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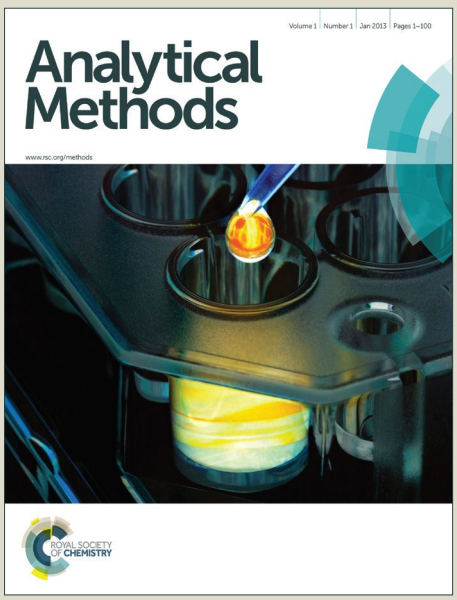

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Simultaneous analysis of tocopherols, tocotrienols, phospholipids, γ-oryzanols and β-carotene in rice by ultra-high performance liquid chromatography coupled to linear ion trap-orbitrap mass spectrometer

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ABSTRACT

A rapid ultra-high performance liquid chromatography linear with ion trap-orbitrap high resolution mass spectrometer (UHPLC-LTQ-Orbitrap HRMS) method for the determination of 21 nutrients in rice was developed. A simultaneous separation of tocopherols (α-, β-, γ-, δ-), tocotrienols (α-, β-, γ-, δ-), phospholipids, γ-oryzanols and β-carotene was achieved in less than 13 min. The detection was performed using a LTQ-Orbitrap MS detector in full scan with positive ion mode. This method was validated according to linearity, limits of detection and quantitation, reproducibility and recoveries. A regression coefficient (r^2>0.99) was obtained within the range of 0.05-10 μg mL^{-1} for tocopherols, tocotrienols and β-carotene, 0.1-50 μg mL^{-1} for phospholipids and 0.001-10 μg mL^{-1} for γ-oryzanols. The method gave detection limits (S/N, 3) of 0.2 to 1.9 ng mL^{-1} and quantitation limits (S/N, 10) of 0.7 to 6.3 ng mL^{-1}. Relative standard deviations, which were applied to estimate repeatability, ranged from 2.3 to 9.6%. Recoveries within a range of 80.6-109.6% for all the analytes were obtained. The mass accuracy for 21 validated compounds was ≤3 ppm. Furthermore, quantitative determination showed that rice processing could cause

content changes of nutritional composition between brown and white rice. Total ion current fingerprint profile (TICFP) could reveal that the significant differentiation of two types of rice samples (brown and white rices) from different regions. This method allowed fast and convenient analysis for the determination of nutrients in rice, which indicated the Orbitrap technology being beneficial for food testing.

Keywords: UHPLC; LTQ-Orbitrap HRMS; rapid; nutrients; rice

1. Introduction

Rice, one of the most important cereals, has been widely used as a staple food for more than half of the world's population, especially for people in the Asian regions [1-3]. Previous phytochemical studies indicated that rice contains high amounts of nutrients (tocopherols, tocotrienols and γ-oryzanols, etc) with diverse biological activities and healthy benefits [4-6]. Tocopherols and tocotrienols, including four isomers of tocopherols (α-, β-, γ- and δ-T) and four isomers of tocotrienols (α-, β-, γ- and δ-T3), have antioxidant abilities in clinical field, such as Parkinson syndrome, HIV and cancer diseases [7,8]. Gamma-oryzanol (γ-oryzanol) plays important roles in antioxidant, anti-inflammatory activities and menopausal disorders [4,5]. Beta-carotene (β-carotene) has been approved for the fundamentality of light energy collection and photoprotection, and the depressed risk of a variety of cancers and certain chronic diseases [7]. Phospholipid has a large number of physiological functions in relation to diverse diseases, including diabetes mellitus, obesity, atherosclerosis, Alzheimer's disease, traumatic brain injury and cancer [9-14]. However, the present studies of using several ingredients in investigating rice were not multi-component researches and could not reflect the overall changes of nutritional composition that occur during the course of processing. Thus, a rapid analytical method for the simultaneous determination of multi-component in rice is urgently essential.

Many analytical techniques such as reverse phase-liquid chromatography (RP-LC), reverse phase-high performance liquid chromatography (RP-HPLC), liquid

chromatography-gas chromatography (LC-GC) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) were utilized for the determination of constituents including carotenoid, vitamin E and γ-oryzanol [7, 15-18]. Much of the original work demonstrated that vitamin E and γ-oryzanol could be quantified by normal phase-high performance liquid chromatographic (NP-HPLC) method [6, 19-23]. However, the issues of NP-HPLC were observed in the process of analysis, which can be concluded as follows: poor reproducibility, long analysis time and low stability [24]. Furthermore, phospholipid was not determined, and a carcinogenic solvent was often contained in the mobile phase [23]. Although several methods have been developed to determine vitamin E, γ-oryzanols and carotenoids in cereals and in rice products, no method was reported for comprehensive and simultaneous separation and quantification of tocopherols, tocotrienols, phospholipids, γ-oryzanols and β-carotene in rice by using reverse phase-ultra high performance liquid chromatographic (RP-UHPLC). In contrast, the advantages of RP-UHPLC were remarkable, which included shorter analysis time, simple solvent composition, low solvent consumption, high sensitivity, resolution and mass accuracy when coupled with a mass analyser of LTQ-Orbitrap MS [25-27]. The features of LTQ-Orbitrap mass detection also vary, with the instruments being time of flight (TOF) and quadrupole-time of flight (Q-TOF) mass spectrometers. The LTQ-Orbitrap MS instrument has significant features an S-lens with up to 10 fold improved ion transmission for the atmosphere, a dual linear ion trap, fast polarity switching, high mass resolution (>15,000 FMWH), high mass accuracy (<2 ppm), a more efficient

higher energy collisional dissociation (HCD) cell interfaced directly to the C-trap and data processing by MetExtract software when compared with TOF and Q-TOF MS [25,28]. All these advantages suggest that RP-UHPLC coupled with high resolution LTQ-Orbitrap mass spectrometry is an advanced, accurate and reliable technique for the comprehensive and simultaneous analysis of multiple compounds in rice.

