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An improved high-performance liquid chromatographic method for simultaneous determination of tocopherols, tocotrienols and γ-oryzanol in rice

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ABSTRACT

An improved normal phase high performance liquid chromatographic (NP-HPLC) method was developed for simultaneous quantification of eight vitamin E isomers (α-, β-, γ- and δ-tocopherols and α-, β-, γ- and δ-tocotrienols) and γ-oryzanol in rice. A complete separation of all compounds was achieved within 25 min using an Inertsil® CN-3, SIL-100A 5 μM (4.6 mm × 250 mm) column and an isocratic elution system of hexane/isopropanol/ethylacetate/acetic acid (97.6:0.8:0.8:0.8, v/v/v/v) at a flow rate varying from 0.7 to 1.5 mL min^{-1}. A linear correlation coefficient ($r^2 > 0.99$) and high reproducibility were obtained at concentrations ranging 0.05–10 μg mL^{-1} for vitamin E isomers and 0.5–500 μg mL^{-1} for γ-oryzanol. This method proved to be rapid, accurate and reproducible.

1. Introduction

Rice bran and brown rice are known to contain high levels of bioactive phytochemicals with diverse biological activities and health benefits. Among these phytochemicals, vitamin E, including the four isomers (α, β, γ and δ) of tocopherols (T) and tocotrienols (T3) [1–3], and γ-oryzanol [4,5] have received the most research attention. As α-tocopherol (α-T) is traditionally believed to possess the highest biological activity [6] and thus most methods available for vitamin E assay were developed exclusively for its determination [7]. However, recent studies have shown that other vitamin E isomers possess similar or better antioxidant, anticancer, anti-inflammatory, immunomodulatory, neuroprotective and hepatoprotective activities than α-T [3,8]. This has resulted in an increased interest in these compounds among the scientific community and the consumers. Consumers are also increasingly aware of health benefits from brown rice and rice bran consumptions. Hence, there is an urgent need to have an efficient method for routine analysis of all vitamin E isomers and γ-oryzanol in rice-based products. Although several methods have been developed to determine all forms of vitamin E in various matrices [2,9–11], no method was reported for the simultaneous separation and/or quantification of all eight vitamin E isomers and γ-oryzanol in rice samples, and for routine analysis of large numbers of samples.

Although RP-HPLC provides a higher column stability, retention time reproducibility, quicker equilibration and shorter analysis time [12,13], and has a more environmentally friendly solvent systems [14] than those used in NP-HPLC, it often fails to separate or poorly separate β-T and γ-T, and β-T3 and γ-T3 [2,15–18]. In contrast, NP-HPLC gives a good separation of β-T and γ-T [13,19,20], and thus is a preferred method for analysis of T and T3 in food samples and vegetable oils. A complete separation of all eight vitamin E compounds has been demonstrated in cereals [1,20–23] and in rice samples [24,25]. However, 1,4-dioxane, a carcinogenic solvent, was often used in the mobile phase of these methods, and γ-oryzanol was not determined. Furthermore, the poor reproducibility, long equilibration times and low stability in the NP-HPLC have always been an issue in the routine analysis [7]. In this study, our aim was to develop a NP-HPLC method for a fast and reliable simultaneous quantification of all vitamin E isomers (T and T3) and γ-oryzanol in rice samples.

2. Materials and methods

2.1. Chemicals

Tocopherols (α-, γ- and δ-T with purity ≥99%, ≥96% and ≥90%, respectively), γ-oryzanol (purity ≥98%), 2,2,5,7,8-pentamethyl-6-chromanol (PMC purity ≥97%; used as an internal standard) and butylated hydroxytoluene (BHT purity ≥99%) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). β-Tocopherol (purity ≥90%) was obtained from Supelco Inc. (Bellefonte, PA, USA). Hexane and isopropanol of HPLC grade were purchased from Merck

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Table 1
Chromatographic conditions.

