

# Metabolite Profiling—A Fractionation Method for Analysis of Major and Minor Compounds in Rice Grains

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ABSTRACT

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A metabolite profiling methodology was developed using rice as model crop. The approach is based on consecutive extraction of lipids and polar compounds and subsequent fractionations of both extracts. Transesterification-solid phase extraction (lipids) and selective hydrolysis of silylated derivatives (polar compounds) are applied to separate major from minor constituents. The method covers a broad spectrum of chemical classes.

Preliminary investigation of the four fractions by gas chromatography/mass spectrometry (GC/MS) analysis of silylated derivatives identified more than 100 compounds. This unbiased approach can serve as an additional analytical tool for the safety assessment of genetically modified crops and may help to increase the chance to detect unintended effects due to the application of recombinant DNA techniques.

Genetic engineering is increasingly applied in modern plant breeding. Commercialization of transgenic crops requires comprehensive premarket food safety assessment. The principle of substantial equivalence (OECD 1993) is a key element of the strategies agreed to by international bodies (OECD 1996; FAO/WHO 1997). This concept is used to identify similarities and differences between genetically modified food and a comparator with a history of safe food use that subsequently guides the safety assessment process (FAO/WHO 2000).

An important part of this strategy is the comparison of the chemical compositions between parental and genetically modified plant, with a special focus on essential nutrients and critical toxicants such as glycoalkaloids in potatoes (Engel et al 1998). This approach has proven to be useful for the safety assessment of the first generation of genetically modified crops (Lavrik et al 1995; Sanders et al 1998; Glennon et al 2000). One of the arguments forwarded by critics of this concept is the biased character of a targeted analysis of single compounds (Millstone et al 1999). To increase the chances of detecting unintended effects caused by the genetic modification, profiling techniques have been suggested as additional analytical tools (Kuiper et al 1999). Gas chromatography/mass spectrometry (GC/MS) (Fiehn et al 2000; Roessner et al 2000) as well as offline liquid chromatography/nuclear magnetic resonance (LC/NMR) (Noteborn et al 2000) have been described as approaches for profiling at the metabolite level.

The objective of this study was to develop a metabolite profiling technique using rice as model crop. In addition to conventional breeding, recombinant DNA techniques are increasingly applied to improve agronomical and nutritional traits of rice (Tyagi et al 2000). One of the most prominent examples is the biosynthesis of  $\beta$ -carotene in Golden Rice (Ye et al 2000). Safety assessment of transgenic rice based on targeted analysis of single compounds has been reported (Momma et al 1999; Hashimoto et al 1999).

This study describes the isolation of extracts of lipids and polar compounds from rice material. The role of rice grains as storage organs results in a complex composition characterized by extreme differences in concentrations of compounds. To meet this challenge, a methodology to fractionate the total rice extracts was developed for GC analysis of a broad spectrum of major and minor constituents.

## MATERIALS AND METHODS

Rough rice (*Oryza sativa* L.) grown in Northern China was used as material for method development. Brown rice samples (round

grain rice from Italy, long grain rice from Spain) used for the applicability studies were purchased in local supermarkets. Reference compounds were obtained from Merck KGaA (Darmstadt, Germany), Fluka (Buchs, Switzerland), Roth (Karlsruhe, Germany), Riedel de Haën (Seelze, Germany), Mallinckrodt-Baker (Deventer, Holland), and Acros (Geel, Belgium).

### Sample Preparation

Rice grains (9–12% moisture) were ground with a cyclone mill (Cyclotec, Foss, Germany) equipped with a 500- $\mu$ m sieve. The flour was immediately freeze-dried using a conventional freeze-drying apparatus (Delta IA, Christ, Germany). The moisture content of the resulting material (<2%) was determined as a loss of weight by drying at 130°C for 2 hr (BgVV 2000).

### Extraction Procedure

Freeze-dried rice flour (2 g) and 5 mL of methanol (Merck) was stirred for 20 min at 30°C in a round flask. After removal of methanol by rotary evaporation, 10 mL of dichloromethane (Riedel de Haën) was added to the flour. The suspension was stirred at 30°C for 30 min and filtered through a filter funnel. The residue was reextracted with another 10 mL of dichloromethane. The combined filtrates were brought to a final volume of 20 mL (lipid extract).

Traces of dichloromethane were removed from the filtration residue under vacuum. For the extraction of polar compounds, the defatted flour was treated with methanol and water (80:20, v/v, 2  $\times$  10 mL) following the procedure described for the lipid extract.

