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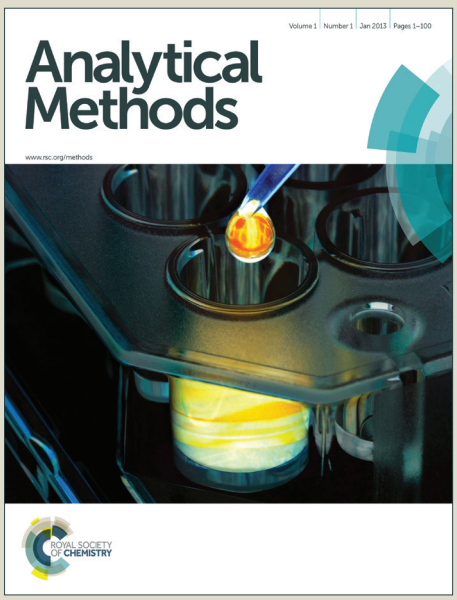

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Simultaneous determination of eight vitamin E isomers and α-tocopherol acetate in functional foods and nutritional supplements by gas chromatography - mass spectrometry

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Abstract: A rapid and efficient gas chromatography-mass spectrometry (GC-MS) has been developed for determination of eight VE isomers including α-, β-, γ-, δ-tocopherols and tocotrienols, as well as α-tocopherol acetate in functional foods and nutritional supplements. The vitamin E isomers in samples were directly extracted without saponification with mixed solvents of methanol and hexane (7:3,v/v). Good separation was achieved using a VF-5MS column (30 m×0.25 mm, 0.25μm) within 13 min. The mass spectrometer was operated in both full scan mode and SIM mode using electron impact ionization. Qualitative detection was based on characteristic ion pairs and retention time. Dibenzanthracene was used as an internal standard for quantification measurement. The linear ranges of the method ranged from 0.1 to 40 μg/mL with the correlation coefficients greater than 0.997. The detection limits ranged from 0.09 ng/mL to 0.46 ng/mL, and the quantification limits were from 0.29 ng/mL to 1.52 ng/mL. The intra-day and inter-day relative standard deviations (RSDs) of the method (for 1μg/mL standard solution) were in the range of 4.9% to 8.0% and 2.1% to 4.9%, respectively. The average recoveries of the method ranged from 83.2 % to 107 %, with the RSDs from 1.1% to 8.4%. The method has been applied for the determination of VE isomers and α-tocopherol acetate in functional foods and nutritional supplements with satisfactory results.

1 Introduction

Vitamin E (VE) is a generic descriptor for all tocopherols and tocotrienols that exhibit α-tocopherol activity. Vitamin E isomers generally refer to the saturated α-, β-, γ-, δ-tocopherols and the unsaturated α-, β-, γ-, δ-tocotrienols. They mainly exist in vegetable seeds and cereal grains, but each isomer's content varies with vegetable varieties and sources. Tocopherols consist of a chromanol ring and a 15-carbon tail. Three trans-double bonds in C-3, 7 and 11 positions of the tail make tocotrienols differ from tocopherols. The differences in number and location of methyl group on the chromanol ring result in isomeric VE (Fig. 1).

The existing studies on the biological functions of VE were mainly focused on tocopherols, especially α-tocopherol, the predominant form of VE, but the studies on tocotrienols have been

increasing since last decade. Researchers found that the distinction in chemical structure would give them unique biological functions, even though the VE isomers with similar chemical structure possess similar antioxidant efficacy. Sen et al.[1] reviewed the potential biological significance of tocotrienols and summarized the functional uniqueness of VE isomers. Both α-tocopherol and γ-tocopherol were found to possess unique physiological functions such as inhibition of cell proliferation, platelet aggregation and monocyte adhesion. Furthermore, γ-tocopherol has the functions of anti-inflammation, cholesterol-lowing, anti-cancer, and neuroprotection, which indicates that it is a better negative risk factor for myocardial infarction and certain types of cancer.[2] Additionally, both γ-tocopherol and δ-tocopherol possess similar anti-proliferative effect, but β-tocopherol does not share this property. In a recent study, the fact that γ-tocotrienol induced cell death in breast cancer cells was associated with suppression of Id1, a key cancer-promoting protein, further substantiating the antiproliferative and chemosensitization effects of γ-tocotrienol.[3] α- and γ-tocotrienols were more effective in inhibiting HeLa cell proliferation, possibly through a pathway that upregulated IL-6 expression and downregulated cyclin D3, p16, and CDK6 expression.[4] Zaiden et al.[5] reported that the ingestion of δ- and γ-tocotrienols might lead to a reduction in triglyceride synthesis and transportation. Total VE and β-tocotrienol intake was found to be associated with a significant reduction in the risk of type 2 diabetes mellitus.[6] To further the knowledge of VE family and evaluate the nutritional value of the functional foods and nutritional supplements, it is necessary to analyze their contents of VE isomers simultaneously.