Time (min)	Flow rate (mL min^{-1})
0	0.7
20	0.7
22	1.5
35	1.5
40	0.7

Mobile phase: 97.6% hexane + 0.8% isopropanol + 0.8% ethylacetate + 0.8% acetic acid.

(Darmstad, Germany). Tocotrienols (α-, β-, γ- and δ-T3; all having a purity ≥97%) were obtained from Davos Life Science Pte. Ltd. (Helios, Singapore). All other reagents used were of analytical grade.

2.2. Standard preparation

Solutions containing individually the α-tocopherol (α-T), β-tocopherol (β-T), γ-tocopherol (γ-T), δ-tocopherol (δ-T), α-tocotrienol (α-T3), β-tocotrienol (β-T3), γ-tocotrienol (γ-T3), δ-tocotrienol (δ-T3) and γ-oryzanol standards were prepared in hexane and were used as stock solutions at concentration 1 mg mL^{-1}. They were kept at −4 °C and protected from light. Working standard mixtures were prepared from these stock standard solutions; they were further diluted with hexane to required concentrations as necessary.

2.3. Chromatographic systems and conditions

The details of the chromatographic conditions are given in Table 1. Briefly, the Hitachi HPLC system (Hitachi, Tokyo, Japan) consisted of a pump (Hitachi L-2130) and a fluorescence detector (Hitachi L-2485) set at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. Chromatographic separation was performed by a normal phase Inertsil® CN-3, SIL-100A (5 μm; 4.6 mm × 250 mm) column coupled with an Inertsil® SIL-100A (5 μm; 4 mm × 10 mm) guard column (GL Sciences Inc., Tokyo, Japan), and a mobile phase composed of hexane/isopropanol/ethylacetae/acetic acid (97.6:0.8:0.8:0.8, v/v/v/v). The flow rate varied from 0.7 to 1.5 mL min^{-1} and the analysis was performed at room temperature. Other solvent systems tested in this study were hexane/isopropanol/ethylacetate (97.8:1.4:0.8, v/v/v) and hexane/ethylacetate/acetic acid (94.6:3.6:1.8, v/v/v), which were adopted from Jennings and Akoh [26], and Nielsen and Hansen [23], respectively. To avoid the effect of oxygen on the fluorescence signal, all mobile phases were well degassed.

Development of the analysis conditions was achieved with the standard mixture of eight vitamin E analogs and γ-oryzanol, and the retention time of every compound was obtained with its individual respective standard. T, T3 and γ-oryzanol in rice samples were identified by retention time and quantified using the linear regression from the calibration curve of the external standards.

2.4. Method validation

To demonstrate selectivity, chromatograms of the individual vitamin E isomers (α-, β-, γ- and δ-T, and α-, β-, γ- and δ-T3) and γ-oryzanol were compared to that of the standard mixture. Repeatability for within-day and between-day was checked through six consecutive injections of the sample solution. Linearity was performed by preparing solutions of working standard mixture at five concentration levels, i.e. 0.05–10 μg mL^{-1} for T and T3, and 0.5–500 μg mL^{-1} for γ-oryzanol. Accuracy was determined as the recovery of known amount of analyte spiked into placebo.

