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Coupled liquid chromatography–gas chromatography for the rapid analysis of γ-oryzanol in rice lipids

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Abstract

An approach based on on-line coupled liquid chromatography–gas chromatography (LC–GC) was developed for the rapid analysis of γ-oryzanol in rice. Total lipids were extracted from rice and subjected to LC–GC without any prior purification. γ-Oryzanol was pre-separated by HPLC from rice lipids and transferred on-line to GC analysis in order to separate its major constituents, 24-methylenecycloartanyl ferulate, cycloartenyl ferulate, campesteryl ferulate, β-sitosteryl ferulate and campestanyl ferulate. The identities of the compounds were confirmed by off-line GC–MS analysis. Total γ-oryzanol content could be quantified by HPLC–UV detection and the distribution of γ-oryzanol constituents could be determined by on-line coupled GC analysis. The proposed methodology paves the way for high-throughput investigations providing information on natural variations in γ-oryzanol content and its composition in different rice varieties.

Keywords: Rice; γ-Oryzanol; Steryl ferulates

1. Introduction

γ-Oryzanol comprises a mixture of phytosteryl ferulates located in rice bran. 24-Methylenecycloartanyl ferulate, cycloartenyl ferulate, campesteryl ferulate, β-sitosteryl ferulate and campestanyl ferulate have been identified as the major components [1]. In addition to the technological usefulness of these compounds, e.g., stabilization of vegetable oils and fats at frying temperature [2], γ-oryzanol has been shown to be suitable as a natural UV filter in sun screen creams [3]. However, the most outstanding properties of γ-oryzanol are its physiological effects. The cholesterol-lowering properties of rice bran and rice bran oil have been attributed to γ-oryzanol [4], and in vitro tests revealed superoxide dismutase-like antioxidative activity of γ-oryzanol [5]. Depending on the composition of the γ-oryzanol preparations, different effects in hyperlipidemic rats were observed [6]. The three major components of γ-oryzanol (24-methylenecycloartanyl ferulate, cycloartenyl ferulate and campesteryl ferulate) were shown to differ significantly in their antioxidative activities against cholesterol oxidation [7]. These data demonstrate the need for appropriate analytical methods to differentiate the individual constituents of γ-oryzanol.

For quantification of total γ-oryzanol in rice bran and rice bran oil, UV-spectroscopy or normal-phase HPLC have been applied. However, these methods are not able to differentiate the individual steryl ferulates [8,9]. Separation of individual γ-oryzanol

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components has been achieved using reversed-phase HPLC [1,10,11]. However, these methods are time-consuming because they require purification of γ-oryzanol by liquid–liquid fractionation followed by low-pressure column chromatography [10], or low-pressure column chromatography followed by preparative normal-phase HPLC [1]. A simpler method proposed would involve purification of γ-oryzanol from rice bran oil by liquid–liquid extraction using a solvent mixture which can be used as mobile phase in the subsequent reversed-phase HPLC step [11]. Analysis of γ-oryzanol by high-temperature gas chromatography requires pre-purification by liquid–liquid fractionation followed by preparative TLC, and, even after silylation, only poor resolution of the trimethylsilyl ether derivatives of 24-methylenecycloartanyl ferulate and cycloartenyl ferulate was achieved [12].

The on-line coupling of a liquid chromatographic pre-separation with capillary gas chromatography (on-line LC–GC) is an elegant and efficient approach for the analysis of minor constituents in complex matrices, because it avoids laborious off-line purification steps [13]. On-line LC–GC has been successfully applied to the analysis of sterols and/or steryl fatty acid esters in oils and fats [14–17]. The objective of this study was to develop such an on-line LC–GC method for the rapid analysis of γ-oryzanol in rice lipids.

2. Materials and methods

2.1. Chemicals

2-Propanol (HPLC gradient grade), hexane (mixture of isomers) and sodium hydroxide pellets (pure) were purchased from VWR International (Darmstadt, Germany). Dichloromethane (DAB, stabilized with 25 mg 1-pentene/l) was obtained from Riedel-de Haën (Seelze, Germany) and *tert*.-butyl methyl ether (Driveron S) was supplied from Oxeno Olefinchemie (Marl, Germany). Hexane, dichloromethane and *tert*.-butyl methyl ether were distilled prior to use. Hydrochloric acid (c.p.) was purchased from Acros Organics (Geel, Belgium). γ-Oryzanol (99.9% purity) was a gift from Henry Lamotte (Bremen, Germany).