The reported methods for analysis of VE isomers include gas chromatography (GC),[7,8] high performance liquid chromatography (HPLC),[9-13] capillary electrophoresis (CE)[14-16] and LC or GC tandem mass spectrum (LC/GC-MS).[17-24] But most of the methods are time-consuming, for instance, GC-MS needs tedious derivatization procedure, which limits the application of GC or GC-MS in the analysis of VE isomers.[7] High performance liquid chromatography, combined with ultraviolet detection (UVD),[9] fluorescence detection (FLD),[10] or electrochemical detection (ECD),[11] is used frequently in measurement of VE isomers. Both normal-phase HPLC (NP-HPLC) and reversed-phase HPLC (RP-HPLC) are reported in the literatures, and NP-HPLC could achieve better separation for all of the VE isomers. But water and polar compounds in sample solution could result in drift of the retention times. What's more, it consumes large amounts of organic solvents and needs longer time to equilibrate the column. Although RP-HPLC has no such drawbacks, it needs frequent cleaning; otherwise the highly lipophilic tocopherols would be adhered to RP column and deteriorate the peak shapes. The standard C_8 and C_{18} columns mostly applied in RP-HPLC could not separate β- and γ- isomers well enough. Due to their hydrophobic character, the VE isomers cannot be separated by aqueous capillary electrophoresis system. Thus non-aqueous capillary electrophoresis (NACE),[14] capillary electrochromatography (CEC)[15] and microemulsion electrokinetic chromatography (MEEKC)[16] have been developed. But most of the methods are reported for the analysis of tocopherols only, rather than all of the VE isomers. UV

detector is the most commonly used for the quantitative detection of the VE isomers, but complicated samples containing large amounts of cholesterol, fatty acids and triacylglycerols could interfere with the detection. To avoid matrix effect, mass spectrometer (MS) or tandem MS (MS/MS) detector is a better choice. GC-MS[17-21] applies higher temperatures of injection port and column oven to gasify and separate VE isomers, which needs pre-column derivatization to turn VE isomers into easily gasifiable derivatives. GC-MS with electron impact (EI) ionization could offer accurate qualitative and quantitative information for tocopherols and tocotrienols. The analysis of tocopherols[7,8,17,19,20,21] and δ-tocotrienol[18] by GC-MS and GC have been reported in recent years. But as far as we know, there is no report on simultaneous analysis of eight VE isomers by GC-MS.

Before extraction of VE isomers from samples, the saponification procedure is usually needed.[20,23-25] However, the direct solvent extraction without saponification has also been reported.[26,27] Pinheiro-Sant'Ana et al.[30] compared and evaluated some saponification methods and a direct solvent extraction without saponification. They observed that the saponification procedure could destroy VE, which led to the decrease of VE contents. The similar conclusions were drawn by other researchers too.[29,30]

The important physiological effects and benefit of VE to human health lead to increase of the consumption of functional foods and nutritional supplements containing VE in the recent years. It is reported that some esters of tocopherols, e.g., α-tocopherol acetate, are more chemically stable and less sensitive to light and oxidants than their parent compounds. So they are usually added to nutritional supplements or health products as sources of VE.

The aim of this study was to develop a GC-MS method without derivatization and saponification procedures for simultaneous determination of the eight VE isomers and α-tocopherol acetate in functional foods and nutritional supplements. The internal standard was applied for quantification to improve the accuracy of the method. The proposed method has been applied for the determination of VE isomers in functional foods and nutritional supplements with satisfactory results.

2 Experiment

2.1 Reagents

The VE isomers including α-tocopherol (97%), β-tocopherol (98.6%), γ-tocopherol (99%), δ-tocopherol (95.5%), α-tocotrienol (98.13%), β-tocotrienol (98.29%), γ-tocotrienol (98.45%), δ-tocotrienol (98.37%), D-α-tocopherol acetate (98.8%) and dibenzanthracene as internal standard were all purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored at−18^{o}C until use. The

solvents used in the experiment including methanol, ethanol, acetonitrile, acetone, ethyl acetate, dichloromethane, isopropanol and hexane were all HPLC grade (Tianjin Damao Chemical, Tianjin, China). Water used in the experiment was produced from a Millipore water purification system (18.2 MΩ cm, Millipore, USA).

All standards were dissolved in ethanol to make the stock solutions at 1 mg/mL, then sealed and stored in refrigerator at 4 ^{o}C. The concentration of dibenzanthracene working solution dissolved in ethanol was 150 μg /mL. Due to the VE isomers were sensitive to light and oxidants, it was necessary to calibrate their concentrations before use by spectrophotometry. Table 1 lists the molar absorptivities ($E^{1\%}$) of the VE isomers for the calibration.[31]

2.2 Instrumentation

The chromatographic separation and detection were performed on a 7890A gas chromatographic system coupled with a 5975C mass spectrometer (Agilent Technologies, USA). Other equipments involved in sample preparation were centrifuge (TGL-16B, Anke, Shanghai, China), ultrasonic cleaner (KQ-250E, Dianshanhu Instrument, Kunshan, China), and analytical balance (BSA224S, Sartorius, Germany).

2.3 Operational conditions for GC-MS

Chromatographic separation was performed on a VF-5MS column (30m×0.25mm, 0.25μm, Varian Instruments, Sunnyvale, CA, USA). Helium (purity 99.999%) was employed as carrier gas and its flow rate was set at 2.0 mL/min. The oven temperature was programmed from 200 ^{o}C, held for 2 min, then to 280 ^{o}C at 20 ^{o}C/min, held for 2 min, finally to 285 ^{o}C at 1 ^{o}C/min and held for 1 min. The injector temperature was set at 300 ^{o}C. The sample solutions were injected in splitless mode with the injection volume of 1 μL.

The mass spectrometer was operated in both full scan mode and SIM mode using electron impact ionization (EI, 70 eV) with a mass range of 40-500 m/z. The temperature of transfer line, ion source and manifold were 300 ^{o}C, 230 ^{o}C and 150 ^{o}C, respectively. The solvent delay time was 5 min. The retention time, precursor and characteristic ions for each analyte were listed in Table 2.

The instrumental control, data acquisition and data processing were performed with Agilent MSD Chemstation E.02.00 software.

2.4 Samples preparation

Eleven samples, including 2 brands of tablet, 8 brands of soft capsule and 1 brand of hard capsule were purchased from different local drug stores (Chengdu, China). All of these samples were made in China and claimed to contain VE.

