Alkyl- and Alkenylresorcinols of Wheat Grains and their Chemotaxonomic Significance

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Resorcinolic lipid contents and homologue compositions in extracts isolated from soft winter, soft spring and hard (durum) wheat grains were evaluated by both instrumental and chromatography means. Resorcinol concentrations determined in wheat were diverse and varied in samples harvested within two consecutive vegetative years, whereas their homologue profiles were found to be rather invariable. The predominant alkylresorcinols identified in wheat grains were saturated 1,3-dihydroxy-5-*n*-heneicosylbenzene and 1,3-dihydroxy-5-*n*-nonadecylbenzene. 1,3-Dihydroxy-5-*n*-heptadecylbenzene and 1,3-dihydroxy-5-*n*-tricosylbenzene were also determined, whereas 1,3-dihydroxy-5-*n*-pentadecylbenzene and 1,3-dihydroxy-5-*n*-pentacosylbenzene were present in these extracts only in spurious amounts. Furthermore, our results show that alk(en)ylresorcinols may be useful as chemotaxonomic markers for a distinction between soft and hard wheat plants. Cluster analysis with Ward's amalgamation algorithm and five different distance linkage types clearly discriminated particular wheats into species- and cultivar-specific clusters, whereas the use of principal component analysis allowed us to specify, which of the variables analysed were decisive. This approach may be useful for both plant breeders and taxonomists to classify wheat species/cultivars.

Key words: Resorcinolic Lipids, Phenols, Cereals

Introduction

Grasses, especially cereals, have attracted an attention of the human race since the ancient antiquity. Species like wheat, rice, corn, rye, oat and barley are a basis of food of millions people all over the world. An optimization of food compositions may be achieved, among others, through properly selected and executed agricultural crop systems and practices. Such strategies require also an extensive knowledge of plant breeding and, in general, of botany and plant taxonomy. However, a conventional approach to the plant classification does not fully reflect phylogenetic relationships within the plant kingdom. This problem also concerns the phylogeny of grasses. For example, several attempts about the evolution of the *Triticum* genus have been reported in the literature. A presumable phylogenetic tree of this taxon has been constructed on the basis of different analytical methods applied as well as of different types of compounds analysed, like nucleic acids (Chen *et al.*, 1994; Hsiao *et al.*, 1995; Sasanuma *et al.*, 1996; Muniz *et al.*, 2001), proteins (Marchylo *et al.*, 1989; Ciaffi *et al.*, 1997) or lipids (Dutta and Appelqvist, 1996; Armanino *et al.*, 2002; Ruibal-Mendieta *et al.*, 2002). Even though these latter chemicals constitute rather a minor group in wheat grains, they have various functions and are often of great importance both from physiological and nutritional points of view (Morrison, 1985). Moreover, certain lipids exhibiting antioxidant properties may actively contribute to safe food storage (Wessling *et al.*, 2001). The biochemistry of lipid constituents in wheat was extensively studied and the conclusion was drawn that this species is one of the richest in 5-*n*-alkylresorcinols and 5-*n*-alkenylresorcinols (Verdeal and Lorenz, 1977; Hengtrakul *et al.*, 1990, 1991; Al-Ruqaie and Lorenz, 1992). However, only one paper has drawn attention to the complete analysis of resorcinolic lipid homologues occurring in a limited number of studied wheat cultivars (Hengtrakul *et al.*, 1991). Since this class of naturally occurring polyketide-

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derived, non-isoprenoid phenolic lipids has recently brought attention to researchers (Gembeh *et al.*, 2001; Chaturvedula *et al.*, 2002; Valcic *et al.*, 2002; Zarnowski and Kozubek, 2002; Liukkonen *et al.*, 2003; Miche *et al.*, 2003), and because many other arable plants have been studied for the presence of resorcinols (Zarnowski and Kozubek, 1999; Zarnowski *et al.*, 2001, 2002), the knowledge of the biochemistry of these lipid compounds in wheat can be also utilised to a larger extent. During the recent past, new analytical approaches on new molecules as well as on chemotaxonomy have been emerging. The occurrence of resorcinolic lipids seems to be also an useful feature in chemotaxonomic studies. Statistic-based analyses could assign resorcinol-containing plants to particular taxa or might be helpful to distinguish species within the same taxonomy unit. Because compositions of chemical substances *in planta* depend also on environmental factors, for comparative studies it is always essential to investigate their influence on the physiology and biochemistry of plant organisms. With reference to resorcinolic lipids, this phenomenon has also been demonstrated recently in barley (Zarnowski *et al.*, 2002).

