Food Chemistry 138 (2013) 2079–2088

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Performances of CN-columns for the analysis of γ-oryzanol and its *p*-coumarate and caffeate derivatives by normal phase HPLC and a validated method of quantitation

Michele D'Ambrosio *

Laboratory of Bio-Organic Chemistry, Department of Physics, University of Trento, via Sommarive 14, 38123 Trento, Italy

ARTICLE INFO

Article history:
Received 29 March 2011
Received in revised form 28 June 2012
Accepted 7 December 2012
Available online 20 December 2012

Presented in part by M.D. at the *16th* International Symposium on Separation Science, Rome, Italy, 6–10 September 2010.

Keywords:
γ-Oryzanol
Phytosterols
Ferulate esters
p-Coumarate esters
Caffeate esters
Rice bran
Corn
Wheat
Rye
CN column

ABSTRACT

γ-Oryzanol is an important phytochemical used in pharmaceutical, alimentary and cosmetic preparations. The present article, for the first time, discloses the performances of NP-HPLC in separating γ-oryzanol components and develops a validated method for its routine quantification. The analysis is performed on a cyanopropyl bonded column using the hexane/MTBE gradient elution and UV detection at 325 nm. The method allows: the separation of steryl ferulate, *p*-coumarate and caffeate esters, the separation of *cis*- from *trans*-ferulate isomers, the splitting of steroid moieties into saturated and unsaturated at the side chain. The optimised method provides excellent linear response (R^2 = 0.99997), high precision (RSD < 1.0%) and satisfactory accuracy (R^* = 70–86%). In conclusion, the established method presents the details of the procedure and the experimental conditions in order to achieve the required precision and instrumental accuracy. The method is fast and sensitive and it could be a suitable tool for quality assurance and determination of origin.

1. Introduction

Phytosterols occur in plants either as free or conjugated forms, the latter comprising steryl esters of fatty or phenolic acids and steryl glycosides (Moreau, Whitaker, & Hicks, 2002). A complex mixture of esters, between hydroxycinnamic acids (CAD) (Fig. 1, **R**) and sterols based on the cholestane (Fig. 1, **C**) or the parent cycloartane (Fig. 1, **A**) skeletons, is collectively termed as "γ-oryzanol". The main source of γ-oryzanol is the bran of some grains, in particular brown and red rice (Lerma-García, Herrero-Martinez, Simó-Alfonso, Mendonça, & Ramis-Ramos, 2009). Brans from cereals, such as rye, wheat, triticale, corn, Job's tears and oats, contain less γ-oryzanol than rice bran (Hakala et al., 2002; Seitz, 1989). Rice bran oil (RBO) accounts for 15–20 wt.% of rice bran and dissolves γ-oryzanol (0.9–2.9% oil) and vitamin E (0.10–0.14% oil) (Lerma-García et al., 2009).

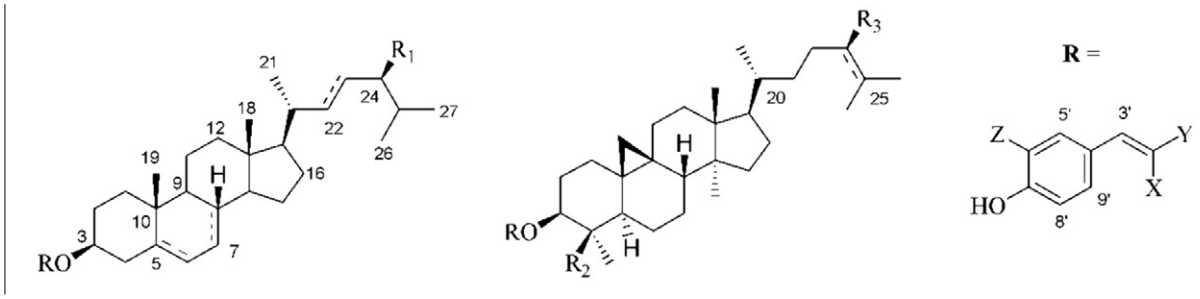
Interest in RBO and γ-oryzanol (Lerma-García et al., 2009) has been growing because of their technological importance and potential health benefits. RBO is used as functional food, in fact γ-oryzanol appears to lower the plasmatic level of low-density cholesterol. Anti-oxidant and free-radical scavenging properties are attributed to the ferulate moiety of γ-oryzanol molecules. So RBO and γ-oryzanol are used as additives in the alimentary industry. γ-Oryzanol gives RBO a very good shelf-life and thermal stability even at frying temperature so that it acts as a natural preservative of refrigerated cooked food during storage time. RBO and γ-oryzanol act as suitable natural UV filter in sun screen creams.

Large number of commercial products which contain γ-oryzanol as an ingredient, require a fast and reliable procedure of detection and quantification. This is a hard task because of the complexity and variability of its composition. There is also the necessity to perform analyses on different types of samples such as cereal bran, crude or refined oils, by-products from cereal processing, foods, etc.

Several analytical methods have been proposed for the quantification of γ-oryzanol and for the identification of its constituents in samples of bran or oil (Lerma-García et al., 2009). Since the specific extinction coefficient ε is a known constant (Hakala et al., 2002), the simplest way to calculate the total content of γ-oryzanol in a rice bran oil sample is to measure its UV absorption. This

* Tel.: +39 0461 281509; fax: +39 0461 281696.
E-mail address: michele.dambrosio@unitn.it

0308-8146/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2012.12.008



C1 R_1= =CH_2 24-Methylencholesteryl
C2 R_1= Me Campestanyl
C3 R_1= Me, Δ^5 Campesteryl
C4 R_1= Me, Δ^7 Campest-7-enyl
C5 R_1= Et Stigmastanyl (Sitostanyl)
C6 R_1= Et, Δ^5 Sitosteryl
C7 R_1= Et, Δ^7 Sitost-7-enyl

A1 R_2= Me R_3= H Cycloartanyl
A2 R_2= Me R_3= H, Δ^{24} Cycloartenyl
A3 R_2= Me R_3= =CH_2 24-Methylencycloartanyl
A4 R_2= H R_3= =CH_2 Cycloeucalenyl
C8 R_1= Et, $\Delta^{5,22}$ Stigmasteryl
C9 R_1= Et, $\Delta^{7,22}$ Stigmast-7-enyl

Z= OMe Ferulate
Z= OH Caffeate
Z= H *p*-Coumarate
X= H, Y= CO *trans*-
X= CO, Y= H *cis*-

Fig. 1. Molecular structures of main components of γ-oryzanol: alcohols, possessing a cholestane (C) or a cycloartane (A) skeleton, are esterified by a hydroxycinnamic acid (R).

method doesn't provide any information about the structure of individual components and much care must be taken to achieve reliable measurements. In fact, other UV absorbing compounds in the oil matrix may interfere with the ferulate chromophore.

Another possible method (Miller & Engel, 2006) used an on-line coupled liquid chromatography–gas chromatography (LC–GC) technique: a not very widespread equipment. The total amount of γ-oryzanol was measured by LC-UV detection and the relative proportion of individual components by GC-FID detection. The analysis takes long time and the resolution seems to be poor (Table 1, entry 1).