### Fractionation and Analysis of Lipids

Lipid extract (5 mL) was evaporated to dryness and redissolved in 300  $\mu$ L of methyl-tert-butylether (MTBE, Oxeno, Marl, Germany). To this solution, 200  $\mu$ L of dry methanol and 50  $\mu$ L of sodium methylate, 5.4M in methanol (Fluka) were added. After reaction for 1 hr at room temperature, 1 mL of dichloromethane and 2 mL of aqueous 0.35M HCl were added. After vigorous shaking, the upper phase was discarded. The lower phase containing the trans-methylated lipids was evaporated to dryness by rotary evaporation and subjected to solid phase extraction (SPE).

A small amount of anhydrous Na<sub>2</sub>SO<sub>4</sub> was placed on top of a 500-mg silica gel SPE cartridge (LiChrolut, Merck) conditioned with one column volume (CV, 2.5 mL) of *n*-hexane (Mallinckrodt-Baker). Transmethylated lipids were redissolved in 250  $\mu$ L of dichloromethane and transferred to the SPE cartridge. The fatty acid methyl ester fraction (fraction I) was eluted with hexane and MTBE (100:2, v/v, 2  $\times$  3 mL). Subsequently, the fraction of minor polar lipids (fraction II) was eluted with hexane and MTBE (70:30, v/v, 2  $\times$  2.5 mL). Elutions from the SPE cartridge proceeded by gravity flow into 10-mL round flasks. The eluates were evaporated to dryness by rotary evaporation under vacuum. The fatty acid methyl ester fraction was redissolved in 150  $\mu$ L of dichloromethane, and 1  $\mu$ L was injected into the gas chromatograph.

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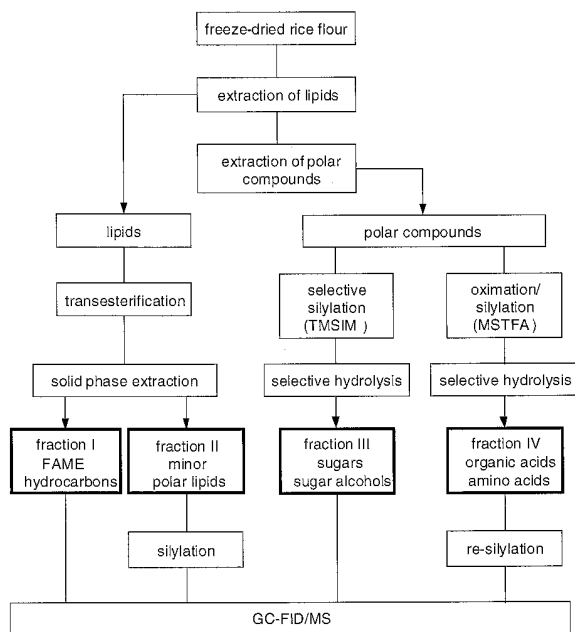
Fraction II containing polar lipids was redissolved in 100  $\mu\text{L}$  of dry pyridine (Fluka) and 50  $\mu\text{L}$  of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA, Merck) was added. After flushing with argon, the flask was tightly sealed with a glass stopper and allowed to stand for 15 min at 70°C in an oil bath. The sample was then cooled to room temperature and 1  $\mu\text{L}$  was analyzed by gas chromatography.

### Fractionation of Polar Extract

Polar extract (4 mL) was concentrated in a rotary film evaporator and dried over phosphorus pentoxide. The dry residue was redissolved in 100  $\mu\text{L}$  of a solution of hydroxylammoniumchloride (Merck) in pyridine (2 mg/mL). After heating for 30 min at 70°C, 50  $\mu\text{L}$  of MSTFA was added and the sample was allowed to stand for another 15 min at 70°C. The silylated sample was diluted with 400  $\mu\text{L}$  of *n*-hexane and 200  $\mu\text{L}$  of water was added for selective hydrolysis of silylated organic and amino acids. After vortexing, the mixture was allowed to stand for 5 min. The upper phase containing oximated and silylated sugars was discarded and residual sugars were reextracted from the aqueous phase by treatment with another 400  $\mu\text{L}$  of *n*-hexane. The lower aqueous phase containing free organic and amino acids was concentrated in a rotary film evaporator and dried over phosphorus pentoxide. For silylation the residue was redissolved in 100  $\mu\text{L}$  of dry acetonitrile (Merck) and further treated as described for fraction II.

