Carboxylic Acids in Wheat, Rye and Barley

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Hordeum vulgare, *Secale cereale*, *Triticum aestivum*, Gas Chromatography–Mass Spectrometry, Mono-, Di-, and Tricarboxylic Acids

Organic acids from wheat (*Triticum aestivum*), rye (*Secale cereale*), and barley (*Hordeum vulgare*, var. "Gerbel" and "Igri") were analysed by gas chromatography–mass spectrometry. Two carboxylic acid fractions were obtained by chromatography on Sephadex LH 20: fraction A contained mainly aliphatic acids, fraction B contained mainly aromatic acids. The identified compounds (50 aliphatic and 32 aromatic acids) are listed in Tables I and II. Quantitative analysis was achieved by calibration of the gas chromatogram with authentic compounds or – if not available – with closely related compounds. The possibility to identify the origin of cereal food-stuff by analysis of the organic acids is discussed.

Introduction

Plant tissues contain a variety of non-volatile organic acids. Some were well known as intermediates of the primary metabolism. Others which can be attributed to the secondary metabolism may be of chemotaxonomic interest. Some acids are accumulated in large amounts and serve as osmotically active anions in the plant cell. However, far more acids occur only in small amounts or even in traces.

Previous investigations on organic acids in higher plants concentrated upon products of the tricarboxylic acid cycle on the one hand [1–5] and on phenolic carboxylic acids on the other hand [6–9]. Only few data are available on organic acids in cereals, some of the data are contradictory [4].

During our investigations on natural inhibitors of germination and growth [10, 11], we determined several aliphatic and aromatic acids in cereals. Continuing these investigations, we now identified several acids which had not yet been detected in cereals. In this paper, quantitative analysis of the identified compounds in wheat, rye, and barley is described.

Materials and Methods

Extraction of plant material

500 g of dried dehulled seeds from wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), and 200 g of dried seeds from barley (*Hordeum vulgare* L.), var. "Gerbel" and "Igri" were ground in a Waring blendor. The ground material was extracted with water (2 l and 1 l, respectively) for 48 h in a Soxhlet extractor. The extracts (1.5 l and 0.75 l) were adjusted to pH 7 with saturated $NaHCO_3$ and extracted with ether. The ether solution which contained mainly neutral compounds was discarded. The aqueous solution was acidified to pH 1 and extracted with ether in a perforator.

Column chromatography

The dried ether solution was evaporated *in vacuo*, the residue was dissolved in 3 ml of methanol and chromatographed on a Sephadex LH-20 column (Pharmacia), using the following conditions: column: glascolumn 600 × 50 mm; mobile phase: water; elution volume: 1200 ml; flow rate: 30 ml/h; detection: measuring the absorption at 230 nm ("Spectrochrom M, F. Gilson).

Two fractions (A and B) were cut according to the signals on the UV detector, the water was removed by lyophilisation and the lyophilisates were methylated by adding diazomethane in diethylether. After the formation of nitrogen had stopped, the unused diazomethane was removed under reduced pressure. The remaining carboxylic esters were cleaned by destillation at 0.5 torr and 120°.

Gaschromatography – mass spectrometry

The fractions were investigated by GC and GC-MS. The carboxylic acid esters were separated on a Carlo Erba Model 2300 gas chromatograph equipped with a flame ionisation detector. The glascapillary column (25 m) was filled with OV 1. The flow rate of the carrier gas was 2 ml min^{-1} of

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helium. The injection temperature was 275°, the detector temperature was 275°. The oven temperature was programmed at the start of the run from 50 to 240° at 3°/min. The carboxylic acids were identified by comparing the retention times of the methylesters with those of the authentic substances or standard acid methylesters and by GC-MS. GC-MS analyses were performed on Varian Mat 312 mass spectrometer with a combined DI/CI ion source and a Varian Model 3700 gaschromatograph. The mass spectrometer was operated under the following conditions: electron energy: 70 eV; ion source: 220°; scan: 34.5–400 m/z. The conditions for GC were the same as described before.

