determining the most effective extraction method.

2. Materials and methods

2.1. Chemicals and instruments

Analytical grade ethyl acetate (EtOAc), n-hexane, isopropanol and water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Ethanol, butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and tocopherols were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tocotrienols were supplied by Davos Life Science (Tuas, Singapore).

The tocopherol and tocotrienol content of soybeans were determined by HPLC. HPLC was performed with a normal-phase HPLC system comprising a solvent delivery pump (515; Waters, USA) equipped with a spectrofluorometric detector (2475; Waters, USA) and a Lichrosorb Si60 column (4.6 mm × 250 mm, 5 μm; Hibar, Darmstadt, Germany). Extracts (10 μL) were injected into an analytical Lichrosorb Si60 column with an isocratic phase of n-hexane:isopropanol (99:1, v/v) and a flow rate of 1.5 mL min^{-1}. The excitation and emission spectra were 290 and 330 nm, respectively. The injection volume was 10 μL, and the column temperature was regulated at 30 °C.

2.2. Samples

Transgenic soybeans stably expressing the *OsHGGT* gene were obtained from the T4 generation of two lines: a homozygous line expressing *OsHGGT* under the control of the seed-specific rice globulin promoter (*Glb-HGGT*) (B20) and a homozygous line expressing *OsHGGT* under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter (*35S-HGGT*) (C5). Wild type soybean (WT, cv. Iksannamulkong) was included in these experiments. All the soybeans were harvested on 24 October 2010 at National Institute of Crop Science, the Rural Development Administration (RDA) farm, Suwon, Korea. The soybeans were stored at 4 °C before the experiment. The conditions for germination were as follows: seeds were soaked in 75% ethanol for 1 min and rinsed twice with distilled water. The seeds were then soaked in 20% sodium hypochlorite for 12 min at ambient temperature and thoroughly rinsed three times with distilled water. Seeds were germinated in plastic dishes containing 15 mL of distilled water at 27 °C for 72 h. They were stored at −70 °C for safe preservation until laboratory extraction and analysis.

2.3. Extraction method in soybean seeds

All of the conditions used in the sample pre-treatment procedures were shown in Table 1. The saponification method was modified (Lim et al., 2007). Soybean seeds (WT, B20 and C5) (100 g) were ground at 260 × *g* for 200 s in an auto-mill disintegrator (Tokken, Japan). Ground soybeans (2 g) were added to a 15 mL aliquot of ethanol containing pyrogallol (PG, 6 g 100 mL^{-1}, w/v) in a saponification vessel and vortexed for 30 s. After sonication for 5 min, 5 mL of potassium hydroxide (33.6 g L^{-1}) was added and the vessel was flushed with nitrogen gas for 1 min. An air condenser was attached and the contents were digested at 70 °C for 50 min in a shaking water bath. After the samples had been cooled for 5 min in an ice bath, 20 mL of sodium chloride (20 g L^{-1}) was added, and then vortexed for 30 s. The mixture was extracted three times with 20 mL n-hexane:EtOAc

Table 1
The conditions used in the sample pretreatment procedures.

	Saponification	Rapid Soxhlet	Ultrasonic	Direct
Ratio of sample weight/solvent	2 g/100 mL	2 g/140 mL	2 g/10 mL	1 g/5 mL
Solvent extractant	EtOH (with 60 g L^{-1} PG), KOH (33.6 g L^{-1}), NaOH (20 g L^{-1}), n-hexane:EtOAc (with 0.1 g L^{-1} BHT)	n-Hexane:EtOAc (with 0.1 g L^{-1} BHT)	n-Hexane (with 0.1 g L^{-1} BHT)	EtOH (10 g L^{-1} PG)
Number of extraction step	8	3	3	3
Total amount of solvent (mL)	600	840	60	30
Extraction temperature (°C)	70	180/120	40–50	–
Total time (min)	164	110/192	60	10