Polar extract (1 mL) was concentrated in a rotary film evaporator and dried over phosphorus pentoxide. Pyridine (100  $\mu\text{L}$ ) and trimethylsilylimidazole (50  $\mu\text{L}$ , TMSIM, Fluka) were added for silylation which was performed as described for fraction II. The silylated sample was diluted with 200  $\mu\text{L}$  of *n*-hexane and 200  $\mu\text{L}$  of water was added for selective hydrolysis of silylated organic acids and amino acids. After vortexing, the open flask was allowed to stand for 5 min and 1  $\mu\text{L}$  of the upper phase was injected into the gas chromatograph. Recovery of sugars was determined by the addition of defined amounts of reference compounds to the freeze-dried flour (1.5 mg/g of flour) at the start of the extraction procedure.

To determine the repeatability (intralaboratory precision) of the fractionation method, five aliquots of a total extract were subjected to the described procedure and the resulting fractions were analyzed by GC with flame ionization detection (FID). Data are reported as relative standard deviations (RSD) of the peak areas obtained.



**Fig. 1.** Metabolite profiling method. FAME = fatty acid methyl esters; TMSIM = trimethylsilylimidazole; MSTFA = *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide. GC-FID/MS = gas chromatography using flame ionization detection or mass spectrometry.

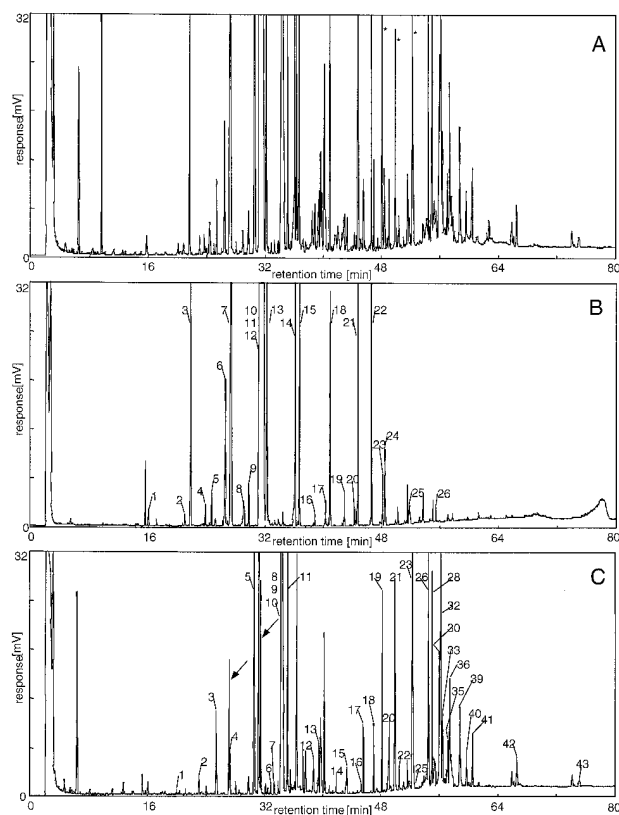
For comparison, two brown rice samples were extracted and fractionated in triplicate. Chromatographic analysis was performed by GC/FID. Confidence intervals of mean peak areas for each sample and significant differences between samples were determined using Student's *t*-distribution ( $P < 0.05$ ).

### Gas Chromatography

GC was performed on a Carlo Erba Mega HRGC 5160 (ThermoQuest, Austin, TX) equipped with a flame ionization detector. The chromatography column was a DB-1, 60 m  $\times$  0.32 mm i.d. fused silica capillary coated with a 0.25- $\mu\text{m}$  film of polydimethylsiloxane (J&W Scientific, Folsom, CA). Split injection (split flow 30 mL/min) was performed at 280°C, and the column temperature was programmed from 100 to 320°C (30-min hold) at 4°C/min. Hydrogen was used as the carrier gas at a constant inlet pressure of 100 kPa.

GC with mass spectrometer (GC/MS) was performed on a GC 8000 Top with a Finnigan Voyager (ThermoQuest) as the mass selective detector. The MS interface temperature was set to 260°C. Full scan mass spectra were recorded at an electron energy of 70 eV (source temperature 200°C) within a scan range of 40–700 mu. The chromatography column was the same as used for analysis by GC/FID. Split injection (split flow 30 mL/min) was performed at 250°C, and the column temperature was programmed as described above. Helium was used as the carrier gas at a constant inlet pressure of 165 kPa.

Rice constituents were identified by comparing retention times with those of silylated and methylated reference compounds or by comparing mass spectra with the entries of mass spectra libraries (NIST 1995; MPI 2000).



**Fig. 2.** Gas chromatography using flame ionization detection of a rough rice lipid extract spiked with  $\alpha$ -,  $\gamma$ -, and  $\delta$ -tocopherol. **A**, Total lipid extract after transesterification and silylation, spiked tocopherols indicated by asterisks. **B**, fraction I (numbers in Table I). **C**, fraction II (numbers in Table II), residual fatty acid methyl esters indicated by arrows.