Quantitative GC analysis of the carboxylic acid esters was performed by calibrating with authentic compounds (A) or related substances (B, see Tables I and II).

Results and Discussion

The procedure described in Materials and Methods is especially useful for analysis of aliphatic carboxylic acids which are concentrated in fraction A of the chromatography on Sephadex LH 20. The gas chromatogram of this fraction from caryopes of wheat, rye or barley shows the presence of about 100 compounds. Some of these are trace compounds which can be detected only at higher sensitivity of the detector. A typical example of such a gas chromatogram is given in Fig. 1 for wheat. Compounds of fraction A which were identified by gas chromatography-mass spectrometry are listed in Table I. The identified compounds of fraction B (aromatic compounds) are listed in Table II except azelaic acid (**60**) and methylaconitic acid (**61**) which are listed in Table I together with the other aliphatic acids although they are found in fraction B of the chromatography on Sephadex LH 20.

An essential step of the separation and identification procedure is the preparation of the methyl esters of the carboxylic acids. Formation of the volatile methyl esters allows their separation from non-volatile contaminants by destillation (see Materials and Methods) and furthermore, is essential for analysis by gas chromatography and mass spectrometry. The method of choice for derivatization is the reaction with diazomethane. This reaction is rapid, its completion can easily be recognized, it can be performed also at micro scale. However, some side reactions of diazomethane have to be considered. Besides carboxylic acid groups, also phenolic hydroxy groups should be methylated. We found that this reaction is incomplete: even after 12 h reaction with diazomethane, free phenols are still present besides phenol methyl ethers. Aliphatic hydroxy groups which should not be methylated can react – at least in traces – to methyl ethers [11]. Methyl ether compounds (*e.g.* **18**) may, therefore, not occur naturally but may be produced by the described procedure. Other artifacts are produced from α,β-unsaturated carboxylic acids. By addition of diazomethane at the double bond, pyrazoline derivatives are formed which can be decomposed during gas chromatography to cyclopropane derivatives and 3-methylalkene carboxylic acid methyl esters [12]. The fractions which contained fumaric and maleic acid dimethylesters (**13** and **14**) also contained the pyrazoline derivative **52**, (*E*) and (*Z*) cyclopropane dicarboxylic acid dimethylesters **21** and **22**, and the methyl derivatives of **13** and **14**, namely fumaric and methyl maleic acid dimethylesters **19** and **20**. The concentration of **13** and **14** given in Table I are the sum of detected amounts of **13**, **14**, **19**, **20**, **21**, and **22**.

Because (*Z*, *E*) isomerization can occur during the derivatization only the total of **13** plus **14** is given.

The previously described occurrence of aconitic acid in cereals [2, 3] could not be confirmed by our analysis. Instead, relatively high concentrations of methylaconitic acid (**51**) were detected in all cereals. **51** was probably derived from aconitic acid by reaction with diazomethane; authentic aconitic acid also yielded **51** under the described conditions.

The main acids found in common in the grains of wheat, rye, and barley are acids of the tricarboxylic acid cycle, namely malic (**17**), citric (**44**), succinic (**12**), and fumaric acid (**13**). The concentrations of these acids are in the same order of magnitude as in previous reports [1, 2, 4]. Contrary to previous results [1, 4], we did not find oxalic acid (**1**) in all cereals but only in rye. Such differences could be due to different conditions for cultivation or different varieties. We found an about 10-fold concentration of most acids in the barley variety "Gerbel" compared with the variety "Igri". Other acids which were found in all cereals are N-methylpiperidine-2-carboxylic acid (**27**), 2-hydroxyglutaric acid (**35**)

Table I. Identification and quantitative determination of aliphatic carboxylic acids from cereals. The acids were methylated with diazomethane and investigated by gas chromatography–mass spectrometry. All values are μmol/g dry weight. A: Identification by comparison with authentic compound in GC (retention time) and MS (fragmentation); calibration in GC with authentic compound. B: Identification by MS (fragmentation); calibration in GC with related compound as indicated in brackets.