In this paper we recapitulate the alkyl- and alkenylresorcinol homologue composition and content in grains of soft spring and soft winter wheat (*Triticum aestivum* L.) as well as of hard wheat (*Triticum durum* Desf.), harvested within two consecutive vegetative years. The wheat cultivars were grown in Poland to obtain data relevant for local nutrition requirements. On the basis of the results presented herein, a new chemotaxonomical approach to the classification of wheat species/cultivars is also reported. We also focused on principal component analysis that allowed us to decrease a number of qualitatively determined chemicals (resorcinolic homologues) while preserving the same level of wheat cultivar differentiation. This is the first complete study on the resorcinolic lipids in wheat grains and, simultaneously, the first study applying various techniques of statistical analysis.

Experimental

General

All solvents and reagents of A-grade quality were purchased from Polskie Odczynniki Chemiczne (Gliwice, Poland), except *N*-methyl-*N*-trimethylsilyl-trifluoroacetimide (MSTFA) provided by Sigma (Poznan, Poland), and Fast Blue B $\times$ BF_4 released by Chemapol (Prague, Czech Republic). TLC plates used in this study were supplied by Merck (Darmstadt, Germany). Standards of authentic rye resorcinols were kindly provided by Prof. A. Kozubek (Institute of Biochemistry and Molecular Biology, University of Wroclaw, Wroclaw, Poland).

Grain samples

One variety of soft spring-crop wheat (cv. *Jasna*), one of winter-crop wheat (cv. *Kobra*) and one variety of hard wheat (cv. *Tetradur*) were studied. All grain samples were cultivated on neighbouring field plots at the Plant Breeding Experimental Station, Agricultural University, Wroclaw, Poland. Complete cultivar vouchers are deposited in this institution and are available for inspection on request. Plant material was collected within two consecutive vegetative years, in 2000 and in 2001. Fully matured grains were harvested and after drying stored in moisture-proof containers. All in all, six different samples (three different wheat grain varieties $\times$ two vegetative years) were subjected to laboratory analyses. Each sample was analysed in triplicate.

Isolation and purification of resorcinolic lipids

The fractions of resorcinolic lipids were isolated from whole intact grains. Each of 40-g samples was soaked completely with an equal volume of acetone. Extraction was carried out for 24 h at room temperature. Next, the acetone extract was filtered through a filter paper in order to remove any solid particles and was stored at 4 °C. The grain sample was extracted twice more with the same amount of acetone for 24 h each. All collected acetone filtrates were combined together and the solvent was removed *in vacuo*. The oily residue was washed with 2-propanol and again concentrated by vacuum evaporation at 40 °C. This step allowed the extract obtained to become solid. Next, the extract was dissolved in 200 μl of ethyl acetate and immediately applied on a 20 cm $\times$ 20 cm preparative TLC plate precoated with silica gel Si60. Separation was carried out gradually, first in pure chloroform, then in a mixture of chloroform and ethyl acetate (85:15, v/v). After evaporation of the solvents, about 1 cm wise strips of the gel on both sides of the plate were sprayed with an aqueous 0.05% solution of Fast Blue B $\times$ BF_4. Resorcinolic lipids were become

visible as reddish-violet spots and were identified on the basis of R_f values with reference to authentic standards. Parts of the gel containing compounds of interest were scrapped off the plate. The material isolated was then ground in order to obtain loose gel particles. Next, this gel was loaded into a 1 cm × 10 cm column and was eluted with 120 ml of pure ethyl acetate. Collected fractions were combined and dried under reduced pressure. The final extract containing resorcinolic lipids was redissolved in 200 μl of ethyl acetate and applied on a similar preparative TLC plate that was separated by hexane/ethyl ether/formic acid (70:30:1, by vol.). Next steps for resorcinol purification were performed as mentioned above. The fraction of pure alk(en)ylresorcinols was redissolved in 200 μl of chloroform and used for further experiments. Each of the isolations was made in four repetitions.