## RESULTS AND DISCUSSION

To cover a maximum spectrum of rice grain constituents, rough rice was used for method development. Metabolite profiling of cereals such as rice faces the challenge that the composition is mainly determined by high amounts ( $\leq 80\%$ ) of polysaccharides that serve as storage substances. In addition, there are LMW polar constituents belonging to various compound classes such as free sugars, sugar alcohols, organic acids, amino acids, amines, and phenolics. The lipid content is relatively low (2–3%). However, even minor representatives of this class (tocopherols) deserve attention because of their nutritional importance (Juliano et al 1985).

A scheme of the profiling method developed is shown in Fig. 1. It starts with consecutive extraction of lipids and polar compounds from freeze-dried rice flour. Both extracts are characterized by the

presence of major constituents (triglycerides and free sugars, respectively) and a spectrum of minor compounds (organic acids, amino acids or free fatty acids, sterols). Therefore, both extracts were further subdivided by using transesterification and SPE for the lipids and by making use of the relative stability of silyl derivatives for the polar compounds.

### Extraction

Rice flour was freeze-dried to standardize the water content for the subsequent extraction procedure. Several solvents were tested for extraction. The use of ethanol resulted in a complex mixture of lipids and polar compounds as described for barley (Sauter et al 1991). However, considering the concentration differences described above, it seemed more appropriate to prepare separate extracts of polar and lipid constituents.

**TABLE I**  
Compounds Identified in Fraction I

No.	Compound	Identification <sup>a</sup>	No.	Compound	Identification <sup>a</sup>
Fatty acid methyl esters			14	C20:1 (cis 11)	1,2
1	C12:0	1,2	15	C20:0	1,2
2	C14:1	3	16	C21:0	1
3	C14:0	1,2	17	C22:1 (cis 13)	3
4	C15:1	3	18	C22:0	1,2
5	C15:0	1	19	C23:0	1
6	C16:1 (cis 9)	1,2	20	C24:1	1
7	C16:0	1,2	21	C24:0	1,2
8	C17:1	3	24	C26:0	1
9	C17:0	3	25	C28:0	1
10	C18:3 (cis 9,12,15)	1,2	26	C30:0	1
11	C18:2 (cis 9,12)	1,2	Hydrocarbons		
12	C18:1 (cis 9)	1,2	22	Squalene	1,2
13	C18:0	1,2	23	C29:0	1

<sup>a</sup> Identification: 1, according to NIST mass spectra database (NIST 1995); 2, according to retention times of reference compounds; 3, according to metabolite mass spectra library of Max Planck Institute of Molecular Plant Physiology (MPI 2000).

**TABLE II**  
Compounds Identified in Fraction II

No.	Compound <sup>a</sup>	Identification <sup>b</sup>	No.	Compound <sup>a</sup>	Identification <sup>b</sup>
Free fatty acids			Sterols and triterpenic alcohols		
1	C12:0	1,2,5	25	Cholesterol	1,2
3	C14:0	1,2	26	Campesterol	2,3
5	C16:0	1,2,5	27	Campestanol	6
6	C17:0	1	28	Stigmasterol	2,3
8	C18:3 (cis 9,12,15)	1,2,5	29	$\Delta^7$ -Campestenol	6
9	C18:2 (cis 9, 12)	1,2,5	31	Obtusifoliol	7
10	C18:1 (cis 9)	1,2,5	32	$\beta$ -Sitosterol	2,3
11	C18:0	1,2,5	33	Sitostanol	6
12	C20:1	1	34	$\Delta^5$ -Avenasterol	7
13	C20:0	1	35	Gramisterol	7
15	C22:0	1	36	Cycloartenol	6,7
16	C23:0	3	37	$\Delta^7$ -Stigmastenol	6,7
18	C24:0	4	38	$\Delta^7$ -Avenasterol	7
22	C26:0	4	39	24-Methylenecycloartanol	6,7
Alcohols			40	Citrostadienol	7
7	C18:0	3	Other compounds		
14	C22:0	3	2	Methyl <i>p</i> -hydroxycinnamate	1
17	C24:0	4	4	Methyl ferulate	1,2
20	C26:0	4			
24	C28:0	1			
30	C30:0	4			
41	C32:0	4			
42	C34:0	4			
43	C36:0	4			
Tocopherols					
19	$\delta$ -Tocopherol	2			
21	$\gamma$ -Tocopherol	2			
23	$\alpha$ -Tocopherol	2,3			

<sup>a</sup> Metabolites identified as trimethylsilylated derivatives.