2.2. Materials

Plastic sheets (20×20 cm) pre-coated with silica gel containing a fluorescence indicator for thin-layer chromatography (Polygram SIL G/UV_{254}) were purchased from Macherey-Nagel (Düren, Germany). Brown rice samples of the rice varieties ''Cripto'' and ''Balilla'' and rough rice were a gift from Mühldorfer Naturkorn-Mühle (Mühldorf, Germany). A sample of the variety ''Xiushui11'' was provided by Qingyao Shu (Institute of Nuclear Agricultural Sciences, Zhejiang University, China).

2.3. Sample preparation

The rice material was ground using a cyclone mill (Cyclotec 1093, Foss Tecator, Hamburg, Germany) equipped with a 500-μm sieve. Rice flour (150 mg) was weighed into a 11-ml vial. Before sealing the vial, 10 ml of dichloromethane–methanol (2:1, v/v) and a stirring bar were added to the rice flour. Extraction was performed in a water bath at 50 °C under stirring for 45 min. After filtration, the residue was re-extracted twice with 5 ml extraction solvent (each time shaking for 30 s) and filtered again. The combined filtrates were collected in a 25-ml flask and evaporated to dryness by rotary evaporation. The residue was redissolved in 2 ml hexane using an ultrasonic bath and subjected to on-line LC–GC analysis after passing through a membrane filter (0.45 μm, Spartan, Schleicher & Schuell, Dassel, Germany). In order to investigate the influence of the extraction temperature on yield, extraction of flour from rough rice was performed at 20, 50, 80 and 100 °C.

2.4. Instrumentation

2.4.1. On-line LC–GC

The analysis was performed using a fully automated on-line LC–GC instrument (Dualchrom 3000, ThermoFinnigan (C.E. Instruments), Egelsbach, Germany). Solvent was delivered by a 20-ml syringe pump (Phoenix 30 CU). Backflush of the LC column was performed using the backflush valve equipped with a 1-ml loop. Detection was with a variable wavelength detector (microUVIS 20, Linear Instruments, Reno, NV, USA). The GC possessed a fully

automated interface valve system. A loop-type interface equipped with a 560-μl loop and an early solvent vapour exit were used for the transfer of the γ-oryzanol containing fraction from LC. Detection during GC analysis was performed using a flame ionization detector (FID). All components were controlled by the Dualchrom software. LC data acquisition was performed by a recorder (Pharmacia LKB REC102, Amersham Bioscience, Freiburg, Germany) and GC data were acquired by the Chromcard Software (ThermoFinnigan).

2.4.2. GC–MS

GC–MS analysis was performed on a Voyager mass spectrometer linked to a GC 8000 Top gas chromatograph (ThermoFinnigan). *tert.*-Butyl methyl ether solutions of the samples (3 μl; approx. 3 mg γ-oryzanol/ml) were injected into a vaporization injector (set at 280 °C) at a split ratio of 1:10. Individual steryl ferulates were separated on the analytical column used for on-line LC–GC analysis. Helium (75 kPa) was used as carrier gas. The temperature program was 250 °C (2 min), then programmed with 15 °C/min up to 310 °C (2 min) and with 1.5 °C/min up to 340 °C (3 min). The interface and the source were heated to 300 and 280 °C, respectively. Mass spectra were obtained by electron impact ionization at 70 eV in the full scan mode at unit resolution from 20 to 750 Da (scan time, 0.7 s; inter-scan delay, 0.05 s). Data were processed using the MassLab Software (ThermoFinnigan).

2.5. LC–GC conditions

HPLC pre-separation of γ-oryzanol was performed on a silica gel column Eurospher, 250×2 mm I.D., 100 Å pore size, 5 μm particle size equipped with a 20×2 mm I.D. pre-column of the same material (Knauer, Berlin, Germany) using a mixture of hexane–*tert.*-butyl methyl ether–2-propanol (95:5:0.5, v/v/v) as eluent. The flow-rate was set to 200 μl/min and UV detection was at 200 nm and 290 nm. Backflush of the column was performed using the backflush valve equipped with a 1-ml loop. The loop was filled with *tert.*-butyl methyl ether by pneumatic pressure. Backflush started 20 min after injection and was maintained for 7 min.