Most efforts have been devoted to reversed-phase (RP) chromatography (Table 1, entries 2–8) after multi-step procedures of chemical (Hakala et al., 2002; Seitz, 1989; Stöggl, Huck, Wongyai, Scherz, & Bonn, 2005) and chromatographic (Norton, 1995; Xu & Godber, 1999) pre-treatment of the sample (Table 1, D). The analyses by high pressure liquid chromatography (HPLC) were done on different experimental conditions, using C8 (Yu, Nehus, Badger, & Fang, 2007), C18 (Lerma-García et al., 2009) and C30 (Stöggl et al., 2005) columns as a stationary phase (Table 1, B), and several mixing of solvents in the mobile phase (Table 1, C). In this technique, generally, UV detection at 315–325 nm was applied. The identification of ferulate esters (Table 1, E) was best obtained by tandem liquid chromatography–mass spectrometry (LC–MS) techniques (Table 1, entries 5, 6 and 8). Past reports describe the collection of each peak followed by the chemical derivatisation of those compounds and their analysis by proton NMR or GC–MS (Table 1, entries 2 and 4).

The chief objective was to separate γ-oryzanol components where up to ten peaks could be well resolved in one single run (Table 1, G). Many compounds have been identified (Akihisa et al., 2000; Xu & Godber, 1999; Yu et al., 2007) and the most common structures are listed (Table 2, L). By comparison of RP-HPLC chromatograms (Hakala et al., 2002; Norton, 1995; Seitz, 1989), the composition of γ-oryzanol appears to vary in different types of cereal source: (i) cycloartenyl ferulates (Fig. 1, **A2** and **A3**) are predominant in rice but almost absent in other grains, (ii) campestanyl and sitostanyl *p*-coumarates (Fig. 1, **C2** and **C5**) and

Table 1
Comparison of analytical methods of γ-oryzanol by their chromatographic performances.

	A	B	C	D	E	F	G	H	I	J	K
1	Miller and Engel (2006)	GC	H_2, 340 °C	chrom	GC–MS	>33	5	1	N	3	∼Y
2	Seitz (1989)	C18, 3	M/W 97:3; I	chem1	^{1}H NMR GC–MS	>30	3	∼2	N	–	–
3	Norton (1995)	C18 , 5	A/B/Aa/W82:3:2:13; I	chrom	Reference standards	∼30	7	∼2	N	20	N
4	Xu and Godber (1999)	C18, ?	M/A/D/Aa 50:44:3:3; I	chrom	GC–MS of TMS ethers	>40	10	1	N	–	–
5	Hakala et al. (2002)	C18, 5	M/W/Aa 97:2:1; I	chem1	LC–APCI-MS	∼20	4	1	N	22	Y
6	Stöggl et al. (2005)	C30 , 3	M/mtbe 8:2; I	chem2	LC–APCI-MS	>50	9	1	N	–	–
7	Chen and Bergman (2005)	C18, 4	A/M/P/W 50:45:5:5; G	None	Reference standards	>28	5	1	N	2	N
8	Yu et al. (2007)	C8, 5	A/W/Fa 20:80:0.1; G	chem3	LC–ESI-MS	>70	8	2	N	2	N
9	Diack and Saska (1994)	SiO_2, 4	i-octane/Ae 97.5:2.5; I	chem4	None	>30	2	1	N	–	–
10	Heinmann et al. (2008)	SiO_2, 5	H/Ae/Aa 98:1:1; I	None	None	30	2	1	N	32	N
11	Huang and Ng (2011)	CN, 5	H/P/Ae/Aa 97:1:1:1; I	None	None	25	1	1	N	2	Y
12	D'Ambrosio	CN, 5	H/mtbe/Fa 97:2:1; G	None	^{1}H NMR LC–MS	32	>4	>2	Y	7	Y

A, reference; B, stationary phase, μm; C, mobile phase (A acetonitrile, Aa acetic acid, Ae ethyl acetate, B butanol, D dichloromethane, Fa formic acid, H hexane, M methanol, P 2-propanol, W water), isocratic or gradient elution; D, sample pretreatment (chem1: $KOH_{(aq)}$/hexane partition, injection of components dissolved into the basic layer, chem2: dispersion oil/(A/M/P 50:45:5) 4:96 v/v, chem3: extraction MeOH/H_2O then partition CH_2Cl_2/H_2O, chem4: conc. $KOH_{(aq)}$ saponification of triglycerides, injection of components dissolved into the hexane layer, chrom: preparative column over silica gel); E, technique of peak identification; F, total elution time; G, # of peaks; H, # of CAD; I, detection of *cis*-isomers; J, # of real samples quantitated; K, validation tests.

Table 2
Previous and present MS data of natural and synthetic components of γ-oryzanol.

	L		M[a]	N[b]	O[b]	P[b]	Q[b]	R[b]
1	24-CH_2-cholesteryl	C1		573		573	543	559
2	Stigmasteryl or stigmast-7-enyl	C8 or C9	484*			587	557	
3	Cycloartenyl or cycloeucalenyl	A2 or A4	498	601		601*	571	587
4	24-CH_2-cycloartanyl	A3	512	615		615	585	601
5	Campesteryl or campest-7-enyl	C3 or C4	472*	575	575		545	561
6	Sitosteryl or sitost-7-enyl	C6 or C7	486*	{589}*	{589}		559	575
7	Cycloartanyl	A1		603	{603}		573	
8	Campestanyl	C2	474	{577}	{577}		547	563
9	Stigmastanyl (sitostanyl)	C5	488	591	591		561	577

L, steryl moieties and codes of the structures shown in Fig. 1; M, (Xu & Godber, 1999); N, (Yu et al., 2007), ferulates; O, standard ferulates at t_R = 11.5 and 19.0 min; P, standard ferulates at t_R = 12.0 and 20.0 min; Q, synthetic *p*-coumarates; R, synthetic caffeates.
*Means compounds with identical molecular weight eluting as separated peaks.
{} Means compounds with different molecular weight coeluting in one peak.
[a] *m*/*z* of $[M]^+$ of steryl TMS ethers.
[b] *m*/*z* of $[M-H]^-$ of steryl CAD esters.

sitostanyl ferulate (Fig. 1, **C5**) are abundant in corn, (iii) campestanyl and/or sitosteryl ferulates (Fig. 1, **C2** and **C6**) are the major steryl moieties in grains other than rice and corn.

Several studies were oriented towards quantisation (Table 1, J) but the validation tests were not always carried out (Table 1, K). Moreover, *p*-coumarate esters (Table 1, H) and minor *cis* isomers (Table 1, I) co-elute with abundant ferulates and *trans* isomers in RP-HPLC (Norton, 1995; Seitz, 1989; Yu et al., 2007) so that they may affect the actual value of each integrated peak. Often the peaks were not resolved to the baseline of the chromatogram (Hakala et al., 2002); in such a case, total γ-oryzanol was quantitated by pooling all the peak area. Thus the separation into components turned out to be vain (Chen & Bergman, 2005). Our early elutions for the quantitative determination of γ-oryzanol revealed also some experimental drawbacks in the RP-HPLC methods. In fact, apolar stationary phases are highly retentive of triacylglycerides (TAG), free fatty acids (FFA) and waxes. A proper column rinsing, before re-equilibration, demands to change from water miscible to water immiscible eluents. Therefore it cannot be easily carried out and is solvent and time consuming. An efficient clean-up procedure in the preparation of the sample is thus mandatory (Table 1, D) where it is again a solvent and time consuming step. In addition, loss of analyte may occur when using partitioning by separatory funnel (Akihisa et al., 2000; Hakala et al., 2002; Seitz, 1989; Stöggl et al., 2005; Yu et al., 2007) or preliminary normal-phase (NP) chromatography (Norton, 1995; Xu & Godber, 1999). Taking into account the vast number of commercial products containing γ-oryzanol, the more selective extraction with alcoholic solvents may not be always feasible (Yu et al., 2007).