<sup>b</sup> Identification: 1, according to NIST mass spectra database (NIST 1995); 2, according to retention times of reference compounds; 3, according to metabolite mass spectra library of Max Planck Institute of Molecular Plant Physiology (MPI 2000); 4, based on mass spectral data; 5, comparison of mass spectral data to those of a reference compound; 6, comparison of mass spectral data to those reported by Xu et al (1999); 7, comparison of mass spectral data to those reported by Kamal-Eldin et al (1992).

A method commonly applied for total lipid extraction is based on the use of a mixture of chloroform and methanol (Folch et al 1957). Owing to a co-extraction of nonlipids, this procedure involves an additional washing step. This often results in emulsions, rendering precise phase separation difficult and resulting in potential loss of polar constituents. The consecutive use of methanol and dichloromethane proved to be suitable to overcome these drawbacks. By applying dichloromethane alone, complete removal of the lipids was not possible. However, disintegration of the flour matrix by pretreatment with methanol and subsequent use of dichloromethane resulted in exhaustive extraction of lipids as demonstrated by sequential extractions. Residues of the solvent were removed from the defatted flour under vacuum.

In accordance with previously described approaches (Katona et al 1999), a mixture of methanol and water (80:20, v/v) was used for extraction of polar compounds. Attempts to increase the polarity of the solvent by increasing the water content were not satisfactory from an operational standpoint because of foaming during the concentration process under reduced pressure.

### Fractionation

The objective of the fractionation procedure was to separate the abundant triglycerides from minor lipids (sterols, tocopherols). This was achieved by transmethylation of the triglycerides and subsequent SPE on silica gel using elution solvents with increasing polarity (Biedermann et al 1993; Schmar et al 1996).

Base-catalyzed transmethylation converts triglycerides and other fatty acid esters into the respective methyl esters. It can be performed under mild conditions and allows separate analysis of the metabolically important free fatty acids that are not methylated. Furthermore, it is useful for the analysis of the sum of free and esterified minor lipids (sterols, triterpenic alcohols). Fig. 2A shows the GC/FID chromatogram of an unfractionated lipid extract after transesterification and silylation.

SPE of the transmethyated lipid extract using *n*-hexane and MTBE (100:2, v/v) results in a fraction consisting mainly of fatty acid methyl esters (FAME) derived from ester bound fatty acids and hydrocarbons (Fig. 2B). Compounds identified in this fraction by means of GC/MS using an apolar stationary phase are listed in Table I. The isolation of the fatty acid esters in this separate fraction also opens the possibility for further analysis, according to degree of unsaturation, on a more polar column.

TABLE III  
Compounds Identified in Fraction III

No.	Compound <sup>a</sup>	Identification <sup>b</sup>
Sugars		
4	Arabinose	1,2,3
5	Arabinose	
7	Fructose	1,2,3
8	Fructose	
9	Fructose	
10	Galactose	1,2,3
12	Galactose	
10	Galactose	1,2,3
11	Glucose	1,2,3
13	Glucose	
15	Sucrose	1,2
16	Raffinose	1,2,3
Sugar alcohols		
1	Glycerol	1,2
2	Threitol	1
3	Erythritol	1
6	Xylitol	1
13	Inositol	1,2

<sup>a</sup> Metabolites identified as persilylated derivatives.

<sup>b</sup> Identification: 1, according to NIST mass spectra database (NIST 1995); 2, according to retention times of reference compounds; 3, comparison of mass spectral data to those of a reference compound.

By using hexane and MTBE (70:30, v/v) as elution solvent, a fraction containing the more polar lipids was obtained (Fig 2C). Table II lists representatives of the various classes (sterols, tocopherols, free fatty acids, waxy alcohols) identified by means of GC/MS analysis of the silylated (MSTFA) fraction. The amount of fatty acid methyl esters not retained in the previous fraction was <2%.

In the course of the development of the method, it became obvious that separation of tocopherols and fatty acid methyl esters is a critical step in the SPE procedure. Tocopherols are naturally occurring in rice only at low levels. Therefore, the transmethyated lipid extract was spiked with  $\alpha$ -,  $\gamma$ -, and  $\delta$ -tocopherol before fractionation (see asterisks in Fig. 2A). After column chromatography (Fig. 2C), 100% ( $\gamma$ - and  $\delta$ -tocopherol) and 95% ( $\alpha$ -tocopherol) of the added compounds could be recovered in fraction II. This clear-cut procedure was confirmed by silylation of fraction I. Except for  $\approx$ 5%  $\alpha$ -tocopherol, the resulting chromatogram showed no difference to the one shown in Fig. 2B.