		Wheat	Rye	Barley var. Gerbel	Barley var. Igri		
Total aliphatic acids		2115	4264	2745	238		
No.	Compound					Identification	Ref.
1	oxalic acid	–	1.700	–	–	A	[1, 2]
2	2-hydroxy-2-methyl-propanoic acid (α-hydroxy-iso-butyric)	34	0.2	–	–	B(**1**)[a]	[13]
3	2-hydroxy-butanoic acid (α-hydroxy-butyric)	–	2	–	–	B(**5**)[a]	[13]
4	3-hydroxy-butanoic acid (β-hydroxy-butyric)	15	0.3	–	–	B(**5**)[a]	[13]
5	2-hydroxy-3-methyl-butanoic acid (2-hydroxy-iso-valeric)	27	0.3	–	–	A	[13]
6	1,4-dimethylbenzene (*p*-xylene)	+	+	–	–	A	
7	malonic acid	68	227	–	95	A	[1, 2]
8	phosphoric acid				+	B	
9	4-oxopentanoic acid (levulinic)	21	4	–	–	A	[13]
10	2-hydroxy-3-methyl-pentanoic acid	103	274	–	–	B(**84**)[a]	[13]
11	2-hydroxy-hexanoic acid	158	–	4	–	B(**84**)[a]	[13]
12	succinic acid	750	650	466	+	A	[1, 2, 14]
13	fumaric acid						
	+	53	190	75	19	A	[1, 2]
14	maleic acid						
15	2-methyl-butane-dioic acid (methylsuccinic)	3	0.1	0.6	–	A	[14]
16	pentanedioic acid (glutaric)	38	188	–	4.4	A	
17	2-hydroxybutane-dioic acid (malic)	130	154	926	40	A	[1, 2]
18	2-methoxybutane-dioic acid	28.5	20	170	–	B(**17**)[a]	[14]
19	methylbutene-dioic acid (E)	1.5	1.5	–	+	B(**15**)[a]	[12]
20	methylbutene-dioic acid (Z)	+	–	1.3	–	B(**15**)[a]	[12]
21	cyclopropane-1,2-dicarboxylic acid (E)	1.5	1.5			B(**15**)[a]	[12]
22	cyclopropane-1,2-dicarboxylic acid (Z)	1.5	1.5	13	3	B(**15**)[a]	[12]
23	2,3-pentene-dioic acid (E)	1	–	0.6	0.6	B(**16**)[a]	[14]
24	3-methyl-2,3-pentene-dioic acid (E)	–	–	5	+	B(**85**)[a]	[12]
25	3-methyl-2,3-pentene-dioic acid (Z)	–	–	5	–	B(**85**)[a]	[12]
26	2,3-hexenedioic acid	–	3	–	–	B(**86**)[a]	[15]
27	*n*-methyl-piperidine-2-carboxylic acid (*n*-methyl-pipecolic)	3.2	5.3	1.3	0.45	A	
28	cyclobutane-1,2-dicarboxylic acid	1.2	+	23	9	A	[16]
29	cyclobutene-1,2-dicarboxylic acid	2.3	–	–	–	B(**28**)[a]	
30	2-furoic acid	8	–	–	–	A	[17, 18]
31	5-hydroxymethyl-2-furoic acid	6.5	–	–	–	B(**87**)[a]	[17]
32	furan-2,4-dicarboxylic acid	2.2	–	–	–	B(**87**)[a]	[18]
33	2-oxo-tetrahydro-pyranyl-6-carboxylic acid	+				B	[19]
34	2-oxo-tetrahydro-furyl-5-carboxylic acid	21	14	6.3	0.7	B(**17**)[a]	[19]
35	2-hydroxypentane-dioic acid	20	11	23	1.7	B(**17**)[a]	[19]
36	3-hydroxy-3-methyl-pentane-dioic acid (β-hydroxy-β-methyl-glutaric)	195	416	132	11	A	[22]
37	2-isopropyl-2-hydroxy-butane-dioic acid (2-isopropyl-malic)	1.5	–	3	0.5	B(**17**)[a]	[22]
38	3-oxo-hexanedioic acid (3-oxo adipic)	11	–	213	22	A	[19]
39	methionine	10	+	–	–	A	
40	*n*-acetyl-leucine	–	–	15	–	A	
41	2-pyrrolidone-5-carboxylic acid (pyroglutamic)	119	133	98	–	A	
42	4-oxo-octanedioic acid	–	–	–	1.4	B(**38**)[a]	[19]

Table I. (continued).