In this article, the antioxidant constituents in rice were divided into five groups: tocopherols, tocotrienols, phospholipids, γ-oryzanols and β-carotene (Fig. A.1). A rapid RP-UHPLC-LTQ-Orbitrap HRMS method was established for the simultaneous determination of tocopherols, tocotrienols, phospholipids, γ-oryzanols and β-carotene using PFP column. The method described has been carefully validated and subsequently employed to quantify multiple constituents and compare their fingerprint profiles in two types of rice from different regions.

2. Materials and methods

2.1. Chemicals and samples

Methanol and formic acid of HPLC grade used for UHPLC analysis were obtained from Merck company (Darmstadt, Germany). 2,6-Di-tert-butyl-4-methylphenol (BHT, antioxidant, analytical reagent) was obtained from Sigma-Aldrich company (USA). High purity water was prepared using a Milli-Q water purification system (Millipore, Milford, USA). All standard compounds had high purity (>98%). The following standards including: α-tocopherol and γ-oryzanols were purchased from Toronto Research Chemicals Inc. (Canada). Other standards were purchased from Supelco Company, USA (β-, γ- and δ-tocopherols), ChromaDex Inc., USA (α-, β-, γ- and

δ-tocotrienols), ANPEL Laboratory Technologies (Shanghai) Inc. (phospholipids), and Wako Pure Chemical Industries, Ltd, Japan (β-carotene). The pierce LTQ positive ion calibration solution (Thermo Scientific, USA) was used for the calibration of LTQ Orbitrap mass spectrometer that was tuned and calibrated using the calibration solution once a week. Rice samples were produced in Thailand, Hubei province and Northeast region in China.

2.2. Preparation of standard and sample solutions

All standard compounds were dissolved in methanol with 0.05% BHT at a concentration of 1000 μg mL^{-1} to obtain stock solutions and stored at 4 °C. Working solutions were prepared by mixing appropriate amounts of the stock solutions and diluting with methanol. All solutions were stored in amber glass bottles at 4 °C in the dark.

Rice samples were extracted rapidly by one-step methanol extraction method with minor modification [15,29] as follows: The rice samples were accurately weighed (0.1 g) and extracted ultrasonically in 3 mL of methanol containing 0.05% BHT for 5 min at room temperature. Then the extraction solution was filtered using a 0.22 μm millipore filter and transferred into a sample vial as the test solution for UHPLC/MS analysis.

2.3. UHPLC-LTQ-Orbitrap HRMS conditions

UHPLC analysis was achieved using a Thermo Scientific UHPLC system (Thermo Fisher Scientific, USA) equipped with automatic sample injector and Accela 1250 pump containing a binary high-pressure. Separation was carried out on a

poroshell 120 pentafluorophenyl (PFP) column (3.0 mm × 150 mm, 2.7 μm, Agilent, USA) with high efficiency and low pressure for UHPLC at a flow rate of 0.3 mL min^{-1} and a column temperature of 25 °C. Solvent A of water containing 0.1% aqueous formic acid and solvent B of methanol containing 0.1% aqueous formic acid were used as mobile phases of a binary solvent system. A linear gradient elution was conducted under the conditions as follows: 0 min, 85% B; 1 min, 95% B; 7 min, 98% B; 13 min, 100% B. The first 5 min was set to inject in waste mode. The injection volume was 10 μL.

MS analysis was performed on a LTQ XL and LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific) coupled with an atmospheric pressure chemical ionization (APCI) source, direct injection device and automatic calibration technique. The corresponding analysers of LTQ XL and LTQ Orbitrap XL were ion trap (IT) and fourier transform mass spectrometer (FTMS), respectively. The APCI source conditions applied were set as follows: mass range m/z 100-1000, vaporizer temperature 400 °C, capillary temperature 270 °C, source voltage 3 KV, capillary voltage 50 V, tube lens 100 V, mass resolution 30,000 FWHM. The flow rates of sheath, aux and sweep gases were 50, 5 and 0 arb, respectively. The data type, mass range, analyzer and polarity were profile, normal, FTMS and positive, respectively. Collision induced dissociation (CID) was used for APCI-MS^2. The data dependent settings were set as follows, a rapid CID-MS^2 scan of the most intense ions is performed with detection in the ion trap mass analyzer, the collision energy was CID with 35 eV, the ion isolation width was 2 m/z, mass and scan widths were 5%, default

charge state was 1, activation Q was 0.250, normalized collision energy at 35% and activation time of 30 ms, the orbitrap resolution was 7500 FWHM.

2.4. Method validation

The validation of the method was carried out after the optimization of UHPLC-LTQ-Orbitrap HRMS conditions. Linearity was evaluated by preparing different calibration curves within the range of 0.050-10 μg mL^{-1} for tocopherols, tocotrienols and β-carotene, 0.10-50 μg mL^{-1} for phospholipids and 0.0010-10 μg mL^{-1} for γ-oryzanols. The limit of detection (LOD) and limit of quantitation (LOQ) for each compound were determined based on the concentrations (based on peak heights) corresponding to 3× noise and 10× noise, respectively. Repeatability and recovery of nutritional compositions were investigated to validate the UHPLC-LTQ-Orbitrap MS method at three different points–lower level: lower calibration level, medium level: 2 μg mL-1 for tocopherols, tocotrienols, β-carotene and γ-oryzanols, 20 μg mL-1 (total concentration) for phospholipids, and high level: higher calibration level. In the recovery studies, the spiking was carried out by adding 100 μL of the appropriate working mixture to 0.1 g of rice. Then the rice samples were let to stand at room temperature to ensure that the solvent was evaporated and the standard compounds were homogenously distributed through the sample. At last, the rice samples were subjected to the established extraction procedure and UHPLC/MS analysis. The precision of the method was determined by the repeatability studies and expressed as the RSD (%). In parallel, matrix effects were investigated in white and brown rices by comparing the slopes of standards in solvent

with the slopes of matrix-matched standards. The matrix effect (%) was calculated via the equation [(1-slope of matrix standards/slope of solvent standards)] × 100 [25,30].