GC separation was performed on a 27 m×0.25 mm I.D. fused-silica capillary coated with trifluoropropylmethyl polysiloxane with a film thickness of 0.1 μm (Rtx-200MS, Restek, Bad Homburg, Germany), connected in series with a retaining pre-column (3 m×0.25 mm I.D.) having the same coating as the analytical column and an uncoated phenylsilylated fused-silica capillary (3 m×0.53 mm I.D; BGB Analytik Vertrieb, Schloßböckelheim, Germany). Solvent vapours were released during transfer by an early solvent vapour exit, which was installed between the retaining pre-column and the separation column via a Y-piece press-fit connector (BGB Analytik). The solvent vapour exit was switched to a restrictor (1 m×0.05 mm I.D. fused-silica) leaving a small purge flow during analysis after transfer. Hydrogen was used as carrier gas at a flow-rate of 1.9 ml/min, measured at 140 °C. The maximum carrier gas inlet pressure for the transfer was set to 250 kPa at the pressure regulator preceding the Porter flow regulator.

Transfer of the fraction containing γ-oryzanol started 12 min after injection of 10 μl of the sample and occurred by concurrent eluent evaporation at 140 °C. With a delay of 50 s on the reduction of the inlet pressure by 80 kPa at the end of the transfer, the solvent vapour exit was switched to the restrictor.

After holding the transfer temperature of 140 °C for 5 min, the column temperature was programmed to 310 °C at 15 °C/min, held for 5 min and then programmed to 340 °C at 2.5 °C/min, which was held for 3 min. The flame ionization detector was set to 320 °C.

2.6. Quantification of total γ-oryzanol and individual steryl ferulates

Standard solutions of γ-oryzanol in hexane (10–40 μg/ml) were subjected to on-line LC–GC. A calibration curve for the quantification of total γ-oryzanol was derived by linear regression of the peak area obtained by LC analysis and detection at 290 nm. Proportions of individual steryl ferulates were calculated from the peak area ratios obtained by the on-line coupled GC analysis and FID detection.

2.7. Recovery and repeatability

The recovery was evaluated by spiking rough rice samples with known amounts of γ-oryzanol. A 500 μl amount of a solution of γ-oryzanol in hexane (25.6 μg/ml) was added to each of three samples of 200 mg of rough rice flour. After evaporation of hexane by a gentle stream of nitrogen, the samples were analysed as described above. In addition, three samples of the same rice flour were analysed without addition of γ-oryzanol. The recovery was calculated on the basis of the peak area obtained by UV detection after LC analysis of the spiked and unspiked samples. Repeatability was investigated by 5-fold analysis of a rough rice sample.

2.8. Preparation of γ-oryzanol from rough rice for GC–MS analysis

About 40 g ground rough rice were weighed into a 200-ml flask. Before sealing the flask, 100 ml of dichloromethane–methanol (2:1, v/v) and a stirring bar were added to the rice flour. Extraction was performed in a water bath at 50 °C under stirring for 60 min. After filtration the residue was re-extracted twice with 50 ml extraction solvent each time by shaking for 30 s and filtered again. The combined filtrates were collected in a 250-ml flask and evaporated to dryness by rotary evaporation. The residue was redissolved in 10 ml hexane using an ultrasonic bath and transferred to a 25-ml flask. After addition of 10 ml 4 *M* sodium hydroxide solution, the vial was sealed and vigorously shaken. The hexane layer was discarded and the sodium hydroxide solution was washed twice with 10 ml hexane each time. The sodium hydroxide solution was acidified with concentrated hydrochloric acid and extracted with 10 ml hexane. After evaporation to dryness by rotary evaporation the residue was redissolved in 1 ml hexane and subjected to preparative thin-layer chromatography on silica gel using a mobile phase consisting of hexane–*tert*.-butyl methyl ether–2-propanol (80:20:1, v/v/v). γ-Oryzanol was detected by extinction of the fluorescence at 254 nm of the fluorescence indicator within the silica gel. The γ-oryzanol-containing band was scraped off and extracted with *tert*.-butyl methyl ether using an ultrasonic bath. Extracts were subjected to GC–MS analysis.

3. Results and discussion

3.1. Extraction of γ-oryzanol from rough rice

A mixture of chloroform and methanol is commonly used for the extraction of total lipids [18]. Owing to the toxicity of chloroform, the latter is often replaced by dichloromethane. Therefore, a mixture of dichloromethane–methanol (2:1; v/v) was used for the extraction of rice lipids containing γ-oryzanol from rice material. After evaporation to dryness, the residue was redissolved in hexane and subjected to on-line LC–GC without any prior purification steps.