		Wheat	Rye	Barley var. Gerbel	Barley var. Igri		
Total aliphatic acids		2115	4264	2745	238		
No.	Compound					Identification	Ref.
43	1,2,3-propane-tricarboxylic acid	–	–	30	–	A	
44	2-hydroxy-1,2,3-propanetricarboxylic acid (citric)	103	180	402	17.1	A	
45	1-hydroxy-1,2,3-propanetricarboxylic acid (isocitric)	–	–	17	–	A	[15]
	isomer of 45	–	–	8	–		
46	2-methoxy-1,2,3-propanetricarboxylic acid	10	15	20	1.6	B (**44**) [a]	47
47	1,3,5-pentanetricarboxylic acid	–	–	8	–	A	
48	1,3,4-pentanetricarboxylic acid	1.6	–	24	–	A	[11]
	(dihydrohaematinic)	1.6	–	24	–	A	[11]
49	heptanedioic acid (pimelic)	2.7	–	–	–	A	
50	nonanedioic acid (azelaic)	37	7.5	14	2.3	A	
51	methyl-1,2,3-propenetricarboxylic acid (methyl-aconitic) (cis/trans)	126	65	41	8	A	

[a] Reference compounds for GC:
84 2-hydroxy-4-methylpentanoic acid.
85 3-methyl-hexanedioic acid.
86 hexanedioic acid.
87 furan-2,5-dicarboxylic acid.

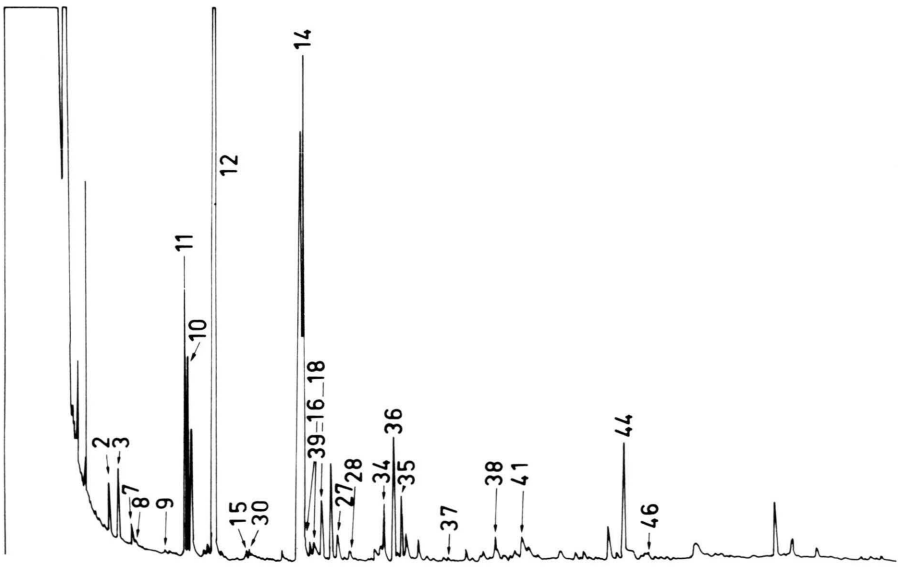

Fig. 1. Gas chromatogram of fraction A (aliphatic carboxylic acid methyl esters) from wheat. Glass capillary column, OV 1, carrier gas He 2 ml · min^{-1}. Injector at 275 °C, FID, detector at 275 °C, column from 50 to 240 °C at 3 °/min. Numbers of compounds correspond to those in Table I.