Column chromatography on silica gel has been used mostly for a semi-preparative collection of γ-oryzanol devoid of TAG and FFA (Norton, 1995; Xu & Godber, 1999). Normal-phase TLC allows the separation of *p*-coumarates from ferulates (Norton, 1995; Seitz, 1989), *cis* from *trans* isomers (Akihisa et al., 2000) and might differentiate between 4-dimethyl, 4-monomethyl and 4-desmethyl steryl esters (Akihisa et al., 2000). Silica columns for HPLC (Table 1, entries 9–12) allow the splitting of γ-oryzanol components into two closely eluting peaks but their composition wasn't proven (Diack & Saska, 1994). While this manuscript was being revised, an improved method, using a NP-HPLC and a cyanopropyl column, was published but γ-oryzanol was eluted as one peak (Huang & Ng, 2011). NP- and RP-HPLC methods have been reported (Heinmann, Xu, Godber, & Lanfer-Marquez, 2008) to determine identical values of the total amount of γ-oryzanol but the authors preferred the NP approach.

In our opinion, RP-HPLC methods are more suitable for qualitative studies of γ-oryzanol components and for a small number of samples. The screening of a large number of samples requires a fast and reliable sample preparation and the possibility of automated and replicated HPLC injections. In NP-HPLC, the huge amount of apolar waxes, TAG and FFA are eluted before the analytes of interest thus eliminating the need of long column rinsing and tedious preliminary cleaning-up of samples. Therefore, our aims are: (1) to explore the potential application of NP-HPLC in separating γ-oryzanol components, (2) to develop and validate a simple and informative NP-HPLC method for the routine quantification of γ-oryzanol.

2. Experimental

2.1. Chemicals

Analytical grade solvents were used for extraction procedures and reactions (VWR International, Fontenay-sous-Bois, France). HPLC-grade hexane, heptane (J. Matthey, Karlsruhe, Germany), 2-propanol (J.T. Baker, Mallinckrodt, The Netherlands), decane, MTBE and formic acid (Sigma Aldrich, Steinheim, Germany) were used for analyses. Standards of ethyl ferulate and umbelliferone were obtained from Fluka (Steinheim, Germany) and γ-oryzanol was obtained from Farmalabor (Canosa (BA), Italy). Reagent grade coumaric acid, caffeic acid, sodium hydroxide, acetic anhydride, oxalyl chloride and sodium borohydride were purchased from Sigma Aldrich (Steinheim, Germany). Silica gel 60 (0.063–0.020 mm) was employed for flash chromatography and silica gel plates 60 F254 were used for TLC and PLC (Merck, Darmstadt, Germany).

2.2. Instrumentations and chromatographic conditions

The preparative HPLC system consisted of a Merck Hitachi model L-7100 pump, L-7400 UV detector, D-7500 integrator and Rheodyne manual injector equipped with a 200 μL loop. The column was a Luna-CN (250 × 10 mm, 5 μm particle size, 100 Å pore size; Phenomenex, Torrance, CA, USA); the initial mobile phase was hexane/MTBE/formic acid 97:2:1 which changed linearly to hexane/MTBE/formic acid 90:9:1 within 23 min at the flow rate of 4 mL/min and detection at λ = 325 nm.

Analytical separations were carried out using an Agilent 1100 series LC system consisting of a binary pump, a vacuum degasser, an autosampler with standard analytical head (100 μL), a column thermostat and a 1200 series diode array detector (DAD). The column oven temperature was fixed at 25 °C; the flow rate was 1.0 mL/min and the injection volume was 10 μL; the DAD was set at 290, 325 and 340 nm. The HPLC instrument was equipped with a Synergi Hydro column (150 × 4.6 mm, 4 μm particle size, 80 Å pore size; Phenomenex, Torrance, CA, USA) for the analyses of com-

ponents in the RP mode; the eluents were 95% methanol–5% water (A) and 100% methanol (B). The initial gradient condition was maintained at 100% A, changing linearly to 100% B in 30 min and then held at this eluent B for 10 min. A Luna-CN column (250 × 4.6 mm, 5 μm particle size, 100 Å pore size; Phenomenex, Torrance, CA, USA) was selected for quantitative measurements in the NP mode; the mobile phase consisted of hexane (A) and hexane/MTBE 1:1 (B), both containing 1% (v/v) formic acid, applied in the following stepped linear gradient: 2–22% B at 0–20 min, 22–100% B at 20–25 min; then 5 min washing at 100% B, 100–2% B at 30–32 min and 13 min as equilibration time. A representative chromatogram is shown in Fig. 2a.

NMR spectra were acquired using a Bruker Avance 400 spectrometer (^{1}H at 400 MHz). Chemical shifts were reported in ppm on the δ scale with the residual solvent signal as internal reference ($CHCl_3$: δ_H 7.26); coupling constants (J) are given in Hz.

LC–MS analyses were performed using an Agilent 1100 series LC system interfaced to a Bruker model Esquire_LC multiple ion trap mass spectrometer equipped with an atmospheric pressure interface electrospray (API-ES) chamber. Conditions for ESI-MS analysis