2.5. Data analysis

UHPLC/MS data were detected and processed using Thermo Xcalibur software V 2.1 including: Qual, Quan and Library Browser in the positive ion mode. The method parameters were set as follows: retention time range 0.00-13.00 min, mass range m/z 100.00-1000.00, microscans 1, max. inject time 500.00, delay 0.00 min, mass tolerance 5.0 ppm, decimals of mass precision 5, intensity range 0.00-100.00%, RDB equiv -1.0-100.0, pullup delay 3000 ms, needle gap valve clean 3 mm. Detector: MS, peak algorithm: ICIS, nitrogen-rule: do not use, inject to: LC Vlv1. Airgap, front and rear volumes were 3, 5 and 5 μL, respectively. Filling and injection speeds were 5 μL/s. Pre and post inject delays were 500 ms. Valve and post clean time solvents 2 were 2 s. Valve and post clean time solvents 1 were 3 and 2 s, respectively. Stator and delay stator wash were 0 and 120 s, respectively. Stator wash time solvents 2 and 1 were 5 s. The following databases have been used for the identification of phospholipids: Human Metabolome Database (HMDB, http://www.hmdb.ca/) and LIPID Metabolites and Pathways Strategy (LIPID MAPS, http://www.lipidmaps.org/). NIST MS Search V 2.0, Mass Frontier V 5.0 and SPSS V 11.5 softwares were used for mass information searching, structural fragment calculations and data processing, respectively.

3. Results and discussion

3.1. Comparison of extraction methods

In this study, one-step methanol extraction method was used for the extraction of compounds in rice. A modification of extraction way was essential to obtain a high extraction efficiency. To establish an optimal extraction method for the analytes, different extraction ways were adopted and tested, such as ultrasonication, oscillation and vorticity. As displayed in Fig. A.2, recoveries within the range of 84.7-98.9%, 81.4-92.8% and 80.6-92.9% were obtained for ultrasonication, oscillation and vorticity methods, respectively. The results suggested that the extraction method of ultrasonication has the ability to obtain a higher recovery of nutritional compounds with higher contents chosen in rice when compared with oscillation and vorticity. The matrix effects of these methods could affect the responses of nutritional compositions with higher contents chosen in rice. In ultrasonication, up to 14.3% of nutritional compositions showed ion suppression ≥30%, almost 85.7% nutrients showed an ion suppression between 3 and 20%. In oscillation, up to 14.3% of nutritional compositions showed ion suppression ≥30%, approximately 85.7% nutrients had an ion suppression between 5 and 30%. In vorticity, up to 14.3% of nutritional compositions showed ion suppression ≥30%, 85.7% nutrients experienced an ion suppression between 7 and 30%. The results indicated that the ultrasonication could obtain lower ion suppression when compared with oscillation and vorticity methods. Thus, the way of ultrasonication was selected as the extraction method in the experiments.

3.2. UHPLC-LTQ-Orbitrap MS

3.2.1. Optimization of UHPLC conditions

The chromatographic separation was performed on an UHPLC system, which was based on a poroshell 120 pentafluorophenyl phase (PFP) column (3.0 mm × 150 mm, 2.7 μm). The comparative chromatograms obtained by using poroshell 120 PFP column and hypersil gold C18 column (2.1 mm × 100 mm, 1.9 μm) were shown in Fig. A.3. As presented in Fig. A.3, only four isomers of vitamin E ion currents could be extracted via using C18 column, whereas PFP column showed better separation efficiency with favourable peak shape. Meanwhile, compounds retained on the PFP column also displayed certain regularities. Tocopherols and tocotrienols were eluted from the PFP column prior to γ-oryzanols and β-carotene, which has a agreement with published literature [7]. Vitamin E and phospholipids retained on the PFP column were quite adjacent since the former has a saturated or unsaturated alkyl side-chain of chromanol ring while phospholipids have two saturated or unsaturated fatty acid chains. The factor of similar polarity might result in the retention time of phospholipids being closed to vitamin E.

The optimization of elution system was aimed at increasing the resolution, decreasing the peak tailing and establishing the optimal peak separation of all compounds including: tocopherols, tocotrienols, phospholipids, γ-oryzanols and β-carotene in rice samples. Different solvent systems such as methanol-10 mM ammonium acetate, methanol-10mM ammonium formate, methanol-0.1% acetic acid, methanol-0.1% formic acid were investigated. By employing solvent system composed of methanol-10mM ammonium acetate, some chromatographic peaks could not be eluted due to the increasement of retention time (Fig. A.4A). While in the

solvent system composed of methanol-10mM ammonium formate, although good peak separation was obtained for eight vitamin E isomers, phospholipids and γ-oryzanols, β-carotene was not separated within 13 min (Fig. A.4B). Furthermore, using methanol-0.1% acetic acid, surface area and peak intensity of all peaks appeared to decrease dramatically with the increasing numbers of run (Fig. A.4C). These suggested that the previous three solvent systems were not suitable for the addition of protonation substance in the mobile phase system, while the solvent system containing methanol-0.1% formic acid (Fig. A.4D) has not only the ability of improving the resolution of adjacent peaks but also is capable of depressing the peak tailing in the extracted ion current (XIC) profile (Fig. 1) of nutritional compositions. Twenty-one compounds (Table A.1) were well separated within 13 min. This system can obtain a good separation and efficiency.