Table II. Identification and quantitative determination of free aromatic carboxylic acids from cereals. The acids were methylated with diazomethane and investigated by gas chromatography – mass spectrometry. All values are µmol/g dry weight. A: Identification by comparison with authentic compound in GC (retention time) and MS (fragmentation); calibration in GC with authentic compound. B: Identification by MS (fragmentation); calibration in GC with related compound as indicated in brackets.

		Wheat	Rye	Barley var. Gerbel	Barley var. Igri		
Total aromatic acids		102	167	62	10		
No.	Compound					Identification	Ref.
52	3,4-dicarbomethoxy-Δ^2-pyrazoline	(+)[b]	(+)[b]	(+)[b]	(+)[b]	A	[12]
53	3,4-dimethoxy-N-methyl-pyrazole	(+)[b]	(+)[b]	(+)[b]	–	A	[12]
54	3-methoxybenzaldehyde	–	–	–	0.03	B(**55**)[a]	[13]
55	3-methoxybenzoic acid	–	–	–	0.4	A	[13]
56	4-hydroxy-3-methoxybenzoic acid (vanillic)	–	–	–	2.5	A	
57	3,4-dimethoxybenzaldehyde (veratrumaldehyde)	–	–	–	0.03	B(**58**)[a]	
58	3,4-dimethoxybenzoic acid	–	–	–	0.8	A	
59	3,4,5-trimethoxybenzoic acid	–	–	–	1.3	A	
60	3,4,5-trimethoxybenzaldehyde	–	–	–	0.05	A	
61	3,4-dimethoxyacetophenone	–	–	–	0.03	A	[11]
62	1-propyl-3,4-dimethoxybenzene	–	–	–	0.01	A	
63	isobutyl-dimethoxybenzene	–	–	–	0.01	B(**62**)[a]	[11]
64	4-hydroxyphenylacetic acid	–	–	9	–	A	
65	2-hydroxy-(2-phenyl-) acetic acid	0.1	–	–	–	B(**64**)[a]	
66	4-methoxy-phenylacetic acid	–	–	2	2.8	A	
67	3-methoxy-phenylacetic acid	2.8	–	–	–	B(**66**)[a]	
68	4-hydroxy-3-methoxy-phenylacetic acid (homovanillic)	–	–	0.5	0.15	A	
69	3,4-dimethoxyphenylacetic acid	0.5	–	0.1	–	A	
70	3,4,5-trimethoxyphenylacetic acid	1.3	–	–	–	B(**59**)[a]	
71	4-hydroxy-3-methoxyphenyl-propionic acid	–	–	0.3	1	B(**88**)[a]	
72	3,4-dimethoxyphenylpropionic acid	–	–	0.2	–	B(**89**)[a]	
73	3,4-dimethoxycinnamic acid (sinapic)	–	–	+	–	A	
74	2-amino-N-acetylbenzoic acid (N-acetyl-anthranilic)	–	–	3.5	–	A	
75	2-hydroxy-3-(phenyl)-propionic acid (phenyllactic)	72	143	–	–	A	
76	2-hydroxy-3-(4-hydroxyphenyl)-propionic acid (*p*-hydroxy-phenyl-lactic)	2	–	–	–	B(**75**)[a]	
77	2-hydroxy-3-(4-methoxyphenyl)-propionic acid (*p*-methoxy-phenyl-lactic)	10	24	–	1	B(**75**)[a]	
78	2-hydroxy-3-(3,4-dimethoxy-phenyl)-propionic acid (3,4-dimethoxyphenyl-lactic)	2	–	–	–	B(**75**)[a]	
79	2-methoxy-3-(4-methoxyphenyl)-propionic acid	2	–	–	–	B(**89**)[a]	
80	2-hydroxy-2-phenylacetic acid (mandelic)	9	–	–	–	B(**64**)[a]	
81	3-indolylacetic acid	–	–	–	0.13	A	[20]
82	3-(3-indolyl)-2-hydroxypropionic acid (indolyllacetic acid)	–	–	42	–	A	[20]
83	3-(3-indolyl)-2-methoxypropionic acid	–	–	4.3	–	B(**82**)[a]	[20]

[a] Reference compounds for GC:
88 3-(4-hydroxyphenyl)-propionic acid.
89 3-(4-methoxyphenyl)-propionic acid.
[b] Derivatives of **13** and **14**; values already contained in the sum **13** + **14**, see Table I.

and its lactone **34**, 3-hydroxy-3-methylglutaric acid (**36**), and pyrrolidone-5-carboxylic acid (**41**). **41** can easily be formed from glutamate, *e.g.* during chromatography on silica gel [4]. Its natural occurrence is, therefore, doubtful. The occurrence of **27**, **34**, and **36** in Gramineae has not yet been described to our knowledge.