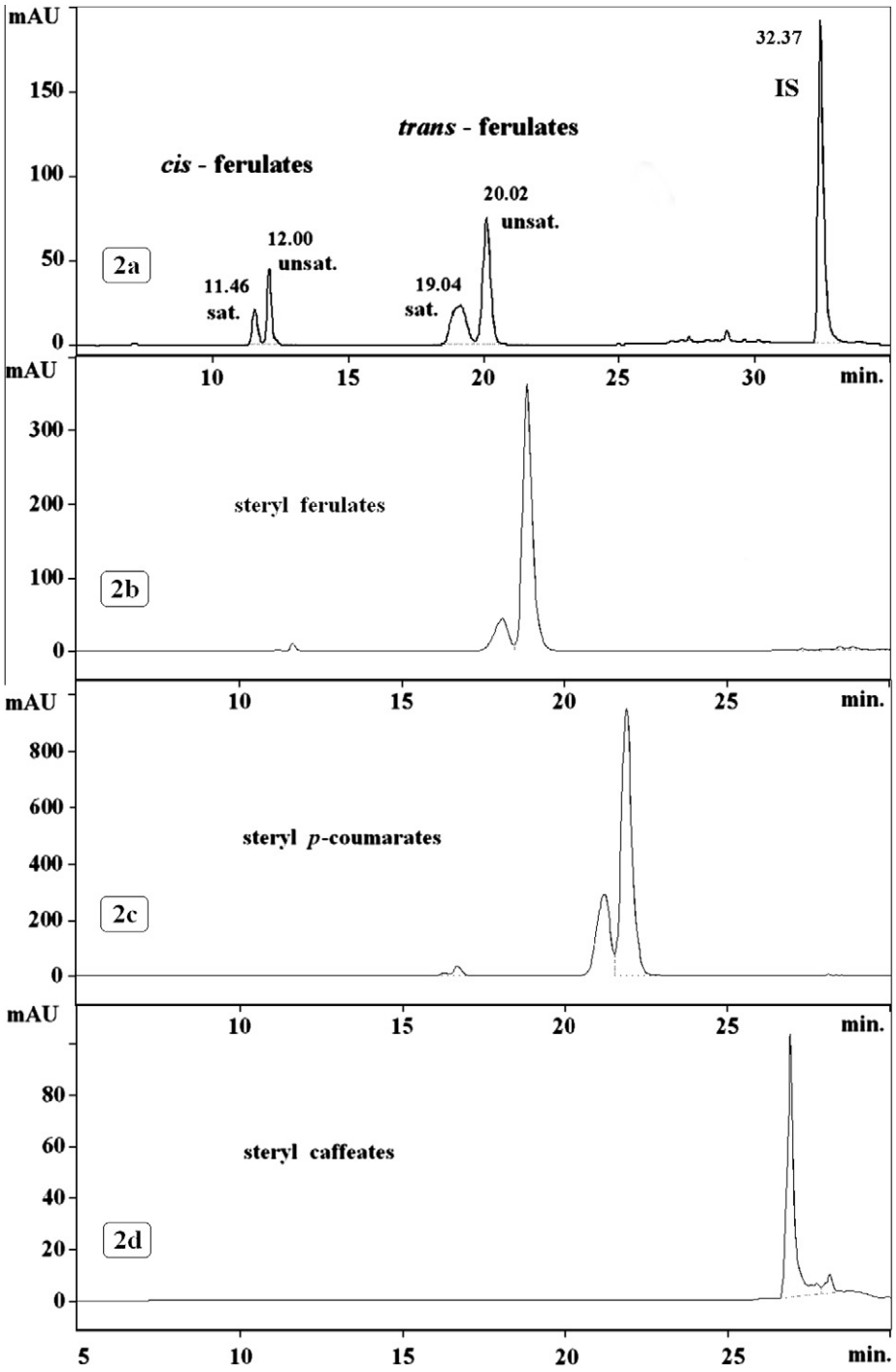


Fig. 2. NP-HPLC runs of: (a) Carnaroli rice bran sample containing a high percentage of *cis*-ferulates, "sat." means steroid moieties possessing a saturated side chain, "unsat." means steroid moieties possessing an unsaturated side chain; (b) standard γ-oryzanol; (c) synthetic steryl *p*-coumarates; (d) synthetic steryl caffeates.

Table 3
Analytical data for the quantification of γ-oryzanol.

	Sample	Carnaroli rice bran	Vialone rice bran	wheat bran	Storo corn flour	Walet rye flour	Supplement tablet	Sunscreen oil
1	Mass of sample (mg)	402.0	399.5	799.4	4000.0	3999.6	423.8	46.0
2	Mass of defatted sample residue (mg)	323.1	328.1	746.2	3775.0	3855.6	364.2	
3	Mass of oil (mg)[a]	66.6	59.9	27.6	118.8	43.4	53.0	46.0
4	Yield of oil (%)	16.6	15.0	3.5	3.0	1.1	12.5	
5	Recovery of oil + defatted sample (%)	97.0	97.1	96.8	97.3	97.5	98.4	
6	Concentration of oil for HPLC analysis (mg/mL)	5.01	4.99	4.93	19.80	9.98	0.57	50.00
7	*cis*-Ferulates (nmol/mL)	2.08	3.30	2.55	9.74	9.73	15.31	0.00
8	RSD%	0.25	0.35	0.17	0.12	0.21	0.33	
9	Cholestane *trans*-ferulate (nmol/mL)[b]	66.73	43.58	67.01	84.44	94.09	86.83	10.13
10	RSD%	0.47	0.39	0.38	0.11	1.06	0.06	0.49
11	Cycloartane *trans*-ferulate (nmol/mL)[c]	121.46	85.55	2.24	4.54	5.77	85.47	77.04
12	RSD%	0.85	0.35	6.37	0.12	0.21	0.08	0.14
13	*cis*-Ferulates (nmol/mg oil)	0.41	0.66	0.52	0.49	0.98	26.70	0.000
14	Cholestane *trans*-ferulate (nmol/mg oil)	13.32	8.73	13.60	4.26	9.43	151.45	0.20
15	Cycloartane *trans*-ferulate (nmol/mg oil)	24.24	17.14	0.45	0.23	0.58	149.07	1.54
16	γ-Oryzanol (∼mg/g sample)	3.78	2.39	0.29	0.09	0.07	24.38	
17	γ-Oryzanol (∼mg/g oil)	22.78	15.92	8.51	2.91	6.41	194.99	1.06
18	Cholestane/γ-oryzanol (%, mol/mol)	35.5	33.7	96.8	94.9	94.2	50.4	11.6
19	*cis*-Ferulates/γ-oryzanol (%, mol/mol)	1.1	2.5	3.5	9.9	8.9	8.2	0.0

[a] "Oil" stands for extract evaporated to dryness or sunscreen oil.
[b] "Cholestane" exactly means: sterols possessing a saturated side chain.
[c] "Cycloartane" exactly means: sterols possessing an unsaturated side chain.

of HPLC peaks in negative ion mode included a capillary voltage of 4000 V, a nebulising pressure of 30.0 psi, a drying gas flow of 7 mL/min and a temperature of 300 °C.

2.3. Samples

Full-fat rice bran (Carnaroli and Vialone Nano varieties) were kindly provided by "FERRON Gabriele e Maurizio snc" (Isola della Scala (VR), Italy). Rye kernels (Walet variety) were provided by "Centro di Sperimentazione Agraria e Forestale" (Laimburg (BZ), Italy). The other samples were purchased from a retail store. Cereal samples were stored at −18 °C before analysis and no stabilisation process was applied. The extracts were stored at −18 °C under nitrogen in white bottles and vials. Samples with high levels of *cis*-isomers were obtained by exposure of samples to sunlight or by UV irradiation.

2.4. Sample preparation

The accurately weighed powder (Table 3, entry 1) was extracted twice with hexane (8 mL/400 mg) at room temperature under magnetic stirring over 3 d. The combined extracts (Table 3, entry 3) were evaporated and the residue was dissolved in hexane at a concentration of 50.0 mg/mL. In order to obtain similar DAD responses, well centred in the calibration curve, different aliquots of each sample (100–400 μL) were evaporated to dryness, dissolved with 500 μL of stock solution IS (umbelliferone) in decane/2-propanol 96:4 and then diluted to 1.0 mL with decane (Table 3, entry 6). Sunscreen oil was simply diluted to 50.0 mg/mL.

2.5. Syntheses of coumarate and caffeate derivatives

Coumarate and caffeate esters of γ-oryzanol steroid moieties have been reported in the literature (Norton, 1995; Seitz, 1989; Yu et al., 2007). Since the samples analysed herein were short of such esters we prepared them by following synthesis.

Sodium hydroxide (4 g, 0.1 mol) was dissolved in water (6 mL) and diluted with ethyl alcohol (24 mL). Standard γ-oryzanol (600 mg, ∼1 mmol) was added to this solution in a 100 mL round bottomed flask and was stirred at 80 °C for 2.5 h. After cooling at room temperature (r.t.), the solution was poured into a separatory funnel, diluted with water (200 mL) and shaken with hexane (3 × 30 mL). The combined organic phase was washed with water to the neutral pH and dried over anhydrous Na_2SO_4. The solvent was finally evaporated to give the sterol mixture (420 mg, ∼99 mmol, 99% yield).

A solution of caffeic acid (180 mg, 1.0 mmol) in dry pyridine (0.3 mL) was added of acetic anhydride (0.47 mL, 5 mmol) and then stirred at r.t. over night. The reaction mixture was gently heated under vacuum to evaporate excess acetic anhydride and pyridine thus obtaining quantitatively (2*E*)-3-[3,4-bis(acetyloxy)phenyl]-2-propenoic acid (264 mg, 1.0 mmol).