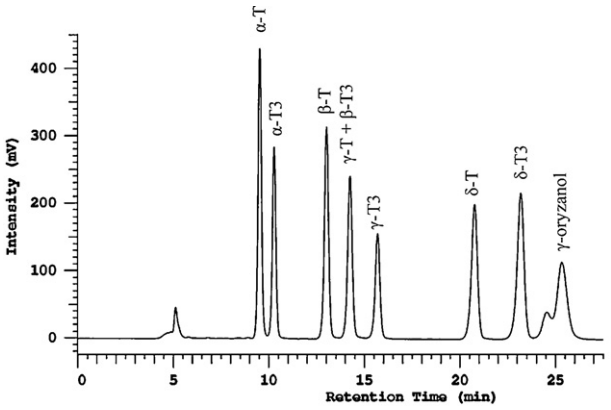

Fig. 1. Chromatogram of a standard mixture. Chromatographic conditions with mobile phase composed of hexane/isopropanol/ethylacetate (97.8:1.4:0.8, v/v/v). α-T: α-tocopherol, β-T: β-tocopherol, γ-T: γ-tocopherol, δ-T: δ-tocopherol, α-T3: α-tocotrienol, β-T3: β-tocotrienol, γ-T3: γ-tocotrienol, δ-T3: δ-tocotrienol, and γ-oryzanol.

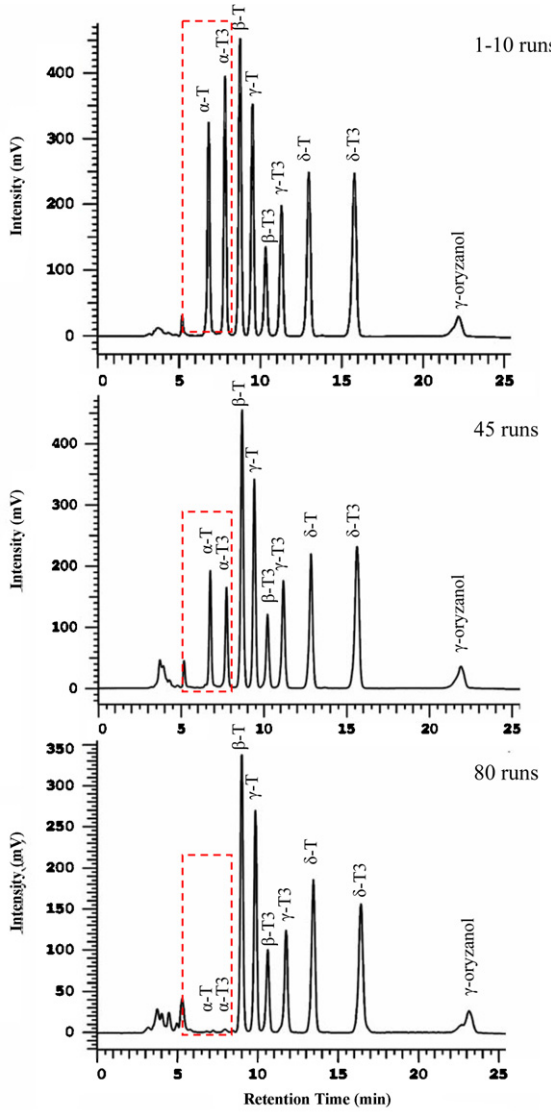

Fig. 2. Chromatograms of a standard mixture. The performance of the chromatographic system with mobile phase composed of hexane/ethylacetate/acetic acid (94.6:3.6:1.8, v/v/v). α-T: α-tocopherol, β-T: β-tocopherol, γ-T: γ-tocopherol, δ-T: δ-tocopherol, α-T3: α-tocotrienol, β-T3: β-tocotrienol, γ-T3: γ-tocotrienol, δ-T3: δ-tocotrienol, and γ-oryzanol.