In order to investigate the influence of the extraction temperature on yield, extractions at 20, 50, 80 and 100 °C were performed. Comparison of HPLC peak areas demonstrated 50 °C to be the optimum temperature. Both, increasing and decreasing the extraction temperature led to decreased extraction efficiencies (mean±confidence interval; $n=3$, $P<0.05$): 91.3%±1.4 (20 °C), 94.7%±3.8 (80 °C) and 81.0%±8.6 (100 °C). Previous investigations of the extraction of γ-oryzanol from rice bran with hexane–2-propanol mixtures at 30, 45 and 60 °C reported a trend towards a positive relationship between γ-oryzanol concentration in extracted oil and extraction temperature [19]. The authors attributed this effect to a lower viscosity of the extraction solvent at higher temperatures resulting in a better penetration of the matrix. Obviously a negative effect on the extraction of γ-oryzanol from rough rice flour dominates at higher temperatures above 50–60 °C.

3.2. On-line LC–GC analysis

Separation of γ-oryzanol from rice lipids was achieved by normal-phase HPLC using hexane–*tert*.-butyl methyl ether–2-propanol (95:5:0.5, v/v/v) as eluent (Fig. 1a). Peak assignment was performed via a γ-oryzanol standard. γ-Oryzanol tends to elute as a tailing peak in normal-phase liquid chromatography due to its dissociable phenolic group. Tailing could