This protected caffeic acid was suspended in dry CH_2Cl_2 (4 mL), stirred under N_2 at r.t. and added of oxalyl chloride (2 mL, 2 mmol, 2 M in CH_2Cl_2). After 30 min all the solid dissolved so the solution was evaporated to dryness *in vacuo*. The resulting solid was dissolved in dry CH_2Cl_2 (4 mL). Then a solution of sterols from γ-oryzanol (660 mg, ∼1.56 mmol) and pyridine (0.097 mL, 1.2 mmol) in 1 mL of dry CH_2Cl_2 was added dropwise. The mixture was stirred for 24 h at r.t. then filtered. The precipitate was washed with CH_2Cl_2 and discarded. Silica gel (1 spoon) was added to the clear solution and the solvent was evaporated. The desiccated slurry was applied to a silica column and chromatographed with a gradient of diethyl ether in hexane to isolate (2*E*)-3-[3,4-bis(acetyloxy)phenyl]-2-propenoate of the sterols (100 mg, ∼0.149 mmol, 15% yield).

$NaBH_4$ (57 mg, 1.5 mmol) was added to a solution of those protected esters in dry THF (5 mL) and the mixture stirred for 24 h. Then acetone (2 mL) was poured into the flask and stirred for 1 h. The solution was concentrated and the residue was applied to a preparative plate of silica gel and eluted with hexane/ethyl acetate 1:1. The band at $R_f = 0.65$ furnished pure caffeate esters of the sterols (43 mg, ∼0.073 mmol, 49% yield).

The above procedure was also applied to coumaric acid (164 mg, 1.0 mmol) to prepare (2*E*)-3-[4-(acetyloxy)phenyl]-2-propenoic acid (184 mg, 0.89 mmol, 89% yield). The subsequent treatment with oxalyl chloride and one-pot esterification of the sterols (604 mg, ∼1.42 mmol) gave pure (2*E*)-3-[4-(acetyloxy)phenyl]-2-propenoates (84 mg, ∼0.14 mmol, 15% yield) after separation by silica column. The deprotection step was similarly carried out in order to obtain pure coumarate esters of the sterols from γ-oryzanol (34 mg, ∼0.06 mmol, 43% yield).

2.6. Method validation

2.6.1. Standard solutions and calibration curves

The internal standard (IS) umbelliferone (50.7 mg) was dissolved in 2-propanol in 50 mL volumetric flask. Then 2.7 mL of solution were poured in 50 mL volumetric flask, evaporated to dryness and dissolved in decane/2-propanol 96:4 to prepare a stock solution IS which was used for sample preparation. A second solution of IS stock at halved concentration and decane/2-propanol 98:2 was obtained by diluting 5.0 mL of IS stock solution with pure decane in 10 mL volumetric flask. This IS stock was used for serial dilutions of the calibration curve. The external standard (ES) ethyl ferulate (52.5 mg) was dissolved in 2-propanol in 50 mL volumetric flask then 100.0 μL were poured into a 4 mL volumetric flask, evaporated to dryness and dissolved with the IS stock to prepare the highest of seven different concentration levels. Standard γ-oryzanol was used for recovery studies. All stock solutions were stored in brown bottles and vials at 4 °C.

2.6.2. Linearity

Linearity was established by injection of the seven standard concentrations with four replicates. The calibration graph was plotted using linear regression of the mean Signal Ratio (area ES/area IS) *vs* concentration ES (nmol/μL).

2.6.3. Limits of detection and quantification

The sample solution Vialone Nano was further diluted in decane/2-propanol 98:2 in order to determine the limit of detection (LOD) and quantification (LOQ) values which were established at a signal-to-noise ratio $S/N = 3$ and $S/N = 10$ respectively.

2.6.4. Accuracy

Recovery tests were studied at four concentration levels (~25%, 60%, 100% and 130% of the expected oryzanol content), each sample was analysed in four replicates and the amount of γ-oryzanol was calculated from the calibration curve. Two sets of experiments were performed: (1) by spiking oil aliquots after the extraction procedure and (2) raw material aliquots before the extraction procedure. The oil extracted from 2010.0 mg of Carnaroli rice bran was divided into five identical aliquots, four of which were spiked with one each of the above defined concentration levels of γ-oryzanol. After analysis, the accuracy was assessed using the equation:

$$\text{Calibration recovery} R^{C}\ (\%) = (\text{amount fortified}_{POST} - \text{amount original}) \times 100/\text{amount of spike}$$

An accurate amount (one for each level) of commercial γ-oryzanol in hexane was added to 400 mg of Carnaroli rice bran and evaporated, the raw material was left for one hour then extracted and analysed as described above. The accuracy was assessed using the equation:

$$\text{Apparent recovery} R^{*}\ (\%) = (\text{amount fortified}_{PRE} - \text{amount original}) \times 100/\text{amount of spike}$$

2.6.5. Precision

The repeatability of the chromatographic method was tested using measurements of the intra- and inter-day variability. The precision was examined using standard solutions at the concentration of 1.18×10^{-4} M which were injected six times. Coefficients of variations were calculated for both retention times (t_R) and integration area. The intra-day variability was determined by analysing the solution within one single day; the inter-day reproducibility was performed on three different days, each time with a newly prepared mobile phase and sample.

3. Results and discussion

3.1. Isolation and structural characterisation of γ-oryzanol components

3.1.1. Preparative NP-HPLC

A sample of standard γ-oryzanol (9 mg) was dissolved in decane and irradiated at UV light ($\lambda = 254$ nm) for one hour then subjected to NP-HPLC at semi-preparative scale; the profile of the chromatogram was similar to that shown in Fig. 2a and sample components were separated into four peaks. Owing to their low intensity, the peaks at $t_R = 11.5$ and 12.0 min were initially collected together and those three fractions used to acquire NMR spectra. Each of the four peaks was also collected and then investigated by RP-LC–ESI-MS technique.

3.1.2. NMR spectroscopy

The elucidation of ^{1}H NMR spectra of those three fractions (see Suppl. Mat., Fig. 1) proved that our chromatographic method (Fig. 2a) allows the separation of *cis*- from *trans*-ferulates and each group of esters is split into cholestane and cycloartane skeletons (Fig. 1). The peak collected at $t_R = 20.0$ min revealed diagnostic resonances which account for: one inductively deshielded proton ($\delta = 4.71$, dd, $J = 11.0$, 4.5) at C3 and the methylene protons ($\delta = 0.36$, d, $J = 4.1$; $\delta = 0.60$, d, $J = 4.1$) at C19 of the cyclopropane ring (structures A), one olefinic proton ($\delta = 5.10$, tm, $J = 7.0$, 2.5) at C24 and the allylic methyls C26 ($\delta = 1.68$, br.s) and C27 ($\delta = 1.61$, br.s) (structure A2), the exomethylene protons ($\delta = 4.67$, m, $J = 1.2$; $\delta = 4.72$, br.s) at the C24 = CH_2 and the methyls C26 and C27 ($\delta = 1.02$ and 1.03, d, $J = 6.7$) (structure A3) (Chen et al., 2008). The peak at $t_R = 19.0$ min showed: deshielded multiplets assignable to the proton ($\delta = 4.7$–4.8, m) at C3 and many shielded methyls at high field ($\delta = 0.65$–0.95) (structures C), the olefinic proton ($\delta = 5.40$, br.d, $J = 5.0$) at C6 (structures C3, C6 and C8) (Chen et al., 2008). Both ^{1}H NMR spectra proved that the peaks at $t_R = 19.0$ and 20.0 min consisted of *trans*-ferulate esters on the basis of characteristic chemical shifts and coupling constants: δ 7.60 (d, $J = 15.9$, H-3′), 7.08 (dd, $J = 8.2$, 1.8, H-9′), 7.04 (d, $J = 1.8$, H-5′), 6.91 (d, $J = 8.2$, H-8′), 6.30 (d, $J = 15.9$, H-2′), 3.94 (s, MeO–) (Chen et al., 2008). The peaks at $t_R = 11.5$ and 12.0 min were also examined by ^{1}H NMR spectroscopy and revealed the same diagnostic resonances attributed to the cholestane and cycloartane skeletons. On the contrary, the proton pattern observed at low field fitted a *cis*-ferulate moiety: δ 7.74 (d, $J = 1.8$, H-5′), 7.13 (dd, $J = 8.2$, 1.8, H-9′), 6.88 (d, $J = 8.2$, H-8′), 6.78 (d, $J = 13.0$, H-3′), 5.84 (d, $J = 13.0$, H-2′), 3.93 (s, MeO–).