2.4.1 Sample treatment with direct extraction. The ground tablet and capsule content were blended well. 0.1 gram of sample was weighed into a 5-mL centrifuge tube with stopple and 2 mL of mixed solvents of methanol and hexane（7:3, v/v) was added and mixed well. Then 20 μL of internal standard (150 μg/mL) was added. The solution was vortex mixed for 2 min and then ultrasonically extracted for 5 min. After the solution was centrifuged for 5 min at 12000 rpm, 1μL of the supernatant solution was injected for GC-MS analysis.

2.4.2 Sample treatment with saponification. The analytes were extracted according to the method reported by Shammugasamy et al.[20] with minor modification. The procedure was as follows: the ground samples (0.1 g) was saponified under nitrogen in a 5 mL centrifuge tube with stopple. The saponification solution was consisted of 2 mL of ethanol (95%), 0.5 mL of potassium hydroxide (60%), 0.5 mL of sodium chloride (10 g/L) and 20 mg ascorbic acid as antioxidant. The tube was incubated in water bath at 70 oC for 40 min and mixed every 5 min during saponification. After that, the tube was cooled to room temperature, vortex mixed for 10 s, and centrifuged at 12000 rpm for 5 min. 1mL of suspension was take out into another 5 mL centrifuge tube and extracted twice with 2 mL of diethyl ether. The organic layer was collected and then washed with water (2 mL) until the pH of water phase was about 7. Then 0.10 mL of organic phase was evaporated to dryness under nitrogen and the residue dissolved in ethanol (1 mL) for analysis.

2.5 Internal standard curves

A mixed working solution at 100 μg/mL, except for α-tocotrienol at 200 μg/mL and δ-tocotrienol at 150μg/mL, was prepared from stock solution with ethanol. The mixed working solution was diluted to 6 gradient concentrations containing 3μg/mL of dibenzanthracene. The injection volume was 1μL throughout the experiment. Table 3 presents the internal standard calibration equations for all of the VE isomers and their correlation coefficients (r).

2.6 Sample analysis

Samples were analyzed under the optimized conditions described in section 2.3. Qualitative determination was based on characteristic ion pairs and retention time using selective ions monitoring (SIM) mode. The internal standard calibration equations were used for calculation of VE isomer concentrations in sample solution and then the VE isomer contents in sample were calculated using the following equation.

$$C = \frac{aV}{m}$$

Where C is the content of analyte in sample (mg/kg); a is the concentration of analyte calculated through the regression equation of the internal standard curve (μg/mL); V is the extraction volume (mL) and m is the weight of sample (g).

3 Results and discussion

3.1 Sample extraction

3.1.1 Extraction solvent, solvent volume and ultrasonication time. The selection of a suitable extraction solvent is particularly important for the direct solvent extraction of VE isomers from samples. Methanol, ethanol, acetonitrile, acetone, ethyl acetate, dichloromethane, isopropanol and hexane were compared for their extraction efficiencies. The experimental results showed that methanol had the highest extraction efficiencies for VE isomers except α-tocopherol acetate, but methanol could not dissolve the oily samples well. Hexane could extract α-tocopherol acetate more efficiently than other solvents. So the mixture of methanol and hexane was used as the extraction solvent.

The effect of proportions of methanol and hexane on extraction efficiency was compared. The results showed that the miscibility was influenced by the proportion of methanol and hexane, when the proportion of methanol in the mixture was more than 60% (*i.e.*3:2), the miscibility of the two solvents was satisfactory. The effects of methanol proportion (6:4 - 9:1, v/v) in the mixed solvent on the detected amount and recoveries of each analyte were studied on sample No. 4. Fig. 2 indicates that the maximum amount and satisfactory recoveries (81.9% - 109%) were achieved by use of 7:3 methanol-hexane as extraction solvent. Therefore, the mixture of methanol and hexane (7:3,v/v) was used as extraction solvent in the following study.

The effect of volume of extraction solvent on the extraction efficiency was studied. Considering that VE contents in functional foods and nutritional supplements were generally high, 0.1 gram of sample No. 4 was used in the following experiments. Different volume (2.00 mL, 5.00 mL and 10.0 mL) of extraction solvent were compared for their extraction efficiencies. The results showed that the amounts of analytes extracted were basically constant as the volume of extraction solvent increased, which indicated that 2.00 mL of extraction solvent was enough to extract the analytes from 0.1 g sample.

The effect of ultrasonication time (0, 5, 10 and 20 min) on the extracted amounts of the analytes was evaluated. The results indicated that 5 min of ultrasonication time was enough for complete extraction of the analytes from different forms of samples.

3.1.2 Extraction efficiency. In order to assess the extraction efficiencies of the mixture

of methanol and hexane (7:3,v/v), 0.1 g sample No. 4 was extracted ultrasonically for 5 min with 2 mL the mixed extraction solvent for three times. After each extraction, the supernatant solutions obtained were collected separately for GC-MS determination. Five analytes, i.e., δ-tocopherol, β-tocopherol, γ-tocopherol, α-tocopherol and α-tocopherol acetate, were detected and the extraction efficiencies of all the five analytes ranged from 95.8 % to 100 % with single extraction. Therefore, all the samples were extracted with 2.00 mL mixed extraction solvent for just one time in the following experiment.