Fractionation of the polar extract was performed to separate free sugars present as major polar LMW compounds from minor constituents such as organic acids and amino acids. Because silylating reagents strongly differ in their suitability for different compound

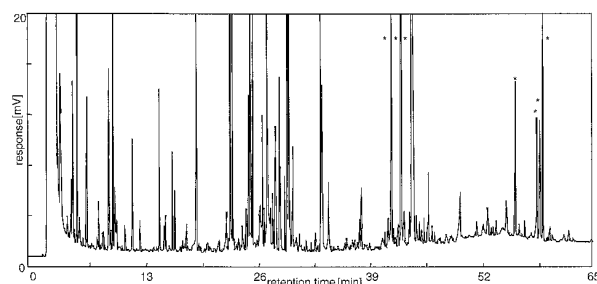


Fig. 3. Gas chromatography using flame ionization detection of a total methanol-water extract of a rough rice sample after silylation with MSTFA; peaks of incompletely silylated sugars indicated by asterisks.

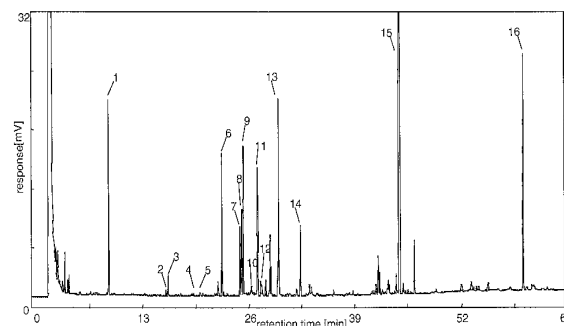


Fig. 4. Gas chromatography using flame ionization detection of fraction III (numbers in Table III).

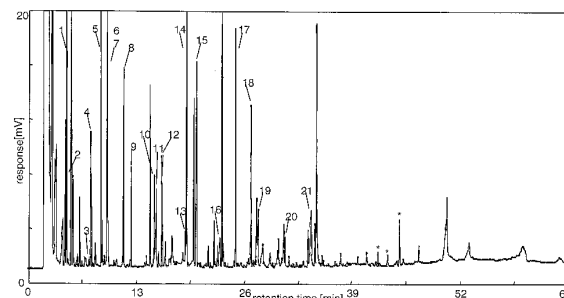


Fig. 5. Gas chromatography using flame ionization detection of fraction IV (fourfold enriched) (numbers in Table IV), residual sucrose indicated by asterisks.

classes (Heberle et al 1995), fractionation would allow an appropriate choice of reagents to ensure complete derivatization.

The fractionation of the polar extract is based on the relative stability of the R-Si(CH<sub>3</sub>)<sub>3</sub> group of sugars/polyols and amino acids/organic acids to aqueous hydrolysis, a principle developed for the analysis of amino acids in human blood (Clay et al 1979). Mixing the silylated polar extract with *n*-hexane and water results in the formation of two phases. The silylated sugars and polyols, which are relatively stable to hydrolysis, are enriched in the hexane layer. The aqueous phase contains the free amino acids and organic acids that are readily released from their unstable trimethylsilyl derivatives.

In view of the above-mentioned differences in suitability of silylating reagents, several reagents and combinations thereof were tested. Figure 3 shows a gas chromatogram of the total polar extract obtained from rice flour after derivatization with MSTFA. Sucrose and raffinose are not completely persilylated, resulting in multiple peaks for these compounds. Trimethylsilylimidazole (TMSIM) is a more powerful silylating agent for sugars (Heberle et al 1995). It persilylates mono-, di-, and trisaccharides quickly under mild conditions. Silylation of the polar extract with TMSIM and subsequent treatment with a hexane and water mixture resulted in a fraction containing only persilylated sugars and polyols (Fig. 4). Metabolites identified in a preliminary GC/MS study are listed in Table III. Recoveries ranging from 84% (raffinose) to 97% (fructose) demonstrate the suitability of this selective hydrolysis of silylated compounds for separation of mono-, di-, and trisaccharides from other polar constituents.