Quantitative determination of resorcinolic lipids

Alkylresorcinol contents in the extracts isolated were measured using the microcolorimetric method (Tluscik *et al.*, 1981). Briefly, the sample analysed was put into a clean dry tube and the solvent was evaporated with a stream of nitrogen gas. To the dry residue 4 ml of the reagent prepared by a 5-fold dilution with *n*-propanol of 0.05% (w/v) Fast Blue B × BF_4 in 5% acetic acid were added. The content was thoroughly vortexed and left in the dark for an hour. The sample was read at 520 nm against the reagent blank. The content of alkylresorcinols was estimated using a calibration curve (1–10 μg) prepared by a suitable diluted stock solution of recrystallized pure 5-*n*-pentadecylresorcinol (Aldrich Chemical Co. Milwaukee, WI) as a reference compound. Each determination was carried out in triplicate.

Conversion of resorcinolic lipids into TMS-derivatives

The resorcinol mixture was reextracted with ethyl acetate containing 1% acetic acid . After removal of the solvent, the residue was dissolved in 100 μl of pure ethyl acetate. Next, 70 μl of the sample was transferred into a glass GC microvial (∅ *ca* 2 mm, 5 cm), the solvent was removed and 50 μl of MSTFA was added. The tube was sealed, mixed gently and allowed to stand for 30 min at 70 °C. Such the prepared sample was used for further instrumental analyses.

Identification and determination of alkyl- and alkenylresorcinol homologue compositions

1 μl of the derivatized sample was injected into a HP 5890 Series II gas chromatograph equipped with a DB-5MS column (J & W Science, Ringoes, NJ, USA, ∅ 0.25 mm × 30 m, 0.25 μm film thickness) and connected to a HP 5973 mass spectrometer. Analysis was done at 70 eV and helium was used as carrier gas with a flow rate of 1 ml min^{-1}. Oven temperature was programmed as follows: 90 °C for 1 min, then 30 °C min^{-1} up to 230 °C, 8 °C min^{-1} to 310 °C and hold at 310 °C for 10 min. The sample injection temperature was 280 °C. Identification of each resorcinol homologue was deduced from the molecular ion and common base peak ions at *m/z* 267 and 268, which are characteristic of ditrimethylsilyl-resorcinol derivatives. Indeed, the peak at *m/z* 267 is due to the dihydroxytropylium ion formed by direct β-fission, while the base peak at *m/z* 268 is due to the McLafferty rearrangement occurring *via* transition complex formation of a hydrogen atom of the side chain. The 267/268-abundance ion ratio of 1 to 4 or of 1 to 5 is in agreement with a *meta* position of the hydroxyl groups in the aromatic ring (Vincieri *et al.*, 1981). The retention times and molecular ions were 11.8 min (464 [M^+], $C_{15:0}$), 13.3 min (492 [M^+], $C_{17:0}$), 14.8 min (520 [M^+], $C_{19:0}$), 16.5 min (548 [M^+], $C_{21:0}$), 18.7 min (576 [M^+], $C_{23:0}$) and 21.9 min (604 [M^+], $C_{25:0}$), respectively. The relative compositions of homologues were estimated from areas of particular peaks in ion chromatograms.

Statistics

The data obtained were processed using Statistica for Windows version 5.1 (StatSoft Ltd., London, UK). Two general statistical approaches were used in this study: cluster analysis (CA) and principal component analysis (PCA). CA was used to classify the objects examined into groups (clusters), and dendrograms were constructed using most probably the most popular Ward's amalgamation algorithm. This algorithm is based on measurements of the distance between clusters utilizing analysis of variance approach (Ward, 1963). The distance between clustered objects was measured using five various methods of linkage measure (simple and square Euclidean distances, Manhattan distance, Chebyshev distance, 1-r Pearson distance). PCA (unrotated principal component

loadings) was applied as an alternative method to CA, however offering somewhat different possibilities of data evaluation (Tranter, 2000). This analysis was applied in order to check, which of considered variables were significant and decisive for this kind of chemotaxonomical studies. In both cases the proper matrices were constructed on the basis of the data obtained including total contents of resorcinols and their homologue profiles found in each of the samples studied (both vegetative years were separately specified). The mean values were also added in order to show the correctness of these analyses.