3.2 Optimization of the chromatographic conditions

The capillary column, oven temperature, injector temperature, flow rate of the carrier gas and the split mode for the best separation of the VE isomers were investigated respectively. Four capillary columns with different polarity, namely DB-WAX (30m×0.25mm, 0.25μm, Agilent Technologies, USA), DB-FFAP (30m×0.25mm, 0.25μm, J&W, CA, USA), DB-5MS (30m×0.25mm, 0.25μm, J&W, CA, USA) and VF-5MS were compared. It was found that DB-5MS column could separate most of the analytes well. However, the resolution of VF-5MS column for γ-tocopherol and δ-tocotrienol (see Fig. 3) was superior to that of DB-5MS, so VF-5MS was chosen as the separation column. Because of the characteristic ion pairs of the two isomers were different, so the identification and quantification of the two analytes were not affected by the overlapping peak. The injector temperature was set at 300^{o}C. The influence of the flow rate of carrier gas (0.8 mL/min, 1.0 mL/min, 1.5 mL/min, 2.0 mL/min and 2.2 mL/min) on relative abundance and resolution was investigated as well. The results showed that the retention times would shorten and the peak shape of the analytes would get better with the increase of flowrate. But when the flowrate reached 2.2 mL/min, the resolution of β-, γ- tocopherol and δ- tocotrienol would be deteriorated. Therefore, 2.0 mL/min was selected as the flow rate of carrier gas in the following study. Splitless mode was also demonstrated with higher sensitivity and better peaks for most of the analytes. The total ion chromatogram of the nine mixed standards and internal standards obtained in full scan mode under the optimum conditions is shown in Fig. 3. From Fig. 3, we can see that within 13 min, all the target analytes were baseline separated except β-tocotrienol, γ-tocotrienol and α-tocopherol acetate.

3.3 Optimization of mass spectrometry conditions

The EI-MS conditions including the temperature of transfer line, ion source and manifold were carefully optimized. The results revealed that the sensitivities of the analytes reached the maximum when the temperature of transfer line, ion source and manifold were 300^{o}C, 230^{o}C and 150^{o}C, respectively. The choice of characteristic ions was based not only on specificity but also intensity and mass. Characteristic ions in the SIM mode for each analyte were shown in Table 2. As shown in Fig. 3, β-, γ- tocotrienol and α-tocopherol acetate could not be separated on the

selected column. However, in consideration of their different characteristic ions as shown in Table 2, the identification and quantification of the three analytes were achieved by selection of different characteristic ions.

Under optimized conditions, the method had a satisfactory performance with reasonable retention time (approximately 13 min), which was much shorter than the conventional HPLC method.

3.4 Selection of internal standard

For GC-MS analysis, the use of an internal standard was desirable to compensate for deviation caused by sample treatment and injection inaccuracy, as well as the signal variation during ionization process. Stable isotope labeled analytes were the preferable internal standard for mass quantitative detection. Other researchers have used VE related compounds, such a α-tocopherol acetate or α-tocopherol succinate as internal standards.[8,9] But these compounds might also present in functional foods and nutritional supplements, they are not suitable to be used as internal standards.

In this study, experiments have proved that dibenzanthracene could be used as internal standard. From Fig. 3, we can see that the retention time of dibenzanthracene was appropriate, in addition, it was well separated from other analytes and the correlation coefficients of the internal standard curves for all the analytes were greater than 0.997, so it was appropriate to be an internal standard in the analysis of the VE isomers.

3.5 Method Validation

The performance of the method has been verified and the linear ranges, the internal standard calibration equations, the correlation coefficients, limits of detection (LODs) and limits of quantification (LOQs) were listed in Table 3. From Table 3, we can see that the correlation coefficients (r) of the method were greater than 0.997. To investigate the statistical significance of the calibration curves, fit test and significance test in regression model were performed using SAS 8.1. The results showed that all the F values were < 0.001, which means that the linearity existed with the confidence level > 99.99%. The LODs of the method ranged from from 0.09 ng/mL to 0.46 ng/mL and the LOQs were between 0.29 ng/mL to 1.52 ng/mL. The intra-day and inter-day precisions of mixed standard solutions (1μg/mL) were less than 8.0 % and 4.9 %, respectively. The recovery experiments were performed by the addition of three levels of mixed standard solutions to one soft capsule sample and one tablet sample, and analysis with the proposed method. The contents of some isomers exceeded the linear ranges, so appropriate dilution before the addition of standards was needed for accurate quantification. The average recoveries of the method were from 83.2% to 107 % and RSDs were not more than 8.4% (Table 4).

3.6 Comparison of pretreatment methods

The performances of the developed method were compared with those of saponification assisted extraction described in Section 2.5.1.2, which was the classical method for the analysis of fat soluble vitamins.[20] Considering that α-tocopherol acetate was completely transformed to α-tocopherol during saponification process, it was appropriate to apply the amount of total VE for comparison. The presence of VE isomers were mostly in good agreement with the two methods as shown in Table 5. The Wilcoxon test showed that there were significant differences between the total VE contents of eleven samples with different matrices obtained (one brand of powder capsule, two brands of tablets, and eight brands of oily liquid capsules) by the two methods ($P<0.01$). The detected amounts of developed method were overall higher than those of saponification method. According to our analysis, VE isomers might have degraded during the saponification procedure. But more studies are needed to clarify the reasons. In addition, δ-tocopherol was not detected in sample No. 9 after saponification. Whether δ-tocopherol had been transformed to other forms or degraded during saponification procedure were still unclear.

3.7 Application of the Method

Eleven samples were analyzed with the developed method. The results were summarized in Table 5 and the related SIM chromatograms of the representative samples were shown in Fig. 4. The VE contents of some samples exceeded the linear ranges, so appropriate dilution before injection was needed for accurate quantification. α-Tocopherol and α-tocopherol acetate were detected in all samples, which indicated that both of them are most added in functional foods. Their contents were apparently higher than other tocopherol forms. α-Tocopherol was traditionally considered the most efficient form among the VE isomers for its prominent antioxidant activities and it was added as the main composition in functional foods and nutritional supplements frequently. However, in view of stability, α-tocopherol acetate was also preferred by producers. Other isomers (δ-tocopherol and γ- tocopherol) existed in much lower contents and β-tocopherol was rarely detected in the samples. However, no tocotrienols were detected in all samples.