3.1.3. LC–MS spectroscopy

The four single peaks isolated by preparative NP-HPLC, were investigated by RP-LC–ESI-MS technique observing negative ions. As expected, the reversed phase chromatography (Fig. 3) splits up each fraction obtained from normal phase into several peaks. The chromatograms of peaks at $t_R = 19.0$ and 20.0 min aren't shown because they appeared superimposable (identical pattern and slight shifts in the retention times) to those at $t_R = 11.5$ min (Fig. 3a) and 12.0 min (Fig. 3b) respectively. The RP-HPLC chromatogram of standard γ-oryzanol is shown in Fig. 3c.

The results of MS data are summarised in Table 2 which lists the structures according to the elution order with increasing retention times. Columns M (Xu & Godber, 1999) and N (Yu et al., 2007) list the steryl moieties identified by previous authors. We used a method which parts γ-oryzanol into six major peaks; ten molecular ions

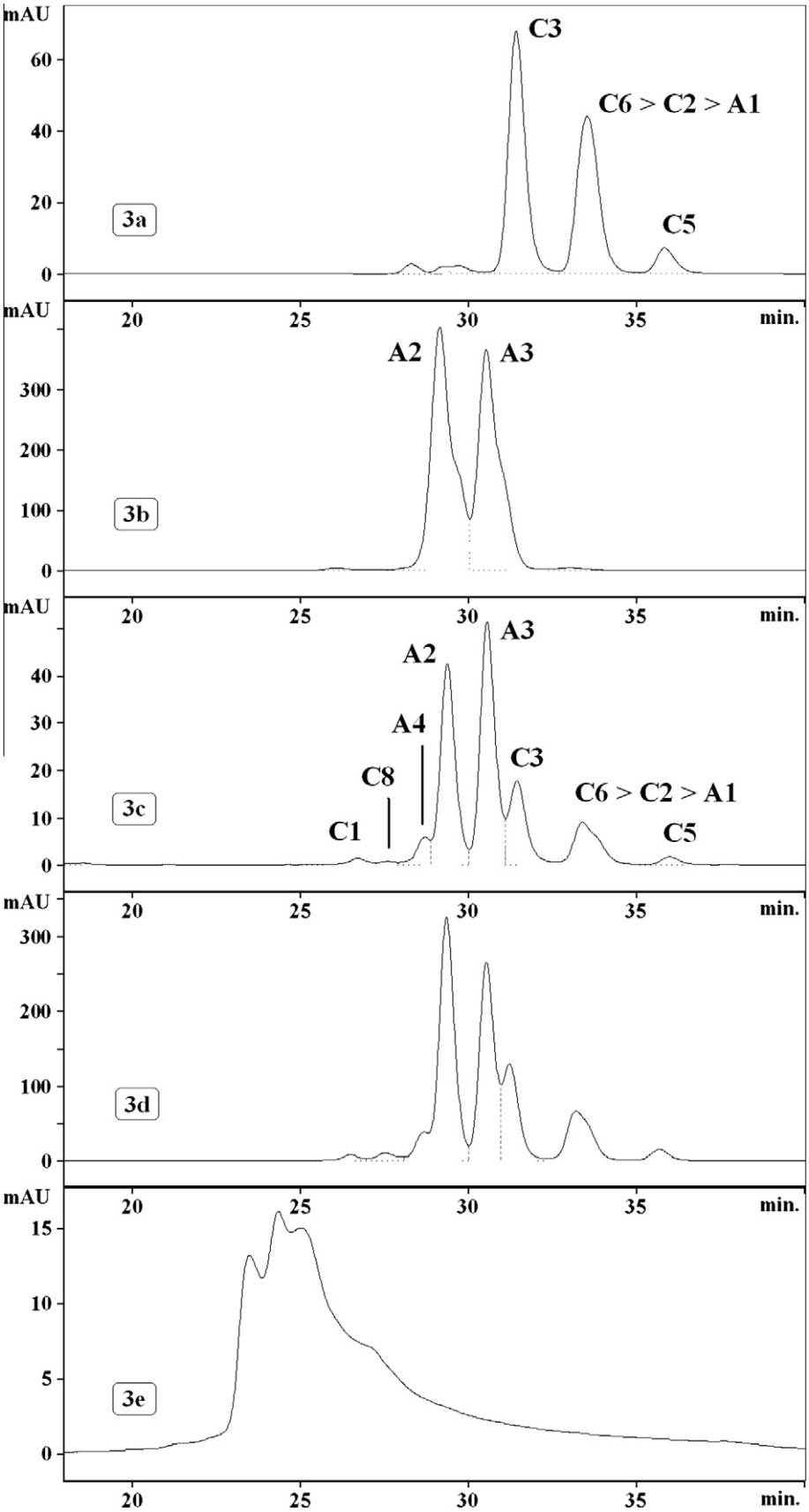


Fig. 3. RP-HPLC runs of: (a) peak collected at t_R = 11.5 min, steryl *cis*-ferulates possessing a saturated side chain, (b) peak collected at t_R = 12.0 min, steryl *cis*-ferulates possessing an unsaturated side chain, (c) standard γ-oryzanol, (d) synthetic steryl *p*-coumarates, (e) synthetic steryl caffeates.

were detected and assigned to the most likely structures by comparison with previous literature data. These compounds belonged to two groups corresponding to each NP-HPLC fraction: peaks at t_R = 11.5 and 19.0 min (col. O) and peaks at t_R = 12.0 and 20.0 min (col. P). The relative proportion of coeluting components in braces at entries 6, 7 and 8 (col. O) was established by integration of MS extracted ion chromatograms thus corresponding to 49%, 19% and 32% respectively.

NMR spectroscopy characterised those NP-HPLC fractions in terms of major steroid skeletons and also distinguished between *cis* and *trans* isomers. The sensitive MS technique allowed to identify minor or even trace components which constitute each chromatographic peak. In fact, the presence of some less abundant compounds (Table 2, entries 1,2 and 7) demonstrates that the fractions being eluted later (t_R = 12.0 and 20.0 min) (Table 2, P) contain steroid moieties which possess a double bond at the side chain whereas the fractions being eluted sooner (t_R = 11.5 and 19.0 min) (Table 2, O) contain compounds which are saturated at the side chain and, possibly, also at the polycyclic ring system. Taking into account that compounds at entries 1, 2 and 7 (Table 2) are very minor components of γ-oryzanol, the NMR attribution of cholestane and cycloartane skeletons to the NP-HPLC fractions is still acceptable.