Results and Discussion

We have demonstrated that all of wheat cultivars tested herein contained alkylresorcinols and can be classified as a high-resorcinol species. Results of quantitative analyses of resorcinolic lipids in the wheat samples expressed as milligram per kilogram of dry weight of grains are presented in Table I. Alkylresorcinol concentrations found ranged approximately from 185.5 to 239.8 mg kg^{-1} (or alternatively from 0.019 to 0.024% of grain dry weight). This range varied depending on a type of wheat as well as on a vegetation year and remains in a good agreement with the data reported by Hengtrakul *et al.* (1990). However this work was focused only on analyses of resorcinolic lipid amounts in different wheat cultivars and grains being at different last maturity stages (Hengtrakul *et al.*, 1990).

It is a common knowledge that a biochemical profile of a plant organism is not exclusively dependent on information included in genes, but is also affected by many environmental factors, like agronomy or climatic conditions. Due to various existing difficulties, these factors can not be often defined precisely and can thus be appointed only by a process of trial and error. Such, but a very preliminary work has been done, however only with reference to rye (Vieringa, 1967). In the present study, we have found that amounts of resorcinols in the wheat cultivars varied within two consecutive vegetation years, while an intensity of these changes was diverse. It is noteworthy that these plants grew on adjoining field plots and were subjected to the same agricultural practices. Therefore, these noticeable changes in resorcinol contents can be only explained as caused by specific to individual years climatic conditions. Depending on a specificity of particular varieties, these unique and complex conditions may improve, impair or cause no effects on the biosynthesis process of resorcinolic lipids. Due to resorcinolic lipid concentration can be subjected to a strong influence of climate conditions and may vary with time, our results unambiguously disprove an idea presented by Hengtrakul and coworkers that wheat plants might be classified solely on the basis of resorcinol content (Hengtrakul *et al.*, 1990).

At least nine different resorcinolic homologues were found in all the samples examined (Table I). The predominant alkylresorcinols in wheat grains were saturated 1,3-dihydroxy-5-*n*-heneicosylbenzene ($C_{21:0}$) and 1,3-dihydroxy-5-*n*-nonadecylbenzene ($C_{19:0}$). To a lesser extent 1,3-dihydroxy-5-*n*-heptadecylbenzene ($C_{17:0}$) and 1,3-dihydroxy-5-*n*-tricosylbenzene ($C_{23:0}$) were also determined, whereas 1,3-dihydroxy-5-*n*-pentadecylbenzene ($C_{15:0}$) and 1,3-dihydroxy-5-*n*-pentacosylbenzene ($C_{25:0}$) were

Table I. Content and composition of resorcinolic lipids in wheat grains.

Cultivar	Year of harvest	Content[a] [mg/kg ± SE]	Homologue composition (%)										UI[b]
			$C_{15:0}$	$C_{17:1}$	$C_{17:0}$	$C_{19:1}$	$C_{19:0}$	$C_{21:1}$	$C_{21:0}$	$C_{23:1}$	$C_{23:0}$	$C_{25:0}$	
Jasna	2000	239.8 ± 6.0	0.2	0.1	4.0	0.7	42.7	0.2	47.1	0.3	4.7	*n.d.*	0.013
	2001	234.0 ± 9.1	0.2	0.2	4.0	1.1	43.6	0.3	45.3	*t*	4.9	0.3	0.016
Kobra	2000	230.2 ± 4.3	0.3	0.5	4.6	1.7	42.2	0.4	46.0	0.3	3.7	0.3	0.029
	2001	212.1 ± 3.3	0.4	0.5	4.2	1.7	41.8	0.4	47.9	*t*	3.0	*n.d.*	0.026
Tetradur	2000	185.5 ± 3.4	0.1	*t*	1.1	0.3	20.4	*t*	68.3	*t*	9.6	0.1	0.003
	2001	238.1 ± 5.1	0.3	*t*	0.3	0.3	11.1	0.2	74.8	0.1	12.7	0.1	0.006

[a] Mean of three replications of three independent samples per cultivar.
[b] Unsaturation index represents a ratio of a sum of unsaturated homologues to total resorcinols.
t: Trace (less than 0.05%).
n.d.: not detected.

present only in spurious amounts in these extracts. Both soft winter and spring wheat had very similar homologue patterns, which were highly comparable within two vegetative periods. In both cultivars $C_{21:0}$ and $C_{19:0}$ homologues were predominated among resorcinolic homologues and their average content amounted about 46.6 and 42.6%, respectively. There were also no significant discrepancies found between soft wheat cultivars with reference to patterns of unsaturated homologues. The unsaturation index (UI) values calculated for both soft varieties were higher than those determined in hard wheat. This finding is only partly in an agreement with the data reported by Hengtrakul *et al.* (1991), who demonstrated durum wheat to contain more unsaturated resorcinols in comparison with common soft wheat cultivars. No diunsaturated resorcinol derivatives were found.