4 Conclusions

An easy, efficient and reliable GC-MS method for separation and quantification of eight VE isomers and α-tocopherol acetate in functional foods was developed. The method involved a direct extraction of analytes with the mixed solvent of methanol and hexane (7:3, v/v) without derivatization and saponification procedures. The results showed that there were significant differences between the extraction methods with and without saponification procedure and the developed method obtained higher total VE contents than the method with saponification

procedure. Compared to the HPLC methods, [13,25,28] the developed method has lower LODs and LOQs. The accuracy of the method has been improved by use of internal standard curves for quantification. The method has been successfully applied to the determination of VE isomers and α-tocopherol acetate in a variety of functional foods and nutritional supplements and all the target VE isomers and α-tocopherol acetate were detected except α-tocotrienol, β-tocotrienol, γ-tocotrienol and δ-tocotrienol. The method is simple, time-saving, inexpensive and environmental friendly. It is suitable to the simultaneous determination of VE isomers and α-tocopherol acetate in functional foods and nutrient supplements.

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Figure captions

Fig. 1 Structures of various isomers of tocopherol and tocotrienol.

Fig.2 Recoveries (below) and the detected content (above) of VE isomers using different proportion of methanol - hexane. Data expressed in mean ± SD (n = 3).

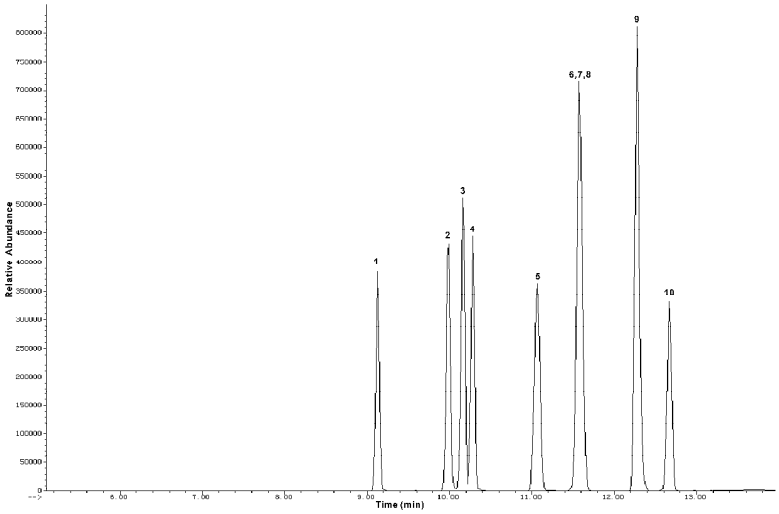
Fig.3 The total ion chromatogram of the mixed standards and internal standards (I.S.).

Peak identifications: 1. δ-tocopherol; 2. β-tocopherol; 3. γ-tocopherol; 4. δ-tocotrienol; 5. α-tocopherol; 6. β-tocotrienol; 7. γ-tocotrienol; 8. α-tocopherol acetate; 9. dibenzanthracene; 10. α-tocotrienol.

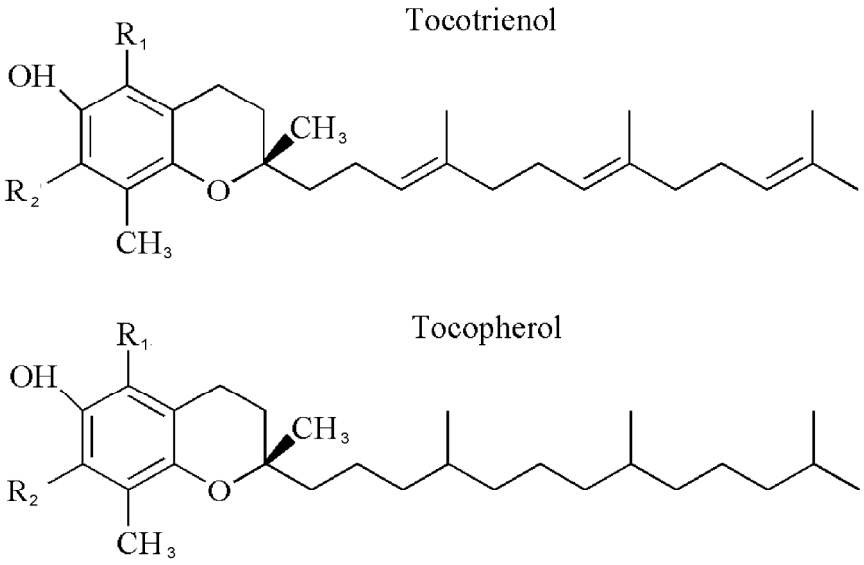
Fig. 4 The SIM chromatograms of the real samples.

The peak identifications are the same as those in Fig. 3.

 refers to the mass spectra of α-tocopherol acetate, which shows only α-tocopherol acetate was detected, and no β- or γ-tocotrienol was detected.