3.2.2. LTQ-Orbitrap HRMS analysis

3.2.2.1. Optimization of LTQ-Orbitrap HRMS conditions

The mass ionization of all compounds was carried out on a LTQ-Orbitrap mass spectrometer combined with an atmospheric pressure chemical ionization (APCI) source. In the experiments, electrospray ionization (ESI) and APCI sources were chosen to evaluate the ionogenic effect of different kinds of ionization sources for components in rice. Eight isomers of vitamin E and β-carotene could not be ionized under the ionization mode of ESI source. Some of them were displayed in Fig. A.5. While the results showed that APCI source without non-ionization phenomenon could make all compounds ionized, the reason might be that vitamin E and β-carotene were

more easily protonated in APCI source under positive ion mode. According to the principle of ionization, APCI source was more suitable to accelerate the ionization of liposoluble constituents (vitamin E and β-carotene) compared with ESI source. Thus, APCI source was employed as the terminal ion source in formal experiments.

Collision induced dissociation (CID) was used for data dependent MS^2 acquisition confirmation. The target of the selection of CID energy was to optimize and obtain the best condition of APCI-MS/MS ionization for bioactive compounds in rice. The fragmentation behaviors with 25 eV appeared a molecular ion peak with high relative abundance, indicating incomplete ionization. The common characters of CID 25 and 45 eV were a variety of background noises and undesired ion peaks arisen in the mass spectra. The results suggested that the collision energy with 35 eV could provide the best fragmentation behaviors of ionization in the positive mode for multiple ingredients in rice.

3.2.2.2. Fragmentation mechanism using high resolution MS

Tocopherols and tocotrienols have a common basic structural perssad with a chromanol ring that located an alkyl side-chain of position 2 of the ring. The structural difference between tocopherols and tocotrienols is that the former is combined with a saturated side-chain while the latter with an unsaturated one. As Figs. A.6 and 7 illustrated, tocopherols and tocotrienols showed an identical fragmentation pathway due to their common basic structural group. The CID of protonated molecular ion $[M+H]^+$ from tocopherols and tocotrienols produced abundant fragmentation ions. Three diagnostic fragments of $[frag1+H]^+$, $[frag2+H]^+$ and

$[frag1+H]^+$ were generated by the protonated molecular ion $[M+H]^+$ of tocopherols and tocotrienols after the disconnection of an alkyl side-chain located in different positions in the APCI-MS^2 spectra.

Fig. A.8 shows the CID pathway of phospholipids investigated in this study. The determination of accurate mass of full scan MS and the fragmentation information of APCI-MS^2 were necessarily applied to the detection and identification of the structures in order to investigate their fragmentation patterns. In positive mode, the adduct ions of $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ and $[M+NH_4]^+$ were the four main formations of phospholipids. Characteristic ion of hydrophilic head at m/z 183 derived from the CID of protonated molecular ion $[M+H]^+$ was simultaneously appeared on the APCI-MS^2 spectra of phosphatidylcholines (PCs). Subsequently, a diagnostic fragment with two structural formations was formed due to the disassociation of the unique fragment ion from the protonated daughter ion $[M+H]^+$.

The APCI of γ-oryzanol compounds easily allowed the identification of six components with m/z values of 602, 616, 576, 590, 604 and 578 to have a loss of ferulic acid molecule at m/z 194 in the full scan mass spectra. Their corresponding ion traces at m/z 409, 423, 383, 397, 411 and 385 indicated their presence as a fragment $[M-C_{10}H_{10}O_4+H]^+$ (molecular ion), which was already described in the published literature [7]. The peaks appearing in the mass spectra m/z 409 could be identified as cycloartenylferulate, m/z 423 as 24-methylencycloartanylferulate, m/z 383 as campesterylferulate, m/z 397 as β-sitosterylferulate, m/z 411 as cycloartanylferulate and m/z 385 as campestanylferulate. The discussion of fragmentation patterns of

γ-oryzanols was divided into three parts because of their common basic chemical structures. The fragmentation patterns of γ-oryzanols were summarized in Fig. A.9. Part 1, campestanylferulate has two primary fragment ions of $[M-C_{10}H_{10}O_4-122+H]^+$ at m/z 263 and $[M-C_{10}H_{10}O_4-62+H]^+$ at m/z 201. Part 2, campesterylferulate and β-sitosterylferulate belonging to one class owing to similar basic structure have different fragmentation pathways. The product ions of m/z 297 and 189 corresponding to the losses of the fragment of $[M-C_{10}H_{10}O_4-86+H]^+$ and $[M-C_{10}H_{10}O_4-108+H]^+$, respectively, were presented in the MS/MS spectra of campesterylferulate. The product ions of m/z 299, 243 and 203 were existed in the fragmentation pathway of β-sitosterylferulate. Part 3, compounds cycloartenylferulate, cycloartanylferulate and 24-methylencycloartanylferulate were classified as one category. The reason was that they not only have a common basic structure but also with the same fragmentation mechanism. The ion fragmentations of m/z 299, 217 and 203 appeared on the MS/MS spectra indicated the existence of cycloartenylferulate, cycloartanylferulate and 24-methylencycloartanylferulate.

Fig. A.10 shows the extracted ion current (XIC) profile, the fragment ions and the CID pathway of β-carotene with a protonated molecular ion $[M+H]^+$ at m/z 537 after the MS and MS^2 experiments. β-carotene with a feature of symmetric structure could yield three major product ions, which were m/z 480, 440 and 412. It is vulnerable to form a three-membered ring in the middle of the symmetric structure after the disconnection of six-membered ring. As a result, the APCI-MS^2 spectrum coupled with CID energy could provide protonated molecular and fragment ions for the

investigation and consequence of fragmentation patterns of tocopherols, tocotrienols, phospholipids, γ-oryzanols and β-carotene.