Due to the traditional methodology for plant classification has increasingly proved to be inadequate, new analytical approaches like work on new molecules as well as on chemotaxonomy have been emerging during the recent past. To date, there are three general chemotaxonomic methods used by researchers to evaluate chemical differentiation between plants: taking one single property, combining chemical characteristics or contrasting competitive alternation (Tétényi, 1980). These theoretical approaches allow plants to be classified in practice by their chemical nature. Such a described above distribution of alk(en)ylresorcinols also suggests that this group of phenols might be an useful chemotaxonomic tool for the differentiation of plants belonging to the *Triticum* genus.

Cluster analysis (CA) with Ward's amalgamation algorithm and five different distance measurement methods yielded a set of dendrograms, which at first glance showed a correct distribution of particular samples into species- and cultivar-specific clusters. Each of five dendrograms created consisted of eight clusters and their compositions were generally equal (Table II). In some cases, however, certain discrepancies between those groups were also observed. The use of simple and square Euclidean distance measurement methods allowed the clusters to be formed (Fig. 1). The distances calculated using 1-r Pearson linkage method revealed slightly different grouping of the studied wheat samples. Moreover the use of different linkage approaches did not result only in diverse grouping of the wheat samples, but values of calculated distances were considerably different. It is also noteworthy that this diversity was clearly distinguishable when absolute values of the data were analysed, but also after their standardization through conversion into relative (percentage) values (Table II).

Principal component analysis (PCA) yielded two principal components (P1 and P2) that explained above 41 and 33% of total variance in the data processed. The first principal component was correlated well with saturated resorcinol homo-

Table II. Distance values between the clusters formed calculated using different distance measurement methods[a].

	Clusters of grouped variables[b]							
Distance	I	II	III	IV	V	VI	VII	VIII
Simple Euclidean	3.09	5.14	8.52	9.11	26.95	38.40	44.92	119.53
	(2.59)	(4.30)	(7.13)	(7.62)	(22.55)	(32.13)	(37.58)	(100.00)
Square Euclidean	9.52	28.56	72.36	83.05	726.46	935.85	2179.37	6813.95
	(0.14)	(0.42)	(1.06)	(1.22)	(10.66)	(13.73)	(31.98)	(100.00)
Manhattan	4.94	8.23	11.09	14.26	36.37	47.43	60.62	257.47
	(1.92)	(3.20)	(4.31)	(5.54)	(14.13)	(18.42)	(23.54)	(100.00)
Chebyshev	2.90	4.83	8.12	9.05	26.30	38.05	42.76	69.49
	(4.17)	(6.95)	(11.69)	(13.02)	(37.85)	(54.76)	(61.53)	(100.00)
1-r Pearson	1.03×10^{-5}	3.21×10^{-5}	4.58×10^{-5}	11.19×10^{-5}	58.48×10^{-5}	83.45×10^{-5}	245.25×10^{-5}	6920.51×10^{-5}
	(0.01)	(0.05)	(0.07)	(0.16)	(0.85)	(1.21)	(3.54)	(100.00)

[a] Numbers represent real distances, whereas those in brackets express relative percentage values.

[b] Roman numbers correspond to created clusters: I – *Jasna* '01 and *Jasna* M; II – *Jasna* '00, *Jasna* '01 and *Jasna* M; III – *Jasna* '00, *Jasna* '01, *Jasna* M and *Kobra* '00; IV – *Kobra* '01 and *Kobra* M; V – *Tetradur* '00 and *Tetradur* M; VI – *Kobra* '01, *Kobra* M and *Tetradur* '01; VII – *Kobra* '01, *Kobra* M, *Kobra* '00, *Tetradur* '01, *Tetradur* '00 and *Tetradur* M; VIII – *Jasna* '00, *Jasna* '01, *Jasna* M, *Kobra* '00, *Kobra* '01, *Kobra* M, *Tetradur* '01, *Tetradur* '00 and *Tetradur* M. For 1-r Pearson linkage method, *Jasna* '00 and *Jasna* M were included into I, whereas V grouped *Tetradur* '01 and *Tetradur* M. For Manhattan distances, *Kobra* '01 and *Kobra* M were placed into III. For Chebyshev distance, VI consisted of *Kobra* '01, *Kobra* M and *Tetradur* '01, whereas *Kobra* '01, *Kobra* M, *Tetradur* '01, *Tetradur* '00 and *Tetradur* M were in VII. Abbreviations: M – mean value.