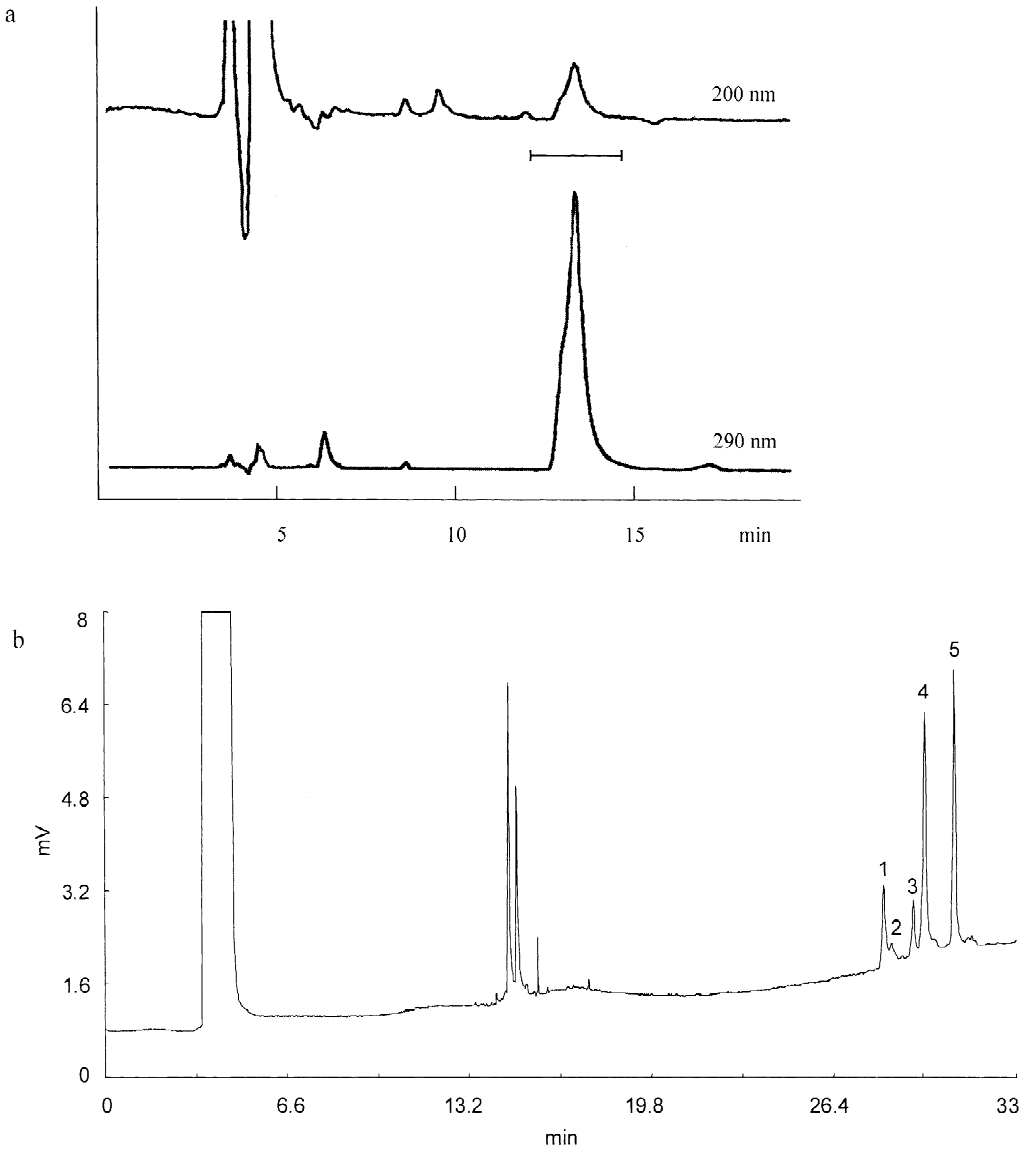

Fig. 1. Analysis of γ-oryzanol extracted from a rough rice sample using on-line LC–GC. Separation of γ-oryzanol from other rice lipids on normal-phase HPLC using UV detection at 200 and 290 nm, respectively (a). The transferred fraction is shown by the indicated time window. GC chromatogram of the on-line transferred steryl ferulates containing fraction (b): campesteryl ferulate (1), campestanyl ferulate (2), β-sitosteryl ferulate (3), cycloartenyl ferulate (4), 24-methylenecycloartanyl ferulate (5) (for conditions see text).

lead to insufficient accuracy of quantification of γ-oryzanol, and could unnecessarily increase the volume of the fraction to be transferred to the GC. Addition of acid to the eluent usually used to avoid tailing of dissociable compounds could not be used because the eluent has to be suitable for the injection into the GC. Using an acidic silica gel (Eurospher), γ-oryzanol eluted in a satisfactory peak shape.

The eluent was monitored by UV detection at 200 and 290 nm. Detection at 200 nm was used to monitor the separation of rice lipids. Steryl ferulates could be specifically detected and quantified at 290

nm due to their absorption maximum at this wavelength [9,20]. External calibration was used for quantification. Concurrent eluent evaporation with the loop-type interface and an early solvent vapour exit was chosen as the transfer technique [21,22] because of the low volatility of steryl ferulates.

A previously reported approach for the gas chromatographic analysis of underivatised steryl ferulates resulted in poor separation into two peaks [12]. Silylation of the phenolic group improved the separation slightly, but, by blocking the polar group before LC–GC analysis, the pre-separation of the minor constituent γ-oryzanol from other rice lipids by normal-phase HPLC would be more difficult. Therefore, the challenge was to improve GC analysis to achieve separation of individual underivatised steryl ferulates. A crosslinked trifluoropropylmethyl polysiloxane fused-silica column (Rtx-200MS) exhibiting mid-range polarity and providing dipole–dipole interactions combined with high thermal stability was found to be suitable for the separation of underivatised γ-oryzanol. The GC separation of the (on-line transferred) γ-oryzanol-containing fraction into its major constituents, campesteryl ferulate, campestanyl ferulate, β-sitosteryl ferulate, cycloartenyl ferulate and 24-methylenecycloartanyl ferulate, is shown in Fig. 1b. Proportions of individual steryl ferulates can be calculated from the peak area ratios of the GC–FID chromatogram.

3.3. Identification of individual steryl ferulates

Identification of individual steryl ferulates was performed by GC–MS analysis of γ-oryzanol prepared from rough rice. After extraction of total lipids, γ-oryzanol was pre-separated from neutral lipids by liquid–liquid fractionation using sodium hydroxide solution and further purified by preparative thin-layer chromatography similar to a procedure described in the literature [12]. Chloroform as mobile phase in thin-layer chromatography was replaced by a less toxic eluent consisting of hexane, *tert.*-butyl methyl ether and 2-propanol. GC–MS analysis of the obtained γ-oryzanol preparation revealed a chromatogram matching the GC–FID chromatogram obtained by analysis of the crude lipid extract by on-line LC–GC. MS spectra allowed the assignment of campesteryl ferulate, campestanyl ferulate, β-sitosteryl ferulate, cycloartenyl ferulate and 24-methylenecycloartanyl ferulate on the basis of published data [12,23]. Characteristic fragment ions are listed in Table 1.