Characteristic for *wheat* are some cyclic compounds (cyclobutene, furan, pyran carboxylic acids, see below) and volatile hydroxy monocarboxylic acids. The following compounds were identified: 2-hydroxy-2-methylpropionic acid (**2**), 3-hydroxybutric acid (**4**), 2-hydroxyisovaleric acid (**5**), 2-hydroxycaproic acid (**10**), and 2-hydroxy-3-methylvaleric acid (**11**). These compounds are found in wheat in relatively high concentrations (20–160 µmol). They are not present in barley but can be detected in rye after concentration of the volatile fraction. The concentration range in rye is 0.2–0.3 µmol except **10** which is present in much higher concentration (274 µmol). Rye contains also 2-hydroxybutyric acid (**3**) which was not found in wheat.

The cyclic compounds of wheat include cyclobutene-1,2-dicarboxylic acid (**29**). The saturated analogue **28** occurs not only in wheat (1,2 µmol) but also in barley in even larger amounts (9–23 µmol). Furan derivatives were only detected in wheat. Furoic acid (**30**) and 5-hydroxymethyl-furoic acid (**31**) may be derived from pentose or hexose by acid treatment. This is not the case for compound **32** which was identified as furan-2,4-dicarboxylic acid by its mass spectrum (Fig. 2). Another cyclic compound which was detected only in wheat was the γ-lactonecarboxylic acid **33**.

Characteristic for *rye* seems to be the hydroxymonocarboxylic acid **3** as mentioned above. On the other hand, the compounds **37**, **38**, and **48** which were found in wheat, barley (see Table I) and in oat [11] were not present in rye.

Of the two analysed varieties of *barley*, var. "Gerbel" contained more acids (total acids 2 mmol) than var. "Igri" (total acids 0.2 mmol). This difference can vary for individual acids: malonic (**7**) and glutaric acid (**16**) were detected in var. "Igri" but not in var. "Gerbel". Characteristic for barley seem to be methylmaleic acid (**20**) and (*E*) and (*Z*) 3-methyl-2,3-pentenedioic acids **24** and **25**, furthermore, the tricarboxylic acids **43**, **47** and two isomers of isocitric acid **45**. The tricarboxylic acid **48** was

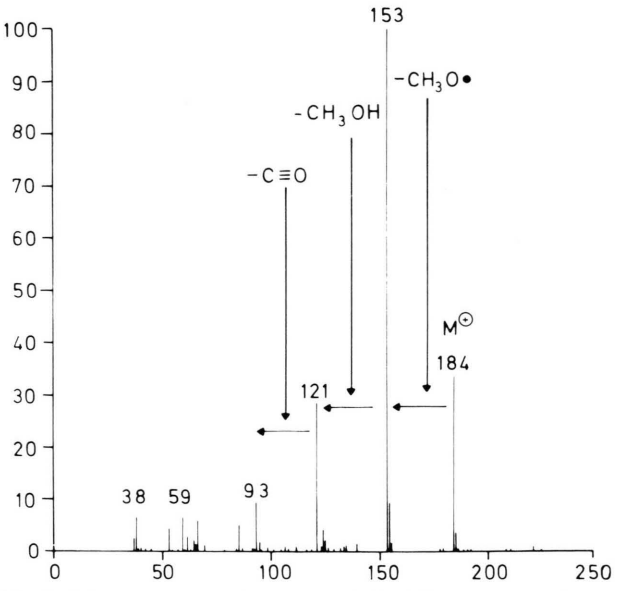

Fig. 2. Mass spectrum of compound **32**. The spectrum is identical with that of authentic furan-2,4-dicarboxylic acid dimethylester (G. Spiteller, personal communication) but different from that of furan-2,5-dicarboxylic acid dimethylester. Characteristic for **32** are the fragments *m/e* 121 for which a bis-ketene structure can be ascribed and *m/e* 93. The spectrum of the 2,5 isomer **87** which cannot form a bis-ketene structure, does not contain the fragments *m/e* 121 and *m/e* 93 but only shows a fragment *m/e* 95.