The amino function of amino acids cannot be silylated using TMSIM (Heberle et al 1995). Its presence even inhibited derivatization of amino acids with MSTFA. Therefore, the analysis of organic acids and amino acids has to start from a separate aliquot of the polar extract. To protect  $\alpha$ -ketoacids from enolization and decarboxylation before trimethylsilylation, an additional oximation step was necessary. A model study with  $\alpha$ -ketoglutarate (oximated with hydroxylamine, silylated with MSTFA) demonstrated that the above-described fractionation strategy was also applicable to oximated derivatives. Addition of water has no effect on the R<sup>1</sup>R<sup>2</sup>C=N-O-Si(CH<sub>3</sub>)<sub>3</sub> group but hydrolyzes the R-C(O)-O-Si(CH<sub>3</sub>)<sub>3</sub> group,

**TABLE IV**  
Compounds Identified in Fraction IV

No.	Compound	Identification <sup>a</sup>
Amino acids and amines		
1	Alanine (2TMS) <sup>b</sup>	1
2	Glycin (2TMS)	1,2
3	Urea (2TMS)	1
4	Valine (2TMS)	1
6	Proline (2TMS)	1,2
8	Serine (2TMS)	1
9	Threonine (2TMS)	1
11	Pyroglutamic acid (2TMS)	1
12	Aspartic acid (2TMS)	1
13	Phenylalanine (2TMS)	1
14	Glutamic acid (3TMS)	2,3
15	Asparagine (3TMS)	1
16	Glutamine (3TMS)	1
18	Allantoin (5TMS)	3
19	Tyrosin (3TMS)	1,2
21	Tryptophane (3TMS)	1
Organic acids <sup>c</sup>		
5	Phosphoric acid	1
7	Succinic acid	1,2
10	Malic acid	1,2
17	Citric acid	1,2
20	Ferulic acid	1,2

<sup>a</sup> Identification: 1, according to NIST mass spectra database (NIST 1995); 2, according to retention times of reference compounds; 3, according to metabolite mass spectra library of Max Planck Institute of Molecular Plant Physiology (MPI 2000).

<sup>b</sup> Number of trimethylsilyl (TMS) groups in parentheses

<sup>c</sup> Metabolites identified as persilylated derivatives.

resulting in an enrichment of the compound in the aqueous layer. Application of this strategy to the polar extract from rice flour and resilylation of the organic acids and amino acids enriched in the aqueous layer (fraction IV) resulted in the chromatogram shown in Fig. 5. Metabolites identified in a preliminary study in this fraction are summarized in Table IV. Sugars as major constituents of the polar extract are almost completely separated from organic acids and amino acids. In turn, no silylated amino acids were detected in the respective hexane layer, which contains silyl derivatives of oximated as well as nonoximated (sucrose and raffinose) sugars.

### Comparative Investigation of Rice Samples

Sufficient repeatability is one of the conditions to be met in order to apply the developed methodology to the differentiation of

**TABLE V**  
Repeatability for Selected Compounds

Compound	RSD (%) <sup>a</sup>	Compound	RSD (%)
Fatty acid methyl esters		Phenolics <sup>b</sup>	
C15:0	11	Methyl ferulate	9
C16:1(cis 9)	12	Sugars <sup>b,c</sup>	
C17:0	11	Fructose	5
C23:0	8	Glucose	3
C24:0	9	Sucrose	5
Hydrocarbons		Raffinose	9
Squalene	16	Amino acids <sup>b</sup>	
C29:0	13	Alanine	10
Free fatty acids <sup>b</sup>		Glycine	10
C18:0	13	Serine	9
C24:0	11	Glutamic acid	9
Alcohols <sup>b</sup>		Asparagine	14
C28:0	7	Tryptophane	19
C32:0	12	Organic acids <sup>b</sup>	
Sterols and triterpenic alcohols <sup>b</sup>		Malic acid	9
Campesterol	13	Citric acid	11
Stigmasterol	14		
$\beta$ -Sitosterol	11		
24-Methylen-cycloartanol	12		

<sup>a</sup> Relative standard deviation ( $n = 5$ ).

<sup>b</sup> Precision data calculated for silylated derivatives.

<sup>c</sup>  $n = 4$ .