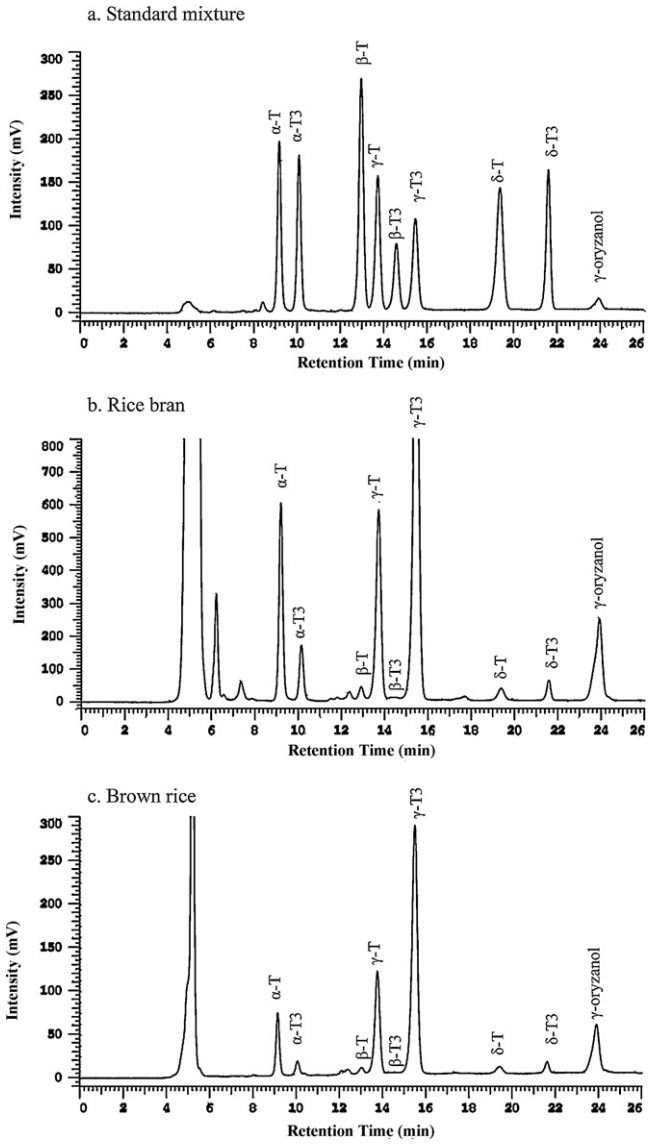

Fig. 3. Chromatograms of the standard mixture, rice bran and brown rice. (a) Standard mixture; (b) rice bran; and (c) brown rice. α-T: α-tocopherol, β-T: β-tocopherol, γ-T: γ-tocopherol, δ-T: δ-tocopherol, α-T3: α-tocotrienol, β-T3: β-tocotrienol, γ-T3: γ-tocotrienol, δ-T3: δ-tocotrienol, and γ-oryzanol.

2.5. Quantification of tocopherols, tocotrienols and γ-oryzanol in rice samples

To evaluate the reliability of the newly established method, rice samples of different grain parts were used. The reliability and repeatability of the developed method was verified by analyzing the recovery of T, T3 and γ-oryzanol contents in rice samples in triplicates. Known amounts of α-T (1 μg mL^{-1}), β-T (1 μg mL^{-1}), γ-T (1 μg mL^{-1}), δ-T (1 μg mL^{-1}), α-T3 (1 μg mL^{-1}), β-T3 (1 μg mL^{-1}), γ-T3 (1 μg mL^{-1}), δ-T3 (1 μg mL^{-1}), γ-oryzanol (10 μg mL^{-1}) and PMC (2.5 μg mL^{-1}) in hexane were added to rice samples prior extraction. The recovery was determined using the formula: recovery = $(A1 - A2)/A3 \times 100\%$; where $A1$ represents the area of a peak obtained from rice sample with added standard individual vitamin E isomer or γ-oryzanol, $A2$ represents the area of the peak obtained from the same rice sample without standard individual vitamin E isomer or γ-oryzanol, and $A3$ represents the area of the peak obtained from the same amount of added individual vitamin E isomer or γ-oryzanol.