A gas chromatography-mass spectrometry (GC-MS) has been developed for the simultaneous determination of eight VE isomers and α-tocopherol acetate in functional foods.



isomer	R_1	R_2
α-tocopherol/tocotrienol	CH_3	CH_3
β-tocopherol/tocotrienol	CH_3	H
γ- tocopherol/tocotrienol	H	CH_3
δ-tocopherol/tocotrienol	H	H

Fig. 1 The chemical structures of the isomers of tocopherol and tocotrienol.
254x245mm (300 x 300 DPI)

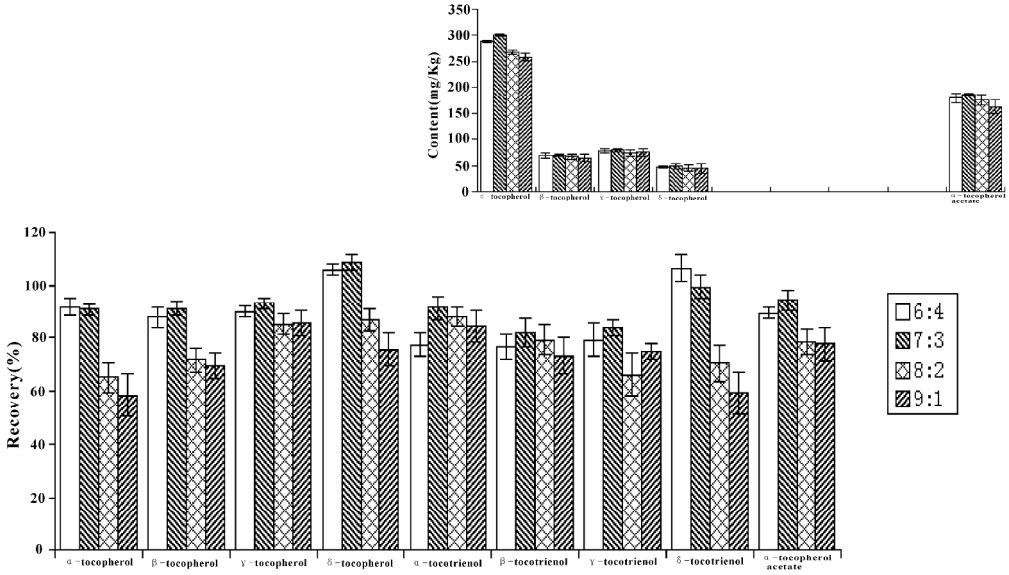


Fig. 2 Recoveries (below) and the detected content (above) of VE isomers using different proportion of methanol - hexane. (mean±SD, n= 3).
1267x785mm (100 x 100 DPI)

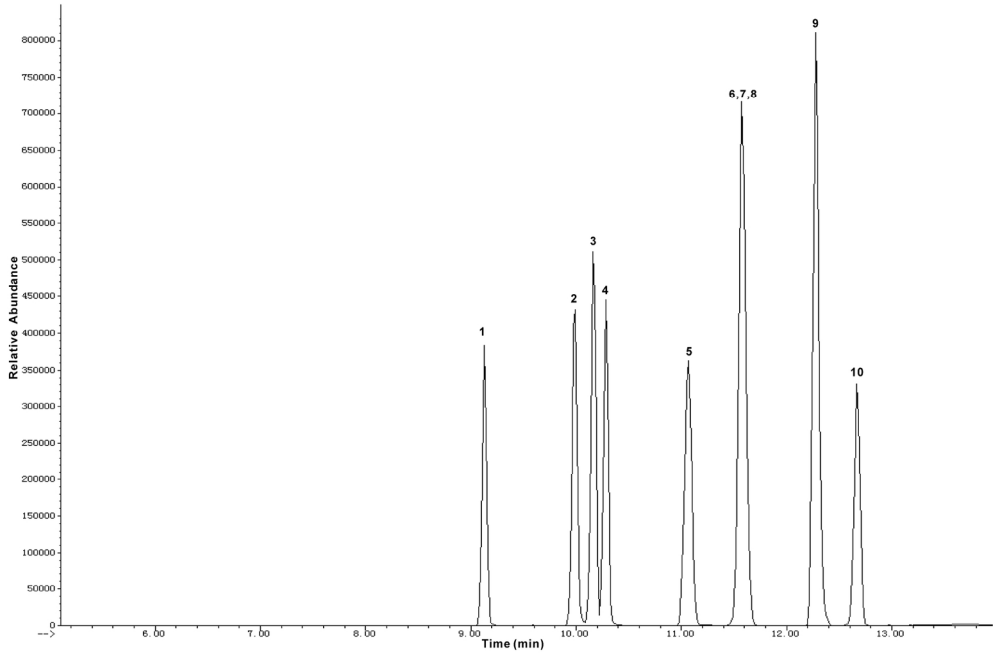


Fig. 3 The total ion chromatogram of the mixed standards and internal standards (I.S.).
Peak identifications: 1. δ-Tocopherol; 2. β-Tocopherol; 3. γ-Tocopherol; 4. δ-Tocotrienol; 5. α-Tocopherol; 6.β-Tocotrienol; 7.γ-Tocotrienol; 8. α-Tocopherol acetate; 9. Dibenzanthracene; 10. α-Tocotrienol.
167x109mm (300 x 300 DPI)