not only found in barley var. "Gerbel" but also in wheat (Table I) and oat [11]. It had previously been identified as germination inhibitor [11].

Fraction B of the chromatography on Sephadex LH 20 contained mainly aromatic compounds, especially phenolic acids. The identified compounds are compiled in Table II. According to the excitation procedure, only free acids were investigated. Many of the phenolic carboxylic acids, *e.g.* ferulic acid, occur mainly in a bound form (60% in oat flour, 84% in wheat flour [8, 9]). This fraction is not investigated here. The free phenolic acids make up only 1/30 to 1/20 of the aliphatic acids.

Characteristic patters for the investigated cereals can also be observed for the aromatic acids. *Wheat* contains a variety of phenylacetic and phenyllactic acid derivatives. Phenyllactic acid (**75**) which acacounts for 75% of the acids of fraction B in wheat and its *p*-methoxy derivative **77** occur not only in wheat but also in rye. The phenyllactic acid derivatives **76**, **78**, **79**, and **80** were only detected in wheat. The phenylacetic acid derivatives **65**, **67**, and **70** are characteristic for wheat. **69** was detected in

wheat and barley. In *rye*, no other aromatic acids were detected besides **75** and **77**. The concentration of these two acids is higher in rye than in any other cereal.

Barley var. "Gerbel" contains a variety of phenylacetic acid derivatives **64**, **66**, **68**, **69**, the phenylpropionic acids **71** and **72**, and N-acetylanthranilic acid (**74**). No phenyllactic acid derivatives were detected. Instead, indolyllactic acid (**82**) is present in relatively high concentration. Its methyl ether **83** is probably formed by the reaction with diazomethane (see above). Barley var. "Igri" contains the phenylacetic acid derivatives **66**, **68**, and **71** (as var. "Gerbel"). In accordance with var. "Gerbel", no phenyllactic acid derivatives were detected. In addition, a variety of benzoic acid and benzaldehyde derivatives (**54**–**60**), dimethoxyacetophenone (**61**), and alkyldimethoxybenzenes (**62** and **63**) were found in var. "Igri". It is remarkable that these derivatives are substituted in position 3 throughout which is rare otherwise. Compounds **61** and **63** had previously been isolated from oat. They have activity as germination inhibitors [11].

Only few of the compounds described in this paper have been detected in cereals before. The aliphatic acids **1**, **7**, **12**, **13**, **17**, **44**, **45**, and **51** were described in wheat, barley, and rye [1, 2]. Sosulski *et al.* [8] identified azelaic acid (**50**) and the aromatic compounds **56**, **59**, **64**, and **72** in flour of wheat, oat, corn and rice.

In summary, number and variety of compounds identified in this paper are large enough to distinguish between the various cereals from which they are extracted. The results with barley point to the possibility that even varieties of one species can be recognized by analysis of their acids but further experiments are needed to establish this possibility. The prospects of such an analysis are manifold. Since the investigated acids are chemically stable compounds it can be expected that the typical patterns persist for a long time. Identification of foodstuff in archeology, i.a. makes use of analysis of fatty acids [23]. Fatty acids are about equally stable as the acids investigated here but the variety of our acids is larger than that of fatty acids. It should be mentioned that many of the compounds detected here in cereals have previously been found in human urine [21, 22]. It is possible that these compounds have been derived from ingested food and are excreted unchanged. This would point to a certain metabolic inertia of these compounds which would even improve the meaning of their analysis.

Acknowledgements

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