3.2. Chromatography and spectroscopy of synthetic coumarate and caffeate derivatives

Both ^{1}H NMR spectra of coumarate and caffeate esters of the sterols from γ-oryzanol revealed the diagnostic resonances attributable to cholestane and cycloartane skeletons as described at the Section 3.1.2. However, the proton pattern observed at low field was in agreement with either a coumarate {δ 7.61 (d, *J* = 15.9, H-3′), 7.44 (d, *J* = 8.4, H-5′ and H-9′), 6.84 (d, *J* = 8.4, H-6′ and H-8′), 6.31 (d, *J* = 15.9, H-2′)} or a caffeate {δ 7.50 (d, *J* = 15.9, H-3′), 7.03 (d, *J* = 1.9, H-5′), 6.91 (dd, *J* = 7.9, 1.9, H-9′), 6.80 (d, *J* = 7.9, H-8′), 6.20 (d, *J* = 15.9, H-2′)} ester.

Synthetic coumarates and caffeates were subjected to analytical chromatography in both NP- and RP-HPLC mode. In the normal phase conditions, *trans*-ferulates eluted at t_R = 17.7 and 18.5 min (Fig. 2b), *trans*-*p*-coumarates separated into two peaks at t_R = 21.2 and 21.8 (Fig. 2c); *trans*-caffeates eluted as one peak at t_R = 26.8 min (Fig. 2d). In the reversed phase conditions the profile of coumarate components (Fig. 3d) was superimposable (identical pattern and slight shifts in the retention times) to those of ferulates (Fig. 3c) whilst caffeate components (Fig. 3e) eluted as broad, tailing peaks at t_R shorter than ferulates. The molecular ions $[M-H]^-$ listed in Table 2 (col. Q and R) were detected by LC–ESI-MS technique thus confirming the presence of *p*-coumarate and caffeate esters of the native steroid moieties.

3.3. Quantitation of γ-oryzanol components optimisation of chromatographic conditions

3.3.1. Column choice

Silica gel was our first choice as stationary phase column, it provided a good separation of oil constituents using an isocratic elution with hexane/2-propanol 99:1. However, the column performances soon revealed themselves to be unreliable probably owing to deactivation of the silica surface from adsorption of water (Diack & Saska, 1994; Hewavitharana, 2003). Subsequently, we tried two cyanopropyl bonded stationary phases. Both gave chromatograms of comparable resolution and were used to elute the sequence of injections for the calibration curve. One of them showed a shorter dynamic range and failure of linearity at low concentration levels. To the best of our knowledge, the only difference between those stationary phases is endcapping of residual free silanols.

3.3.2. Selection of detection wavelength

The detection wavelength was set at 325 nm, close to the λ_{max} of the ferulate chromophore. The *p*-coumarate moiety has strong absorption at 290 nm and marginal absorption at 340 nm: comparison of these chromatograms points to the elution of those very minor esters (Norton, 1995; Seitz, 1989).

3.3.3. Selection of standard compounds

Ethyl ferulate was chosen as an external standard because it possesses the identical chromophore of γ-oryzanol components and has a precise molecular weight (Yu et al., 2007). On the contrary, γ-oryzanol is a mixture of cholestane and cycloartane alkyl ferulates whose actual composition depends on its vegetable origin and hence has no well defined molecular weight. Umbelliferone (7-hydroxycoumarin) was chosen as an internal standard because it shows an UV spectrum similar to the ferulate moiety, elutes far from the peaks of interest and cannot suffer *cis*–*trans* isomerism. Simple coumarin elutes close to ethyl ferulate and might superimpose to the signal of investigated compounds. However, umbelliferone showed low solubility in pure decane, so it was necessary to add 2% (v/v) of 2-propanol and to restrain its concentration.

3.4. Method validation

3.4.1. Linearity, LOD, LOQ and peak resolution

The regression equation of ethyl ferulate was $y = 8.7136x$ in which y is the signal ratio (peak area/IS area) at 325 nm and x is the concentration of analyte in nmol/μL (≡μmol/mL ≡ mmol/L). The determination coefficient was R^2 = 0.99997 and the calibration graph was linear in the range 8.86×10^{-7}–1.18×10^{-4} M. The developed method shows excellent linear response over a large range of concentrations. Good sensitivity was demonstrated with LOD and LOQ at 1.5×10^{-7} and 6.0×10^{-7} M respectively for both cholestane and cycloartane alkyl ferulates. Under the chromatographic conditions employed in the current study, the resolution was >1.5 for the *cis*-ferulate pair of peaks and in the range 1.15–1.20 for the *trans*-ferulate pair.

3.4.2. Accuracy

Apparent recoveries R^* (%) were acceptable in all tested concentrations: 89.9 (25%), 81.3 (60%), 71.8 (100%) and 69.3 (130%). We expected these results because the apparent recovery is related to the overall systematic errors of the whole analytical process and the extraction step is not optimised. According to the suggestion by Spanish analysts (Garrido-Frenich et al., 2006), we also evaluated the term named "calibration recovery" which is related to the systematic measurement errors during the quantification process. The calibration recovery R^C (%) were: 100.1 (25%), 100.7 (60%), 98.9 (100%) and 100.8 (130%). These values mean that the chromatographic system shows a high accuracy during the quantification steps.

3.4.3. Precision

Repeatability was a hard task to achieve and several instrumental options were tried. To our surprise, the problem was seen to originate from the solvent which the sample was dissolved in especially in the case of incompletely filled vials. In fact, hydrocarbons like *n*-hexane, *n*-heptane and isooctane, possess a vapour pressure high enough to slowly concentrate the sample during the time of chromatographic elution thus giving an increasing area of integrated peaks. The evaporation can be cut if vials pending in the autosampler are completely filled. In order to prepare working solutions at a stable concentration, we preferred to solve the prob-

lem by dissolving samples in decane/2-propanol 98:2, the small percentage of alcohol being necessarily added to help the dissolution of umbelliferone. Eventually, the precision at the concentration of 1.18×10^{-4} M was satisfactory because the intra- and inter-day variability of the signal ratios were 1.03 ± 0.004 and 1.03 ± 0.007 with relative standard deviation (RSD) of 0.4 and 0.7% respectively; the intra- and inter-day variability of the t_R were 24.7 ± 0.04 and 24.7 ± 0.09 with RSD of 0.2% and 0.4% respectively.

3.5. Application to cereals and commercial samples

We examined the bran from two varieties of rice, one variety of wheat, the flour of corn and rye, a supplement tablet and a sunscreen oil. The percentage of rice bran oil was similar to previous reported values (Lerma-García et al., 2009) (Table 3, entry 4). The moisture content can be roughly deduced (Table 3, entry 5). Each oil was then diluted to a concentration that gives a signal ratio falling into the linear range of the calibration curve (Table 3, entry 6). The concentration of ferulate esters in our samples was estimated by the regression equation and is precisely expressed in nmol/mL (Table 3, entries 7–12). The data are the mean of four replicates and show a high precision of the HPLC system with RSD < 1 in most cases. Taking into account the oil concentration (Table 3, entry 6), the content of ferulate esters could be related to the mass of oil (Table 3, entry 13–15). Assuming that the molecular weights of cholestane and cycloartane alkyl ferulates average 583 and 609 g/mol respectively, our data were approximately changed to mg/g of γ-oryzanol into the mass of sample or oil (Table 3, entries 16 and 17). The relative proportion of cholestane and cycloartane components points out the vegetable origin (Table 3, entry 18). The percentage of *cis*-ferulate isomers is also shown (Table 3, entry 19).