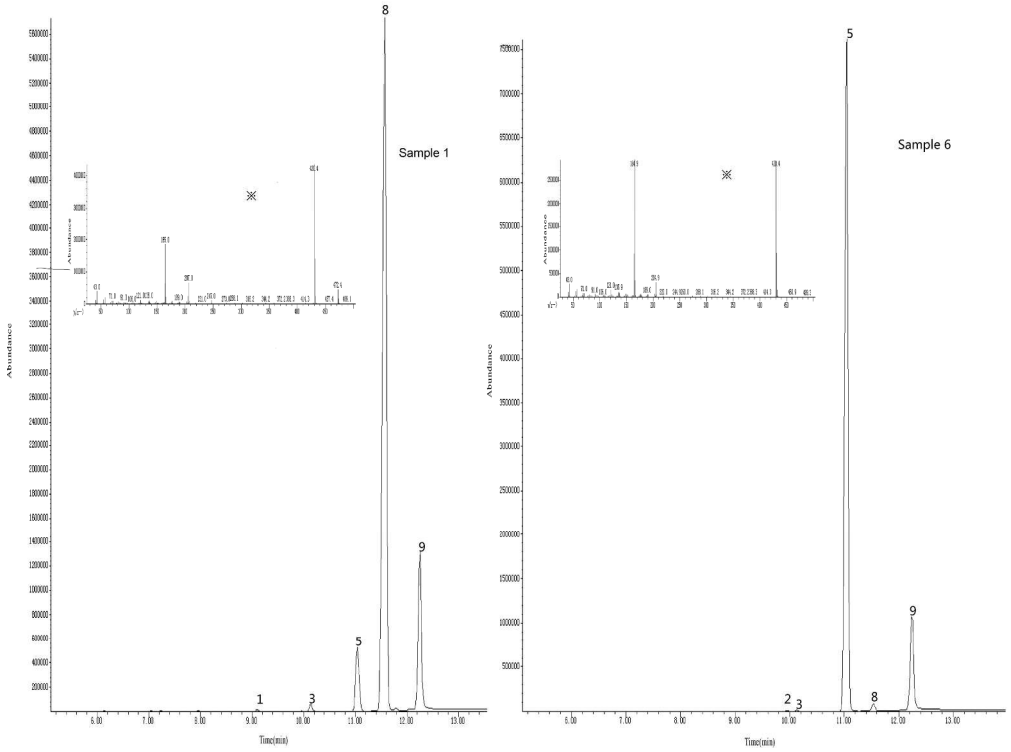


Fig. 4 The SIM chromatograms of the real samples.
The peak identifications are the same as those in Fig. 3. ※ refers to the mass spectra of α-tocopherol acetate, which shows only α-tocopherol acetate was detected, and no β- or γ-tocotrienol was detected.

262x197mm (300 x 300 DPI)

Table 1 Maximum absorption wavelength, molar absorptivities of nine analytes in 96% ethanol and the spectrophotometric concentration of annalytes.

Analyte	λ_{max}(nm)	Molar absorptivity (in $E^{1\%}$)	Spectrophotometric concentration (μg/mL)	Gravimetric concentration (μg/mL)
α-Tocopherol	291.9	75.8	18.47	20.00
β-Tocopherol	295.9	44.0	19.68	20.00
γ-Tocopherol	296.8	89.4	21.30	20.00
δ-Tocopherol	297.1	91.4	20.85	20.00
α-Tocotrienol	291.8	87.3	21.03	20.00
β-Tocotrienol	294.2	91.0	20.83	20.00
γ-Tocotrienol	296.8	87.3	20.28	20.00
δ-Tocotrienol	296.9	90.5	21.04	20.00
α-Tocopherol acetate	284.7	88.1	20.23	20.00

Table 2 Retention time (RT), molecular formula and detection settings for the analytes and internal standard.

Analyte	RT (min)	Molecular formula	Identification ions (m/z)			Quantitation ions (m/z)	Dwell time (ms)
δ-Tocopherol	9.14	$C_{27}H_{46}O_2$	402.3	137.0	177.0	402.3	100
β-Tocopherol	9.98	$C_{28}H_{48}O_2$	416.3	150.9	190.8	416.3	50
γ-Tocopherol	10.17	$C_{28}H_{48}O_2$	416.3	150.9	190.8	416.3	50
δ-Tocotrienol	10.29	$C_{27}H_{40}O_2$	396.2	137.0	177.0	396.2	100
α-Tocopherol	11.06	$C_{29}H_{50}O_2$	430.4	165.0	205.0	430.4	100
β-Tocotrienol	11.59	$C_{28}H_{42}O_2$	428.3	164.9	204.9	428.3	30
γ-Tocotrienol	11.59	$C_{28}H_{42}O_2$	410.3	151.0	191.0	410.3	30
α-Tocopherol acetate	11.59	$C_{31}H_{52}O_3$	472.4	430.4	165.0	472.4	30
Dibenzanthracene	12.29	$C_{22}H_{14}$	278.0	139.0		278.0	100
α-Tocotrienol	12.67	$C_{29}H_{44}O_2$	424.3	165.0	205.0	424.3	100

Table 3 Internal standard calibration equations, correlation coefficients, linear ranges, LODs and LOQs of the method.

Analyte	Internal standard calibration equations	Correlation coefficients (r)	Linear range (μg/mL)	LOD (ng/mL)	LOQ (ng/mL)
α-Tocopherol	y=0.654x-0.257	0.999	0.10-20.00	0.13	0.44
β-Tocopherol	y=0.469x-0.200	0.999	0.10-20.00	0.16	0.52
γ-Tocopherol	y=0.547x-0.238	0.999	0.10-20.00	0.19	0.62
δ-Tocopherol	y=0.365x-0.143	0.999	0.10-20.00	0.35	1.14
α-Tocotrienol	y=0.822x+0.199	0.999	0.20-40.00	0.27	0.87
β-Tocotrienol	y=0.199x-0.113	0.999	0.10-20.00	0.18	0.60
γ-Tocotrienol	y=0.066x-0.036	0.998	0.10-20.00	0.46	1.52
δ-Tocotrienol	y=0.073x-0.007	0.999	0.15-30.00	0.19	0.63
α-Tocopherol acetate	y=0.455x-0.309	0.998	0.10-20.00	0.09	0.29

Table 4 Recoveries and precision of the analytes in different matrices obtained with various formulations (n=3).