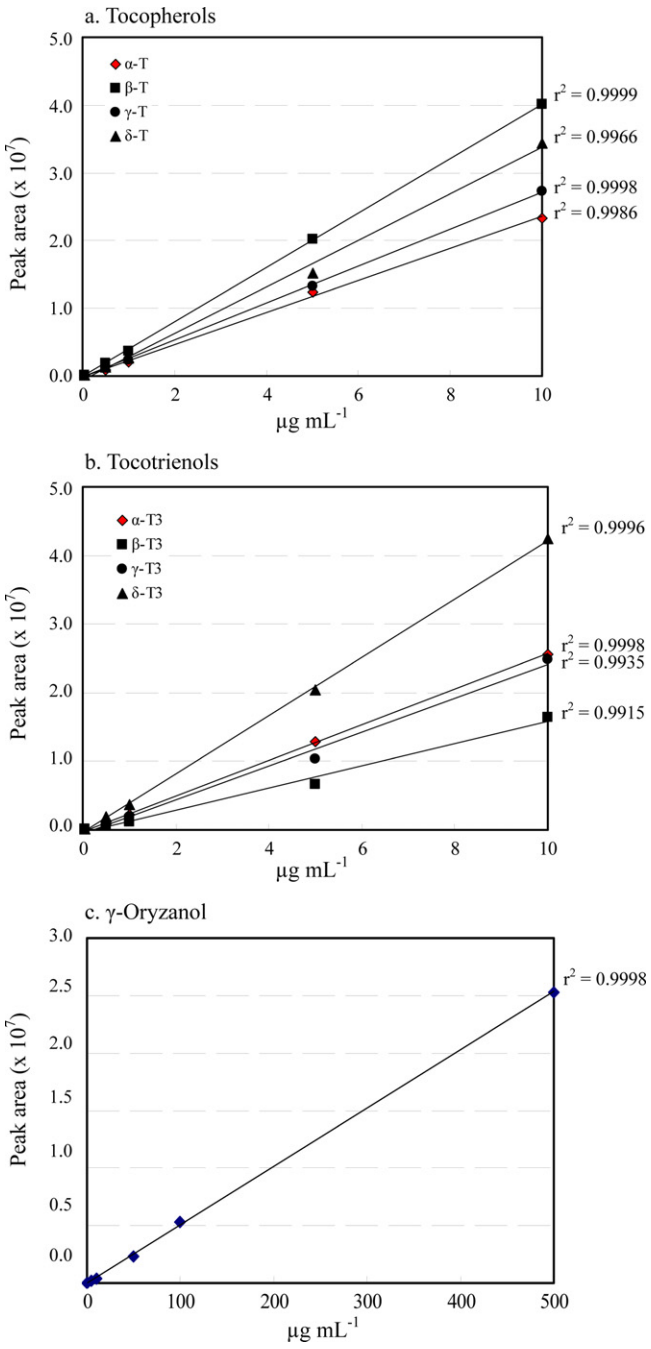

Fig. 4. Linearity of response of nine reference standards. (a) Tocopherols (α-T: α-tocopherol, β-T: β-tocopherol, γ-T: γ-tocopherol, and δ-T: δ-tocopherol) and (b) tocotrienols (α-T3: α-tocotrienol, β-T3: β-tocotrienol, γ-T3: γ-tocotrienol, and δ-T3: δ-tocotrienol), and γ-oryzanol.

2.6. Rice sample extraction

The extraction procedures were adopted from Jang and Xu [18]. In brief, 0.5 g of each rice samples was taken and transferred into a test tube (25 mm × 150 mm) to which 3 mL hexane and 0.1 mL BHT (10 mg mL^{-1}) were added, and the mixture was vortexed for 30 s. The test tubes were then capped and placed in a 60 °C water bath, followed by subjecting to extraction for 20 min with shaking. The hexane layer in each tube was separated by centrifugation at 2000 × *g* for 15 min. The supernatant was collected while the residue was further subjected to similar procedures of extraction twice. The supernatant obtained from three separate extractions

Table 2
The reproducibility of retention time and peak area of tocopherols, tocotrienols and γ-oryzanol.