3.3. Method validation

The linearity was assessed by plotting the peak area of each standard compound and its corresponding concentration. The calibration curves were observed to be linear or quadratic with coefficients of determination (r^2 >0.99), which were obtained for all standard compounds in the required concentration ranges, and the results were reported in Table 1. The limits of detection (LOD) and limits of quantitation (LOQ) for each compound were determined based on the concentrations (based on peak areas) corresponding to 3× and 10× noise, respectively. The method gave low detection limits (0.2-1.9 ng mL^{-1}) of all vitamin E isomers and γ-oryzanols, which indicated higher sensitivity of this method by comparison with published literatures [15]. The low detection limits (0.6-1.4 ng mL^{-1}) of phosphatidylcholines and β-carotene were obtained, and the results also expressed the method with a high sensitivity. The LOQ of all compounds, estimated in the experiments, ranged from 0.7 to 6.3 ng mL^{-1}. To examine the repeatability of the present chromatographic method, repeated runs with the standard mixture were performed. The relative standard deviations (RSDs%) of repeatability for eight vitamin E isomers, phosphatidylcholines, γ-oryzanols and β-carotene were presented in Table 1. As could be observed, the ranges of 0.7-9.6%, 1.2-8.6% and 2.3-9.6% represented high, medium and lower level of analytical curve, respectively. In brown rice, the results indicated that a satisfactory quantitative recovery within the ranges of 83.5-101.9, 81.1-103% and 84.3-106.7 for lower,

medium and high level of analytical curve, respectively. In white rice, the ranges of 81.4-105.3, 83.3-104% and 80.6-109.6 were obtained for lower, medium and high level, respectively (Table A.2). Because of matrix effects, the responses of nutritional compositions either decreased or increased. The matrix could either enhance or suppress ionization of all compounds, its effects might vary from compound to compound and ultimately affect the UHPLC-LTQ-Orbitrap MS quantitative results. To evaluate matrix effects, the slope of the calibration curve obtained, at the same concentration levels, in sample extracts were compared to those of nutrient standards prepared in solvent. The full results were showed in Fig. 2. In white matrices, approximately 9.5% of nutritional compositions showed ion suppression ≥30%, up to 4.8% of nutrients experienced an ion enhancement >10%. Similar results were observed in brown rice, of which 4.8% nutrients were enhanced, and almost 4.8% of nutritional compositions had ion suppression ≥30%. The degree of ion suppression and enhancement from UHPLC-LTQ-Orbitrap MS was not severe. That is logical since matrix effects are not related to a particular type of detector but to the ionization process [31].

Table 1 Linearity, detection and quantitation limits and repeatability for eight vitamin E isomers, phosphatidylcholines, γ-oryzanols and β-carotene.

Compounds	Linear range (μg mL^{-1})	r^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Repeatability (n=6, RSD%)		
					High level	Medium level	Lower level
α-T	0.050-10	0.9946	0.8	2.7	2.3	4.6	2.2
β-T	0.050-10	0.9949	1.9	6.3	4.8	4.2	3.7
γ-T	0.050-10	0.9948	1.2	4.0	4.2	5.6	3.4
δ-T	0.050-10	0.9959	1.1	3.7	4.6	6.7	5.4
α-T3	0.050-10	0.9952	0.7	2.3	1.6	3.2	2.9
β-T3	0.050-10	0.9962	0.6	2.0	2.9	3.5	2.4
γ-T3	0.050-10	0.9962	0.4	1.3	1.7	2.3	1.2
δ-T3	0.050-10	0.9946	0.6	2.0	2.4	2.9	2.7
PC(36:5)	0.10-10	0.9920	1.4	4.7	7.6	6.9	6.1
PC(36:4)	1.0-50	0.9907	1.3	4.3	9.6	8.2	5.6
PC(34:2)	1.0-50	0.9920	1.1	3.7	7.9	9.2	3.5
PC(36:3)	1.0-50	0.9910	0.9	3.0	8.4	8.6	5.0
PC(36:2)	0.10-10	0.9940	0.6	2.0	7.8	9.6	4.9
PC(34:1)	0.10-10	0.9919	0.7	2.3	7.1	7.8	8.6
cycloartenylferulate	0.10-10	0.9935	0.3	1.0	2.6	2.5	3.1
24-methylencycloartanylferulate	0.10-10	0.9946	0.3	1.0	1.5	2.6	2.0
campesterylferulate	0.010-10	0.9947	0.3	1.0	0.7	3.0	2.8
β-sitosterylferulate	0.010-10	0.9974	0.3	1.0	1.5	2.3	2.2
cycloartanylferulate	0.0010-10	0.9957	0.2	0.7	2.0	4.2	2.6
campestanylferulate	0.0010-10	0.9953	0.3	1.0	2.0	3.9	2.4
β-carotene	0.050-10	0.9901	1.0	3.3	2.1	7.0	4.8

3.4. UHPLC/MS quantification and fingerprint profiles of rice samples

3.4.1. Quantification of brown and white rice samples

The validated method was applied to the quantification of two types of rice samples (brown and white rices) that were analyzed in triplicate. Tocopherols, tocotrienols, phospholipids, γ-oryzanols and β-carotene were chosen as the target compounds in all rice samples. Table 2 outlines the quantification of target compounds in brown and white rices from different regions. Compounds α-T, α-, β- and δ-T3, four of vitamin E nutrients, existed in brown rice produced from China, which illustrated that these nutritional compositions were the four major formations in brown rice. Northeast brown rice-1 (NBR-1) and Northeast white rice-1 (NWR-1) samples presented the higher number of target compounds compared to NBRs-2, -3 and NWRs-2, -3 in either brown or white rice sample with the exception of some compounds that were not detected or with a low concentration, which explained the difference of rice-growing districts had significant effects on the content of rice. PC (36:5) could not be detected in each type of sample from brown and white rices, which might not existed in rice. PCs, including PC (36:4), PC (34:2), PC (36:3), PC (36:2) and PC (34:1), have higher contents in brown rice. It can be explained that PCs are significant components and its content can be greatly reduced after being processed as white rice. PCs measured in this study were observed to have two unsaturated fatty acyl residues with a higher number of carbons. These PCs may offer an inhibition for the development of type 2 diabetes mellitus. The acyl-alkyl-phosphatidylcholine was inversely correlated to plasma triglyceride and linked to improve insulin sensitivity.