**TABLE VI**  
Comparison of Peak Areas for Selected Compounds in Two Commercial Rice Samples

Fr.	Compound	Peak Areas [mV $\times$ min] <sup>a</sup>		Ratio of Peak Area <sup>d</sup>
		Sample I <sup>b</sup>	Sample II <sup>c</sup>	
I	Methyl eicosanoate	1,066 $\pm$ 151	1,168 $\pm$ 262	1.1
	Methyl docosanoate	507 $\pm$ 44	522 $\pm$ 108	1.0
	Methyl eicosanoate (*) <sup>e</sup>	1249 $\pm$ 178	879 $\pm$ 189	0.70
	Methyl hexacosanoate (*)	163 $\pm$ 5	225 $\pm$ 51	1.4
	Squalene (*)	760 $\pm$ 57	432 $\pm$ 88	0.57
II	Campesterol	848 $\pm$ 189	962 $\pm$ 56	1.1
	$\beta$ -Sitosterol	2,235 $\pm$ 434	2,133 $\pm$ 225	0.95
	Palmitic acid (*)	5,328 $\pm$ 1,048	15,646 $\pm$ 567	2.9
	Stearic acid (*)	442 $\pm$ 87	1,776 $\pm$ 83	4.0
	$\Delta^1$ -avenasterol (*)	112 $\pm$ 19	87 $\pm$ 12	0.78
II	Xylitol	28 $\pm$ 10	22 $\pm$ 1	0.8
I	Glucose (*)	128 $\pm$ 14	70 $\pm$ 4	0.55
	Raffinose (*)	262 $\pm$ 10	696 $\pm$ 8	2.7
IV	Glycerol (*)	423 $\pm$ 56	2,233 $\pm$ 79	5.3
	Serine	304 $\pm$ 49	243 $\pm$ 72	0.8
IV	Asparagine	234 $\pm$ 121	234 $\pm$ 78	1.0
	Glutamic acid (*)	1,293 $\pm$ 76	939 $\pm$ 315	0.73
	Aspartic acid (*)	892 $\pm$ 93	341 $\pm$ 99	0.38
	Succinic acid (*)	93 $\pm$ 15	263 $\pm$ 34	2.8

<sup>a</sup> Mean  $\pm$  confidence interval ( $P < 0.05$ ) ( $n = 3$ ).

<sup>b</sup> Round grain rice (Italy).

<sup>c</sup> Long grain rice (Spain).

<sup>d</sup> Mean peak area of sample II/mean peak area of sample I.

<sup>e</sup> Asterisk indicates significant differences between mean peak areas detected by Student's *t*-test.

rice cultivars. Data obtained for selected compounds belonging to various classes (Table V) demonstrate the good intralaboratory repeatability of the fractionation approach.

In a preliminary study, two commercially available brown rice samples were analyzed in triplicate by means of the described procedure. In addition to excellent mean peak area matching for methyl eicosanoate, campesterol, asparagine, significant differences between mean peak areas were also detected in all fractions ranging from -22% ( $\Delta^7$ -avenasterol) to +530% (glycerol). The observed differences are significantly higher than the variation of the methodology expressed as confidence intervals (95%) of the peak areas determined for the compounds (Table VI).

## CONCLUSIONS

The first prerequisite of an unbiased profiling approach is the use of a technique isolating a broad spectrum of compounds from the sample matrix. This in turn results in a complex extract exhibiting strong differences in concentrations of abundant constituents on one hand and minor but nutritionally or toxicologically relevant compounds on the other hand. The work-up of such a mixture must always be a compromise because clean-up procedures established for the targeted analysis of single compounds cannot be applied. The usefulness of a subfractionation of the extract has to be evaluated in the light of the additional information gained and the input of work required.

The profiling method allows the separate isolation of lipids and polar compounds. For further fractionation, two routinely applicable approaches were selected. For the lipid extract, column chromatography on commercially available cartridges proved suitable to separate major from minor constituents. For the polar compounds, a strategy based on the hydrolytic stability of silyl derivatives was modified and applied for the first time to an extract from plant material.

This subfractionation is more laborious than the direct analysis of total extracts described previously (Katona et al 1999; Fiehn et al 2000). However, the procedure decreases the probability of coelutions in the resulting gas chromatograms and thus provides the basis to use GC/FID as routine screening mode and GC/MS for final confirmation of differences detected. The method will allow valuable additional information, especially on the minor constituents and, owing to the simplicity of the chromatograms obtained, future automated data treatment and assessment will be facilitated.

Trimethylsilylation turned out to be a universal method for the detection of a broad spectrum of compounds. However, the necessity to select the appropriate silylating reagent became obvious. Compounds exhibiting thermal instability and low volatility cannot be detected by this method. Profiling techniques based on liquid chromatography have the potential to close this gap (Fraser et al 2000).

The GC/MS identification of the chemical structures of about 100 compounds is to be considered as a first step. A more thorough investigation of the fractions obtained will allow further assignments of minor peaks.

Results obtained through comparative investigation of two rice samples and the repeatability data determined so far indicate the potential of the methodology to identify metabolic phenotypes, to differentiate rice cultivars, and to get an idea of the natural degree of variation in the metabolites identified. These aspects are currently under detailed investigation.

The profiling method developed should be applicable to plants with similar distributions of constituents and the principles involved might serve as a basis for compositional analysis of other crops. This unbiased approach can serve as an additional analytical tool for the safety assessment of genetically modified crops. The screening of a broad spectrum of compounds combined with an effective separation of major and minor constituents may help to increase the chance to detect unintended effects due to the application of recombinant DNA techniques.

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