	Within-day ($n=6$)				Between-day ($n=6$)			
	Retention time		Surface area		Retention time		Surface area	
	$x \pm$ SD	% CV	$x \pm$ SD ($\times 10^6$)	% CV	$x \pm$ SD	% CV	$x \pm$ SD ($\times 10^6$)	% CV
α-T	9.36 ± 0.01	0.12	33.69 ± 0.39	1.15	9.27 ± 0.04	0.40	33.88 ± 0.93	2.74
α-T3	10.48 ± 0.05	0.48	35.78 ± 0.26	0.72	10.24 ± 0.07	0.66	33.88 ± 0.99	2.92
β-T	12.58 ± 0.06	0.48	30.48 ± 0.19	0.61	12.53 ± 0.10	0.80	30.67 ± 1.03	3.34
γ-T	13.54 ± 0.06	0.45	20.98 ± 0.14	0.67	13.40 ± 0.09	0.71	20.21 ± 0.66	3.27
β-T3	14.27 ± 0.08	0.54	22.69 ± 0.08	0.33	14.03 ± 0.12	0.84	21.96 ± 0.85	3.85
γ-T3	15.36 ± 0.08	0.52	27.67 ± 0.78	2.83	15.03 ± 0.12	0.81	27.92 ± 1.00	3.58
δ-T	19.21 ± 0.09	0.47	49.96 ± 1.11	2.23	18.75 ± 0.14	0.74	50.44 ± 1.40	2.77
δ-T3	21.35 ± 0.06	0.28	42.54 ± 1.31	3.07	21.10 ± 0.12	0.58	42.81 ± 0.97	2.26
γ-Oryzanol	23.97 ± 0.11	0.47	2.85 ± 0.14	4.78	23.44 ± 0.21	0.92	2.88 ± 0.78	2.70

Tocopherols (α-T: α-tocopherol, β-T: β-tocopherol, γ-T: γ-tocopherol, and δ-T: δ-tocopherol); tocotrienols (α-T3: α-tocotrienol, β-T3: β-tocotrienol, γ-T3: γ-tocotrienol, and δ-T3: δ-tocotrienol).

were combined, filtered and diluted to a volume of 10 mL, of which 20 μL was taken for HPLC analysis.

3. Results and discussion

The present study has achieved in developing a NP-HPLC method for the simultaneous determination of all vitamin E isomers (T and T3) and γ-oryzanol within 25 min. This method was confirmed to be sensitive, reproducible and reliable for the routine analysis of complex rice samples.

To establish the optimal peak separation of all compounds, we have adopted and tested different solvent systems based on published literatures. Consistently, using the solvent system composing of hexane/isopropanol/ethylacetate (97.8:1.4:0.8, v/v/v), γ-T and β-T3 were not separated (Fig. 1). In the solvent system composed of hexane/ethylacetate/acetic acid (94.6:3.6:1.8, v/v/v), although a good peak separation was obtained for all eight vitamin E isomers in the beginning, separation of α-T and α-T3 was noted to deteriorate after 40 runs, and was not separated at 80 runs. Furthermore, the surface area of all peaks appeared to dramatically decrease with increasing numbers of run (Fig. 2). This suggests that the chromatographic system is unstable and is not suitable for routine analysis of a large number of samples. Under the newly developed chromatographic system, besides avoiding the commonly use toxic solvent 1,4-dioxane as a mobile phase, it is stable and reliable in separating all nine important bioactive phytochemicals of rice, namely all vitamin E isomers and γ-oryzanol even over 500 runs (Fig. 3). The order of retention time was α-T, α-T3, β-T, γ-T, β-T3, γ-T3, δ-T, δ-T3 and γ-oryzanol.

Linearity determination was performed for each single vitamin E analogs and γ-oryzanol using five standard mixture solutions at four concentration points ranging 0.05–10 μg mL^{-1} for vitamin E isomers, and 0.5–500 μg mL^{-1} for γ-oryzanol. The regression plot and correlation coefficient are presented in Fig. 4. Results showed that all vitamin E analogs and γ-oryzanol exhibited a good correlation coefficient ($r^2 > 0.99$) at the tested range of concentrations.