3.4. Method evaluation

The calibration curve used for quantification of total γ-oryzanol by normal-phase HPLC showed good linearity, illustrated by a high correlation coefficient (0.998). The recovery (96%) was determined by spiking rough rice samples with 6.4 mg γ-oryzanol/100 g. Reproducibility was evaluated by 5-fold repeated analysis of a rough rice sample. Quantification of total γ-oryzanol could be performed with a relative standard deviation of 2.4% and revealed a total γ-oryzanol content of 37.4±2.5 mg/100 g. Analysis of γ-oryzanol in rice bran by reversed-phase HPLC, after purification of a lipid extract by liquid–liquid fractionation, followed by low-pressure column chromatography revealed a total γ-oryzanol content of about 340 mg/100 g [10]. Based on the fact that γ-oryzanol is located in bran and rough rice contains about 10% bran [24], the

Table 1
Electron-impact ionization mass spectra of steryl ferulates obtained by GC–MS analysis of γ-oryzanol prepared from rough rice (for conditions see text)

Steryl ferulate	MW	Characteristic fragment ions; m/z (relative abundance)
24-Methylenecycloartanyl ferulate	616	439(2), 422(7), 407(6), 379(7), 297(4), 194(25), 177(100), 149(14)
Cycloartenyl ferulate	602	408(8), 393(8), 365(7), 297(3), 203(10), 194(69), 177(100), 149(18)
Campesteryl ferulate	576	382(72), 367(22), 274(24), 255(26), 213(21), 194(88), 177(72), 149(23)
β-Sitosteryl ferulate	590	396(19), 381(7), 275(6), 255(11), 213(8), 194(100), 177(82), 149(17)
Campestanyl ferulate	578	578(4), 384(1), 330(1), 257(2), 215(2), 194(100), 177(25), 150(25)

amount of total γ-oryzanol in rough rice determined by the proposed on-line LC–GC method is in good accordance with these data. Distribution of individual steryl ferulates could be determined by the developed on-line LC–GC method with relative standard deviations ranging from 0.8 to 5.3% ($n=5$). Cycloartenyl ferulate and 24-methylenecycloartanyl ferulate were determined to be the major constituents (36.6±0.3 and 33.5±0.3%, respectively) in the investigated rough rice sample followed by campesteryl ferulate (16.9±0.2%), β-sitosteryl ferulate (8.3±0.3%) and campestanyl ferulate (4.6±0.3%). The proportions determined are similar to those published for rice bran [10].

3.5. Analysis of γ-oryzanol in different brown rice samples

The developed on-line LC–GC method was applied to the analysis of γ-oryzanol in brown rice samples of the varieties ''Cripto'', ''Balilla'' and ''Xiushui11''. Total γ-oryzanol contents differed significantly: the sample of the variety ''Cripto'' contained 62.7±3.2 mg total γ-oryzanol/100 g, whereas the samples of the variety ''Balilla'' and ''Xiushui11'' contained only 39.3±0.8 and 31.0±0.8 mg/100 g, respectively ($n=6$, $P<0.05$). The compositions of the γ-oryzanol in the three rice samples determined by on-line LC–GC are shown in Table 2. Proportions of individual steryl ferulates varied significantly between the different samples. Potential reasons for these variations include differences in genotypes as well as environmental effects and are subject of current investigations employing the newly developed on-line LC–GC approach.

4. Conclusion

The described on-line LC–GC method provides a rapid and effective isolation of γ-oryzanol from crude rice lipid extracts by means of normal-phase HPLC and the separation of individual underivatised steryl ferulates by improved capillary gas chromatographic analysis of the on-line transferred LC-fraction. Analysis of γ-oryzanol in a crude rice lipid extract takes less than 1 h. Future applications of the proposed on-line LC–GC method will assist in providing information on natural variations of γ-oryzanol content and composition in rice due to genotype and environmental effects, as well as information on the fate of individual steryl ferulates during different processing steps of rice material.

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Table 2
Proportions of individual steryl ferulates in rice samples of the rice varieties ''Cripto'', ''Balilla'' and ''Xiushui11'' determined by on-line LC–GC analysis ($n=6$; $P<0.05$)

Steryl ferulate	Proportions of individual steryl ferulates (%)		
	Cripto	Balilla	Xiushui11
Campesteryl ferulate	7.3±0.2	17.9±0.6	19.3±0.3
Campestanyl ferulate	12.5±0.4	5.9±0.1	6.8±0.2
Sitosteryl ferulate	5.3±0.2	8.4±0.2	9.5±0.3
Cycloartenyl ferulate	47.2±0.7	32.7±0.7	37.7±0.6
24-Methylenecycloartanyl ferulate	27.8±0.4	36.0±1.0	26.6±0.5

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