The results were consistent with that reported previously [32]. Thus PCs, as one class of the important nutrients, contained diverse biological activities and health benefits. Six primary compositions of γ-oryzanol were detected in each of brown rice sample. Most of compounds of γ-oryzanol in white rice were quantized with the exception of campestanylferulate in NWR-2, NWR-3 and Hubei white rice (HBWR) and cycloartanylferulate in HBWR and Thailand white rice (TLWR). The amount of β-carotene was determined as the presence of non-detection or low concentration for all rice samples. In general, the amounts of compositions in brown rice samples are higher than those in white rice samples.

3.4.2. Total ion current fingerprint profile of brown and white rice samples

Fig. 3 shows the total ion current fingerprint profile (TICFP) of five brown and five white rice samples using UHPLC/MS. In brown rice, samples 1, 2, 3, 4 and 5 represent NBR-1, NBR-2, NBR-3, Hubei brown rice (HBBR) and Thailand brown rice (TLBR), respectively. In white rice, samples 1, 2, 3, 4 and 5 represent NWR-1, NWR-2, NWR-3, HBWR and TLWR, respectively. The TICFP of brown rice sample produced in Thailand (sample 5) was different from those in China (samples 1, 2, 3 and 4) by comparison of retention time, peak shapes and areas. A study of the overall profile of compounds presented in brown rice indicated that samples 1, 2 and 3 were similar in compositions. Moreover, the TICFP of sample 4 in brown rice differed from rice samples 1, 2 and 3. In the TICFP of white rice, the similarity of samples did not appear to be displayed particularly. Each sample might be different from others. Samples 2 and 4 were quite similar to samples 3 and 5, respectively.

As shown in Fig. 4A, all sample groups in brown rice were systematically classified in five clusters, which were samples 1, 2, 3, 4 and 5. Samples 1, 2 and 3 have a similar degree in their fingerprint pathways of TIC. Although the boundary outline of sample 1 in TICFP was quite close to sample 2, it still existed some differences, thus samples 1 and 2 can be divided into two classes as displayed in Fig. 4B. The reason for the differences above was that samples 1, 2 and 3 came from the same region but from different rice-growing districts. It was proved that the dendrogram produced via hierarchical cluster method could be usefully and intuitively applied to the expression of relationship of different sample groups.

Figs. 4C and D show the classification of sample groups from brown and white rice after the hierarchical cluster based on single linkage using canonical discriminant functions. The classification of discriminant analysis could provide the further confirmation for the similarity and differentiation of TICFP. Obviously, the distances of NBR-1 and NBR-2 were more closer to NBR-3 than HBBR. The cluster of TLBR was far away from other groups. However, the group centroids of NWR-2 and HBBR in white rice were quite close to NWR-3 and TLBR, respectively. The reason might be that brown and white rices presented different processing degree, and the former had a higher content of nutrients with stable preservation than the latter. The results of the confirmation of discriminant analysis were consistent with the classifications displayed in the dendrogram using hierarchical cluster, with 100.0% of original grouped cases correctly classified (Table A.3), which indicates the UHPLC/MS fingerprint profile was a useful and powerful technique in quality control of the cereal

extract.

4. Conclusions

The RP-UHPLC-LTQ-Orbitrap HRMS method for the simultaneous determination of 21 nutrients was firstly reported. The method was successfully applied to identify and quantify eight vitamin E isomers, phospholipids, γ-oryzanols and β-carotene in rice, which were separated within 13 min. Phospholipids were first reported as one of the bioactive nutrients with biological activities in rice. The whole time from sample extraction to UHPLC/MS analysis took less than 30 min. The analysis of TICFP can be used for the generation of an overview of all components in rice samples. Therefore, the method was rapid, effective and reliable for quality control of rice or other foods.

Table 2 Contents of nutritional compositions in brown and white rices ($\bar{x}$ ±sd, μg g^{-1}).