To examine the reproducibility of the present chromatographic method, repeated runs on the standard mixture were performed. The coefficients of variation (% CV) for within-day and between-day runs for the nine bioactive compounds of rice are presented in Table 2. As expected, the coefficient of variation of individual T, T3 and γ-oryzanol for within-day assays was generally lower than for between-day assay. The reliability for most of the analyzed compounds was below 5%. Overall, the variability obtained under the present method was as good as that reported by Amaral et al. [27] and Nielsen and Hansen [23].

In the recovery studies, rice samples were spiked with their respective standard compounds, followed by subjecting to the established extraction procedure and HPLC separation. Results showed that the quantitative recovery of all vitamin E isomers and γ-oryzanol varied from 82.72% to 119.36% (Table 3).

NP-HPLC using silica is well known to have the capability of discriminating isomers [28]. For analysis of vitamin E isomers, a carcinogenic solvent 1,4-dioxane is commonly used in the mobile phase. In this study, we used isopropanol as a modifier in the mobile phase and found that at 0.8% isopropanol produced the best resolution of all analyte peaks. In addition, we added 0.8% acetic acid to reduce peak tailing [29]. Under this condition, with a normal phase Inertsil® CN-3, SIL-100A 5 μM (4.6 mm × 250 mm) column and a mobile phase composed of hexane/isopropanol/ethylacetate/acetic acid (97.6:0.8:0.8:0.8, v/v/v/v), a complete separation and high reproducibility of all nine analyzed compounds was achieved within 25 min.

In RP-HPLC, resolution of β-T and γ-T has often reported to be either poor or co-eluted [2,15–18], whereas in some normal phase methods poor separation has been reported on γ-T and β-T3 [13] and others [22]. These problems may cause a misinterpretation in the identification of the different vitamin E isomers in samples. Comparing with silica C18 stationary phase, higher resolution between carotenoids, tocopherols and γ-oryzanol in crude rice bran oil was obtained using the C30 stationary phase, of which also found to have high resolution of β-T and γ-T [30]. However, tocotrienols were not analyzed in this system. In this study, the chromatographic peaks of both BHT and PMC (the internal standard) were well separated from the other sample peaks. We also confirmed from the blank runs that the solvent front peak was clear from any co-eluting substances in the samples. As a good separation was obtained for all peaks, this suggests that the present method allows an improved accuracy on the quantification of all eight vitamin E isomers and γ-oryzanol in complex rice samples.

Table 3
Recovery yield.

	% recovery	
	Rice bran	Brown rice
PMC	98.15	98.15
α-T	91.87	98.35
β-T	87.16	92.06
γ-T	98.20	96.39
δ-T	108.26	113.74
α-T3	82.72	87.74
β-T3	96.93	103.00
γ-T3	90.16	91.81
δ-T3	85.77	90.07
γ-Oryzanol	119.36	119.02

Values are mean of three analyses ($n=3$); PMC: 2,2,5,7,8-pentamethyl-6-chromanol (as internal standard); tocopherols (α-T: α-tocopherol, β-T: β-tocopherol, γ-T: γ-tocopherol, and δ-T: δ-tocopherol); tocotrienols (α-T3: α-tocotrienol, β-T3: β-tocotrienol, γ-T3: γ-tocotrienol, and δ-T3: δ-tocotrienol).

4. Conclusion

In conclusion, the described chromatographic conditions provide a fast, reliable and relatively inexpensive procedure for simultaneous analysis of all eight vitamin E isomers and γ-oryzanol in complex rice samples. Besides having equivalent or better analytical strengths than other reported systems, it also has the advantage of being reproducible for analysis of large numbers of samples, and hence could be used for routine analysis of these compounds in foods and their physiological metabolic studies in plants.

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