Formulation	Analyte	Background (mg/kg, $\times 10^3$)	Spiked (mg/kg, $\times 10^3$)	Found (mg/kg, $\times 10^3$)	Recovery (%)	Average recovery (%)	RSD (%)
Capsule	α-Tocopherol	5.52	2.00	7.79	113	107	2.6
			5.00	9.75	84.6		4.7
			10.0	17.9	124		3.0
	β-Tocopherol	ND	0.200	0.172	86.2	98.7	6.4
			0.500	0.515	103		3.6
			1.00	1.07	107		2.2
	γ-Tocopherol	0.691	0.200	0.862	85.6	100	3.6
			0.500	1.19	99.9		4.4
			1.00	1.84	115		3.4
	δ-Tocopherol	0.476	0.200	0.648	86.4	92.6	6.4
			0.500	0.928	90.4		4.3
			1.00	1.49	101		4.0
	α-Tocotrienol	ND	0.200	0.164	81.8	89.5	6.5
			0.500	0.456	91.2		3.2
			1.00	0.955	95.5		4.9
	β-Tocotrienol	ND	0.200	0.189	94.6	96.9	3.2
			0.500	0.466	93.2		4.8
			1.00	1.03	103		3.3
	γ-Tocotrienol	ND	0.200	0.153	76.6	83.2	7.1
			0.500	0.406	81.1		5.2
			1.00	9.20	92.0		5.0
	δ-Tocotrienol	ND	0.200	0.156	78.2	92.0	8.4
			0.500	0.516	103		4.4
			1.00	0.949	94.9		6.5
	α-Tocopherol acetate	3.74	2.00	5.40	83.4	96.8	5.1
			5.00	8.58	96.9		3.9
			10.0	14.7	110		3.3
	α-Tocopherol	1.80	2.00	3.86	103	102	5.0
			5.00	7.09	106		4.3
			10.0	11.5	97.1		3.8

Tablet	β-Tocopherol	0.468	0.200	0.652	91.9	95.1	3.3
			0.500	0.920	90.4		2.9
			1.00	1.50	103		1.4
	γ-Tocopherol	0.526	0.200	0.703	88.1	94.5	5.1
			0.500	0.978	90.4		4.9
			1.00	1.57	105		4.6
	δ-Tocopherol	ND	0.200	0.172	86.0	91.1	4.3
			0.500	0.462	92.3		3.8
			1.00	0.951	95.1		2.7
	α-Tocotrienol	ND	0.200	0.177	88.5	91.7	5.9
			0.500	0.468	93.7		3.6
			1.00	0.929	92.9		4.7
	β-Tocotrienol	ND	0.200	0.181	90.6	96.4	5.3
			0.500	0.487	97.5		3.2
			1.00	1.01	101		1.1
	γ-Tocotrienol	ND	0.200	0.176	88.0	91.4	5.4
			0.500	0.475	94.9		2.1
			1.00	0.912	91.2		2.8
	δ-Tocotrienol	ND	0.200	0.186	93.2	97.8	4.4
			0.500	0.509	102		3.9
			1.00	9.81	98.1		2.8
	α-Tocopherol acetate	0.913	0.200	1.11	98.5	98.1	3.9
			0.500	1.42	101		3.7
			1.00	1.86	94.7		2.2

ND, not detected.

Sample No.	Formulation	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	α-Tocotrienol	β-Tocotrienol	γ-Tocotrienol	δ-Tocotrienol	α-Tocopherol acetate	Total VE
1	Soft capsule	5.52 [a]	ND[a]	0.691 [a]	0.476 [a]	ND[a]	ND[a]	ND[a]	ND[a]	37.4 [a]	44.1 [a]
		42.7 [b]	ND [b]	0.640 [b]	0.450 [b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	43.8 [b]
2	Soft capsule	15.4 [a]	0.462 [a]	0.881 [a]	0.572 [a]	ND[a]	ND[a]	ND[a]	ND[a]	0.612 [a]	17.9 [a]
		14.9 [b]	0.440 [b]	0.832 [b]	0.530 [b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	16.7 [b]
3	Soft capsule	12.5 [a]	ND[a]	0.692 [a]	0.492 [a]	ND[a]	ND[a]	ND[a]	ND[a]	2.02 [a]	15.7 [a]
		11.5 [b]	ND [b]	0.647 [b]	0.455 [b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	13.0 [b]
4	Soft capsule	2.98 [a]	0.711 [a]	0.801 [a]	0.498 [a]	ND[a]	ND[a]	ND[a]	ND[a]	18.7 [a]	23.7 [a]
		20.1 [b]	1.16 [b]	1.76 [b]	0.480 [b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	23.5 [b]
5	Soft capsule	0.783 [a]	ND[a]	0.721 [a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	7.50 [a]	9.01 [a]
		8.51 [b]	ND[b]	0.570 [b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	9.07 [b]
6	Tablet	18.0 [a]	0.468 [a]	0.526 [a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	0.913 [a]	19.9 [a]
		16.7 [b]	0.471 [b]	0.556 [b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	17.7 [b]
7	Soft capsule	2.42 [a]	0.560 [a]	0.899 [a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	25.7 [a]	29.6 [a]
		27.7 [b]	0.882 [b]	1.54 [b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	30.1 [b]
8	Soft capsule	4.56 [a]	ND[a]	0.713 [a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	86.1 [a]	91.4 [a]
		88.3 [b]	ND [b]	1.07 [b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	89.4 [b]
9	Hard Capsule	4.81 [a]	ND[a]	0.482 [a]	0.403 [a]	ND[a]	ND[a]	ND[a]	ND[a]	40.9 [a]	46.6 [a]
		44.8 [b]	ND [b]	0.557 [b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	45.4 [b]
10	Tablet	1.90 [a]	ND[a]	0.262 [a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	16.3 [a]	18.5 [a]

		17.6	ND	0.546	ND					ND	18.1
11	Soft capsule	5.98 [a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	60.3 [a]	66.3 [a]
		67.2 [b]	ND [b]	ND [b]	ND [b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	67.2 [b]

ND, not detected.
a, direct solvent extraction
b, extraction after saponification