Investigated compounds	Brown rice					White rice				
	NBR-1a	NBR-2	NBR-3	HBBRb	TLBRc	NWR-1d	NWR-2	NWR-3	HBWRe	TLWRf
α-T	12.23±0.01	7.50±0.10	5.34±0.01	1.68±0.01	1.60±0.04	3.15±0.17	1.75±0.03	1.76±0.10	ND	ND
β-T	Lg	L	L	L	1.67±0.04	ND	ND	ND	ND	ND
r-T	L	NDh	ND	ND	ND	ND	ND	ND	ND	ND
δ-T	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
α-T3	3.71±0.10	3.05±0.15	1.70±0.03	1.86±0.03	2.67±0.04	2.37±0.03	1.58±0.04	1.55±0.01	1.57±0.00	1.61±0.05
β-T3	12.78±0.15	12.25±0.21	10.12±0.30	15.08±0.44	30.81±0.25	8.14±0.11	5.65±0.03	6.57±0.19	8.57±0.39	3.99±0.05
r-T3	ND	L	L	ND	ND	ND	ND	ND	ND	ND
δ-T3	1.70±0.00	1.67±0.09	1.75±0.09	1.76±0.09	1.82±0.03	L	L	L	L	L
PC(36:5)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PC(36:4)	128.79±0.90	60.27±0.90	47.01±0.27	L	L	L	ND	ND	ND	ND
PC(34:2)	117.98±0.84	58.10±1.68	35.97±0.68	29.81±0.17	L	L	L	L	L	ND
PC(36:3)	128.61±0.17	101.54±3.49	89.80±0.17	75.46±0.17	74.52±1.57	69.53±0.52	64.14±0.03	64.41±0.00	ND	ND
PC(36:2)	97.15±0.01	70.38±2.68	53.89±0.27	54.76±0.11	60.14±1.61	32.52±1.70	ND	30.97±0.04	30.66±0.08	ND
PC(34:1)	81.43±0.25	61.58±3.26	44.91±0.25	34.57±1.14	46.39±0.41	17.89±0.11	ND	ND	14.52±0.25	ND
Cycloartenylferulate	85.53±0.33	83.94±0.75	65.85±0.01	11.54±0.24	36.32±0.50	12.02±0.74	4.73±0.07	5.59±0.08	3.74±0.02	3.68±0.04
24-Methylencycloartanylferulate	71.59±0.41	69.21±0.49	54.71±0.25	23.57±0.33	171.01±0.25	11.58±0.16	3.50±0.03	4.94±0.35	3.41±0.07	4.96±0.02
Campesterylferulate	40.34±0.30	38.68±0.44	28.16±0.16	16.63±0.05	45.18±0.11	6.99±0.03	1.63±0.02	2.85±0.01	1.85±0.11	1.68±0.07
β-Sitosterylferulate	34.76±0.12	32.61±0.20	24.43±0.11	9.61±0.05	43.99±0.32	6.16±0.04	1.64±0.01	2.85±0.10	0.88±0.05	1.57±0.06
Cycloartanylferulate	4.65±0.01	4.07±0.03	3.11±0.10	1.17±0.01	3.67±0.08	0.59±0.05	0.11±0.01	0.21±0.01	L	L
Campestanylferulate	0.77±0.02	0.73±0.02	0.55±0.03	0.47±0.02	1.99±0.07	0.41±0.01	ND	ND	ND	0.05±0.00
β-carotene	ND	ND	ND	ND	1.81±0.01	ND	ND	ND	ND	ND

[a, b, c] Northeast, Hubei and Thailand brown rice, respectively.

[d, e, f] Northeast, Hubei and Thailand white rice, respectively.

[g] Too low to be reliably quantified. [h] Not detected.

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Figure Captions

1. Manuscript:

Figure 1. The extracted ion current (XIC) profile of 21 nutritional compositions.

Figure 2. UHPLC-LTQ-Orbitrap MS matrix effects.

Figure 3. Total ion current fingerprint profile (TICFP) of five brown and five white rice samples using UHPLC/MS.

Figure 4. The results of the similarities of UHPLC/MS fingerprint profiles utilizing hierarchical cluster and discriminant analysis (HCDA).

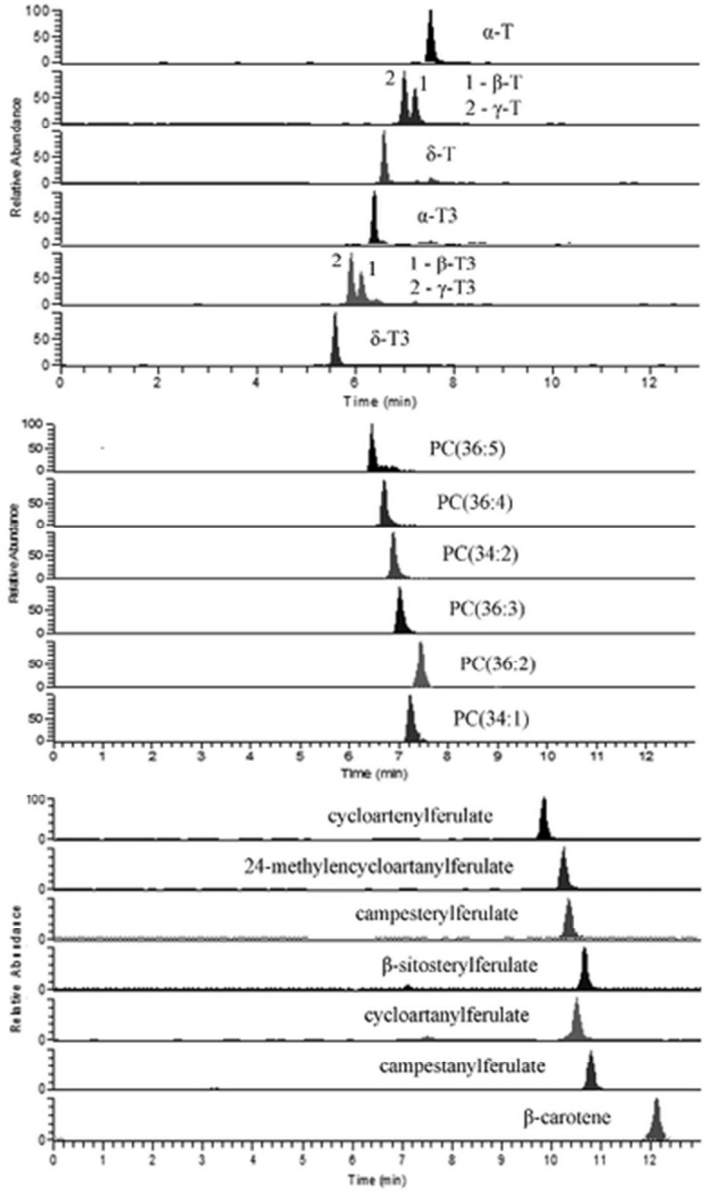

Figure 1. The extracted ion current (XIC) profile of 21 nutritional compositions.
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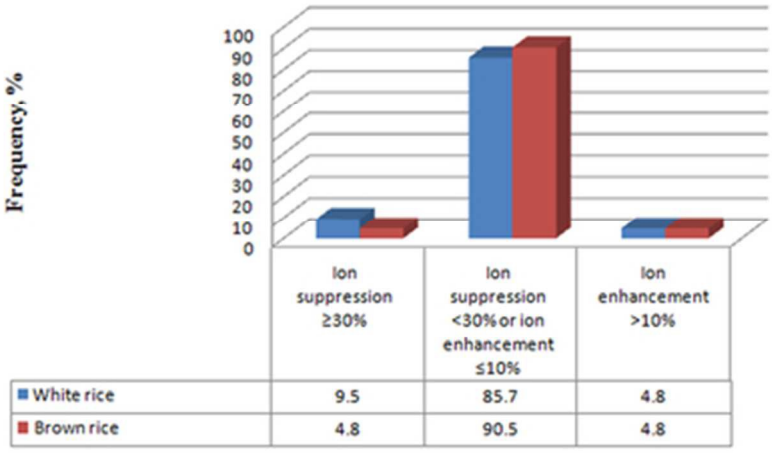