3.6. Comparison of various determination methods

The performances of several chromatographic methods are outlined in Table 1. It is interesting to note that gradient elutions in RP-HPLC (col. C, entries 7 and 8) have been applied to samples with no preliminary treatment of purification (col. D) in order to wash the column out of apolar compounds by 100% organic solvents. The identification of peaks separated by NP chromatography has been performed by the present author for the first time (col. E). In general, the shorter the elution time (col. F) the lesser the number of peaks (col. G) and the worst the baseline separation. Without using IS, the total elution time of the method proposed herein, would have been shorter. Cereal phytosterols can be esterified by different CAD derivatives (col. H). RP-HPLC provides the separation of caffeate (entry 7) from ferulate which coelute with coumarate esters. Coumarates elute as shoulders of large ferulate peaks (col. H, entries 2 and 3) so it is difficult to achieve well resolved peaks and their quantification is not reliable. On contrary, NP-HPLC shows a very good resolution (Fig. 2). The author for the first time identified the peaks of *cis*-ferulate isomers and reported their retention times in the HPLC chromatogram by an NP approach (col. I). In case of quantitative measurements of γ-oryzanol, it is reported the number of cereal samples which were analysed (col. J) and if the validation tests were carried out (col. K).

4. Conclusion

It is rare to find in the literature examples of quantitative analyses by NP chromatography, perhaps because the RP approach is the preferred one. As to concern γ-oryzanol, the inspection of Table 3 suggests the following advantages about the NP over RP-HPLC:

1. Total elution time is comparable or even shorter and a baseline separation is easily obtained.
2. The quantity of each steryl moiety is partially lost but the knowledge of the relative proportion of cholestane/cycloartane alkyl ferulates (Table 3, entry 18) is sufficient and valuable information. In fact, γ-oryzanol contains cycloartane alkyl ferulates in remarkable amount when extracted from rice and negligible from other cereals.
3. Unique clear-cut separation of all the CAD esters is possible. *p*-Coumarates are abundant in corn bran so both points 2 and 3 indicate the extraction source of γ-oryzanol.
4. Exclusive information with regards to *cis*/*trans* isomers is achieved. *cis*-Ferulates were absent in freshly extracted oils so they are artefacts of manipulation and storage. Their presence provides clues about the preservation of products and of γ-oryzanol as their ingredient (Table 3, entry 19).
5. It is evident that only NP-HPLC is promising method to be applied for the routine quantification of a large number of samples without any preliminary step of purification.
6. In the present method, crude oils or extracts can be injected; no long column rinsing is required so that multiple, automated chromatographic elutions can be carried out.

In conclusion, we established a fast and sensitive method for the quantification of γ-oryzanol and it could be a suitable tool for quality assurance and determination of origin.

Acknowledgment

Authors thank the chef Gabriele Ferron (FERRON Gabriele e Maurizio s.n.c.) and Dr. G. Peratoner (Centro di Sperimentazione Agraria e Forestale) for supplying rice and rye samples. The excellent technical assistance of Mr. Sandro Gadotti is greatly appreciated. The author is much indebted to Dr. Petru Harghel for his help in the syntheses. We also thank Dr. A. Luppi and one of the referees for his suggestions on the evaporation of incompletely filled vials. This work has been supported by Provincia Autonoma di Trento (project CRS-2007).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.12.008.

References

Akihisa, T., Yasukawa, K., Yamaura, M., Ukiya, M., Kimura, Y., Shimizu, N., et al. (2000). Triterpene alcohol and sterol ferulates from rice bran and their anti-inflammatory effects. *Journal of Agricultural and Food Chemistry, 48*, 2313–2319.

Chen, M. H., & Bergman, C. J. (2005). A rapid procedure for analysing rice bran tocopherol, tocotrienol and γ-oryzanol contents. *Journal of Food Composition and Analysis, 18*, 139–151.

Chen, C. R., Wang, C. H., Wang, L. Y., Hong, Z. H., Chen, S. H., Ho, W. J., et al. (2008). Supercritical carbon dioxide extraction and deacidification of rice bran oil. *Journal of Supercritical Fluids, 45*, 322–331.

Diack, M., & Saska, M. (1994). Separation of vitamin E and γ-oryzanol from rice bran oil by normal-phase chromatography. *Journal of the American Oil Chemists' Society, 71*, 1211–1217.

Garrido-Frenich, A., Romero-González, R., Martínez-Vidal, J. L., Plaza-Bolaños, P., Cuadros-Rodríguez, L., & Herrera-Abdo, M. A. (2006). Characterisation of recovery profiles using gas chromatography – Triple quadrupole mass spectrometry for the determination of pesticide residues in meat samples. *Journal of Chromatography A, 1133*, 315–321.

Heinmann, R. J. B., Xu, Z., Godber, J. S., & Lanfer-Marquez, U. M. (2008). Tocopherols, tocotrienols and γ-oryzanol contents in japonica and indica subspecies of rice (*Oryza sativa* L.) cultivated in Brazil. *Cereal Chemistry, 85*, 243–247.

Hakala, P., Lampi, A. M., Ollilainen, V., Werner, U., Murkovic, M., Wähälä, K., et al. (2002). Steryl phenolic acid esters in cereals and their milling fractions. *Journal of Agricultural and Food Chemistry, 50*, 5300–5307.

Hewavitharana, A. K. (2003). Simple solutions to problems encountered in quantitative analysis of tocopherols and tocotrienols using silica columns. *Analytical Biochemistry, 313*, 342–344.

Huang, S. H., & Ng, L. T. (2011). An improved high-performance liquid chromatographic method for simultaneous determination of tocopherols, tocotrienols and γ-oryzanol in rice. *Journal of Chromatography A, 1218*, 4709–4713.

Lerma-García, M. J., Herrero-Martinez, J. M., Simó-Alfonso, E. F., Mendonça, C. R. B., & Ramis-Ramos, G. (2009). Composition, industrial processing and applications of rice bran γ-oryzanol. *Food Chemistry, 115*, 389–404.

Miller, A., & Engel, K. H. (2006). Content of γ-oryzanol and composition of steryl ferulates in brown rice (*Oryza Sativa* L.) of European origin. *Journal of Agricultural and Food Chemistry, 54*, 8127–8133.

Moreau, R. A., Whitaker, B. D., & Hicks, K. B. (2002). Phytosterols, phytostanols and their conjugates in foods: Structural diversity, quantitative analysis and health-promoting uses. *Progress in Lipid Research, 41*, 457–500.

Norton, R. A. (1995). Quantitation of steryl ferulate and *p*-coumarate esters from corn and rice. *Lipids, 30*, 269–274.

Seitz, L. M. (1989). Stanol and sterol esters of ferulic and *p*-coumaric acids in wheat, corn, rye and triticale. *Journal of Agricultural and Food Chemistry, 37*, 662–667.

Stöggl, W., Huck, C., Wongyai, S., Scherz, H., & Bonn, G. (2005). Simultaneous determination of carotenoids, tocopherols and γ-oryzanol in crude rice bran oil by liquid chromatography coupled to diode array and mass spectrometric detection employing silica C30 stationary phases. *Journal of Separation Science, 28*, 1712–1718.

Xu, Z., & Godber, J. S. (1999). Purification and identification of components of γ-oryzanol in rice bran oil. *Journal of Agricultural and Food Chemistry, 47*, 2724–2728.

Yu, S., Nehus, Z. T., Badger, T. M., & Fang, N. (2007). Quantification of Vitamin E and γ-oryzanol components in rice germ and bran. *Journal of Agricultural and Food Chemistry, 55*, 7308–7313.