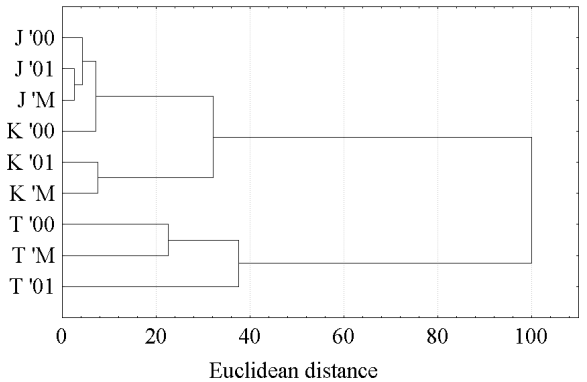

Fig. 1. Grouping of wheat cultivars using cluster analysis (simple Euclidean distances as a distance measure and Ward's amalgamation algorithm) based on total resorcinolic lipid contents and their relative percentage homologue profiles. The scale represents relative (percentage) Euclidean distance values. Abbreviations used: J – *Jasna*; K – *Kobra*; T – *Tetradur*; M – mean value.

logues like $C_{17:0}$, $C_{19:0}$, $C_{21:0}$ and $C_{23:0}$ (0.879653, 0.906917, – 0.905986 and – 0.834594, respectively) (Fig. 2). The second principal component was positively correlated with $C_{15:0}$ (0.929653) as well as with unsaturated $C_{17:1}$, $C_{19:1}$, $C_{21:1}$ (0.822424, 0.775149 and 0.859046, respectively). The homologues $C_{17:0}$, $C_{19:0}$, $C_{21:0}$ and $C_{23:0}$ were negatively correlated due to major differences in their contents in both soft and hard wheat varieties. However PCA is invariant to the mirroring through the origin (Tranter, 2000), so all these four homologues belonged to the same group. Thus, there is no need to evaluate all of these variables to achieve the same level of characterization of objects. Unfortunately, PCA could not be used for differentiation between wheat varieties, because it yielded only one principal component explaining above 94% of total variance in the data (not shown). For that reason, soft and hard wheat varieties could not be resolved using PCA. However, this method also revealed that the minor differences in grain resorcinol compositions between the studied wheat cultivars are sufficient to allow a clear-cut individualization of those wheat species/cultivars. These both resorcinolic lipid-based statistical approaches support chemotaxonomic evidence thus points to different positions of durum and soft wheats within this tribe. What is more important, the results presented above remain in a good agreement with being currently in force an established knowledge of the *Triticum* genus taxonomy. Namely, soft wheats have been classified currently into a separate group, whereas durum has been considered a distinct tetraploid species. The variability of resorcinol contents in soft wheats were, therefore, observed to a lesser extent and their homologue patterns were considerably similar. On the other hand, the species assigned on the basis of genome types to different groups (soft wheats versus durum) had both distinct resorcinolic lipid homologue profiles and unsaturation indices.

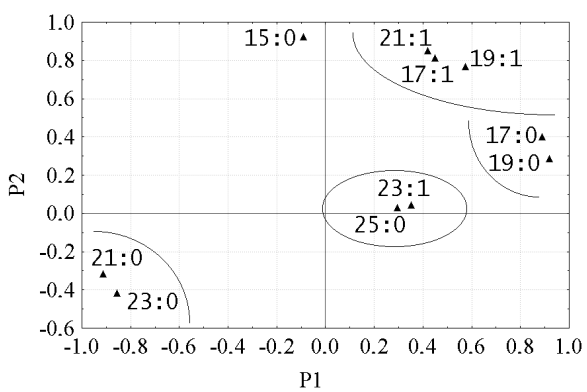

Fig. 2. Grouping of wheat resorcinol homologues using principal component analysis (unrotated principal component loadings) based on calculated principal components P1 and P2. The scale represents real (absolute) values.

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