Figure 2. UHPLC-LTQ-Orbitrap MS matrix effects.
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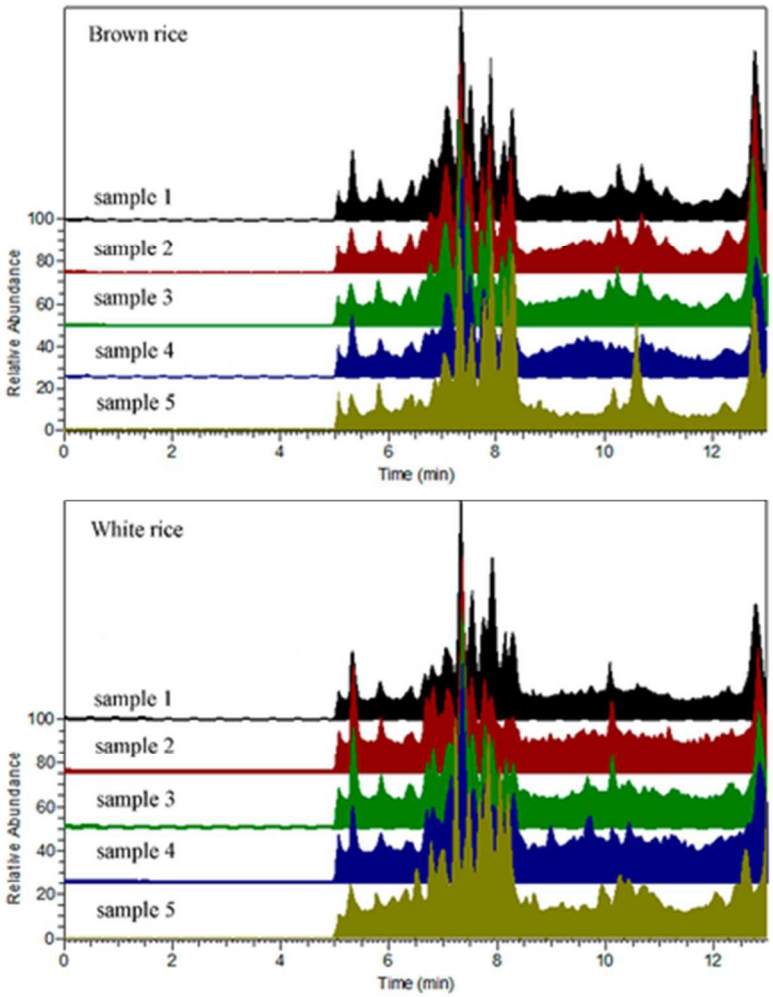

Figure 3. Total ion current fingerprint profile (TICFP) of five brown and five white rice samples using UHPLC/MS.
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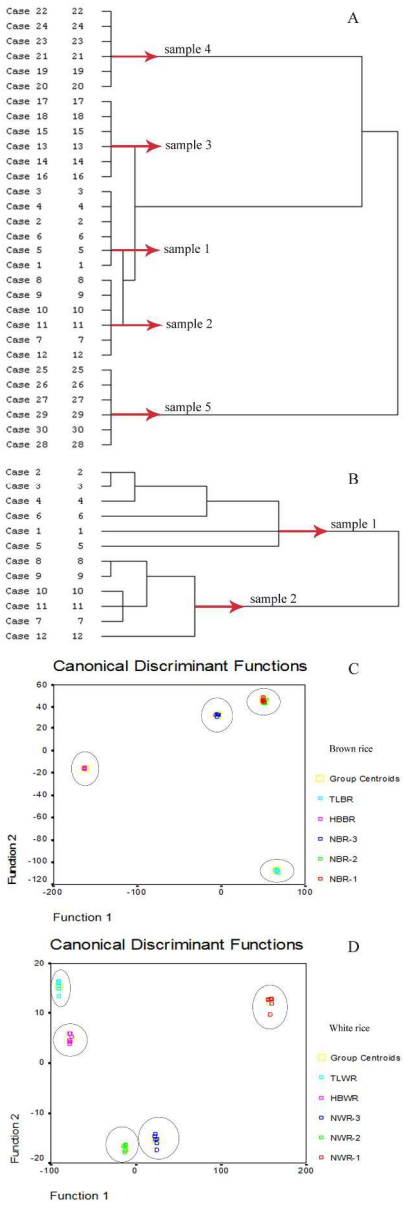

Figure 4. The results of the similarities of UHPLC/MS fingerprint profiles utilizing hierarchical cluster and discriminant analysis (HCDA).
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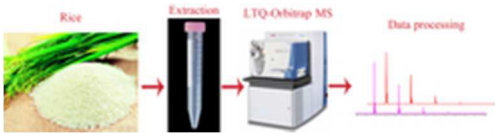

UHPLC-LTQ-Orbitrap MS method was developed for the simultaneous qualitation and quantitation of tocopherols, tocotrienols, phospholipids, γ-oryzanols and β-carotene in rice
20x5mm (300 x 300 DPI)