PHENOLIC ACIDS IN GRAINS OF WILD-TYPE BARLEY AND PROANTHOCYANIDIN-FREE MUTANTS

by

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The phenolic acid composition in grains of wild-type barley and proanthocyanidin-free mutants in different *ant* genes was studied. No qualitative differences were detected between *ant* mutants and their wild-types. Quantitative differences in phenolic acid content were small. The sum of the six most abundant phenolic acids varied between 5 and 12 mg · 100 g⁻¹ of barley (dry weight). Compared to proanthocyanidins and catechin in wild-type barley, the amount of acetone extractable phenolic acids is small, and the presence of simple phenolic acids in proanthocyanidin-free mutants of the genes *ant* 17 and *ant* 18 cannot explain the high polyphenol values measured according to the method of Jerumanis in beers produced from these mutants. Tests according to Jerumanis of fractions obtained from hydrolysed barley grain extracts by HPLC, revealed that the highest polyphenol values were obtained in fractions containing compounds related to flavones. These compounds are more abundant in mutants of the genes *ant* 17 and 18 than in mutants of the gene *ant* 13.

1. INTRODUCTION

Barley grains contain a diversity of phenolic compounds, but until now most studies of these substances have concentrated on the proanthocyanidins, because of their important role in the formation of beer haze (2, 6, 11, 14, 18). Procyanidins and prodelphinidins, often referred to as polyphenols, were rather early recognized as major phenolic components of beer haze (16). The proanthocyanidins have been considered essential for beer flavour and taste (3), but in recent experiments trained taste panels have not been able to distinguish beer brewed with malt of proanthocyanidin-free mutants from proanthocyanidin containing reference beer (5, 7, 8).

Polyphenol content in beer and wort is often determined according to the procedure of Jeru-

manis (1), in which compounds having an o-diphenol structure give rise to a red-brown coloured complex with ferric ions in alkaline solution, but barley grains contain a number of phenolic compounds with other substitution patterns, which do not give a colour reaction under these conditions. In beers made from the proanthocyanidin-free mutants *ant* 17-105, *ant* 18-102 and *ant* 18-111 50-80% of the compounds reacting with ferric ions in the reference beer are present, while beer brewed from *ant* 13-13 barley contains only 15% of these substances which obviously are neither proanthocyanidins nor catechins (23, 24).

Diallelic crosses have identified six *ant* genes in barley, which upon mutation can block the synthesis of catechin and proanthocyanidin

Abbreviations: CMC = carboxymethylcellulose; DHQ = dihydroquercetin; EDTA = ethylenediamine tetraacetate; HPLC = high performance liquid chromatography; TLC = thin layer chromatography.

Benzoic acid derivatives

p-Hydroxybenzoic acid $R=R'=H$

Protocatechuic acid $R=OH, R'=H$

Vanillic acid $R=OCH_3, R'=H$

Gallic acid $R=R'=OH$

Syringic acid $R=R'=OCH_3$

Gentisic acid $R=OH, R'=H$

Cinnamic acid derivatives

p-Coumaric acid $R=R'=H$

Caffeic acid $R=OH, R'=H$

Ferulic acid $R=OCH_3, R'=H$

Sinapic acid $R=R'=OCH_3$

Chlorogenic acid

Figure 1. Structures of phenolic acids.

(12). Grains of wild-type barley as well as proanthocyanidin-free mutants in the different *ant* genes contain complex mixtures of phenolic compounds, such as phenolic acids and flavonoids. Mutants in the genes *ant* 17, 18 and 22 accumulate intermediates in the biosynthetic pathway leading to the proanthocyanidins (15, 24).

Phenolic acids and flavonoids are widespread in the plant kingdom. Except for proanthocyanidins and catechins they seldom occur in free form but usually as glucosides or esters. The hydroxycinnamic acids are important precursors in the biosynthesis of flavonoids and lignins, and some of the phenolic carboxylic acids are present in lignin as esters (10). The chemical

structures of different phenolic acids are shown in Figure 1.

In the present study, the phenolic acid composition in grains of proanthocyanidin-free mutants and their mother varieties was investigated as well as various intermediates and sideproducts of the flavonoid pathway. In order to find the explanation for the surprisingly high polyphenol values obtained with Jerumanis procedure on beers brewed from certain proanthocyanidin-free barley mutants, mutant and wild-type barley grain extracts were separated by high performance liquid chromatography (HPLC) and the fractions tested for their reaction with ferric ions in alkaline solution.

2. MATERIALS AND METHODS

2.1. Plant material

Phenolic acids and flavonoids were analysed in grains of the three wild-type spring barley varieties Nordal, Triumph and Georgie, the anthocyanin-free spring barley variety Alva and the winter barley variety Tipper.

The following proanthocyanidin-free mutants were investigated: *ant* 13-13 (induced in Foma), *ant* 13-226 (induced in Secobra 18193), *ant* 13-101, *ant* 17-104, *ant* 17-142, *ant* 18-102, *ant* 18-141 (induced in Nordal), *ant* 13-152, *ant* 17-148 (= Galant), *ant* 17-151, *ant* 18-186 (induced in Triumph), *ant* 17-192, *ant* 18-196, *ant* 21-194 (induced in Georgie), *ant* 18-234 (induced in Tron), *ant* 19-109 (induced in Alf), *ant* 21-1507 (induced in Amagi-Nijo) and *ant* 22-1508 (induced in Haruna-Nijo).

2.2. Chemicals

All phenolic acid standards and (+)-catechin were obtained from Fluka AG (Buchs, Switzerland). Naringenin was from Sigma (St Louis, USA). Dihydroquercetin was purchased from Serva (Heidelberg, West Germany). Carboxymethylcellulose, sodium salt, low viscosity grade was obtained from BDH Chemicals Ltd (Poole, England).

2.3. Extraction procedures

Barley grains (10 g) were ground into a powder by a Brown Type Mx 32 homogenizer. The freshly ground flour was shaken with 100 ml 75% (v/v) acetone for 1 hr. The acetone was filtered off on a Büchner funnel and the barley flour reextracted twice. The extracts were com-

Table I

Average elution volumes with HPLC and R_f values with TLC of phenolic acids and flavonoids. UV fluorescence after NH_3 treatment and colours after spraying with ethanolic FeCl_3 are also listed. (CAW = chloroform-acetic acid-water; BzAW = benzene-acetic acid-water).

Compound	Elution volume (ml)	CAW	R_f value BzAW	CH_3COOH	UV fluorescence	Colour with ethanolic FeCl_3
gallic acid	13.50	0.17	0.10	0.53	purple	greyish black
protocatechuic acid	16.70	0.44	0.34	0.58	purple	greyish black
gentisic acid	21.20	0.60	0.48	0.76	turquoise	purple
p-hydroxybenzoic acid	21.20	0.77	0.74	0.71	purple	orange
chlorogenic acid	22.70	0.20	0.03	0.72	yellow-green	blue-grey
vanillic acid	24.30	0.89	0.89	0.68	purple	brown
caffeic acid	25.60	0.48	0.32	0.34	yellow-green	blue-grey
syringic acid	27.30	0.91	0.89	0.63	purple	purple
p-coumaric acid	37.40	0.82	0.78	0.54	purple	brownish orange
ferulic acid	42.40	0.90	0.89	0.50	blue	greyish brown
sinapic acid	44.90	0.92	0.85	0.42	turquoise	greyish purple
o-coumaric acid	47.40	0.85	0.79	0.58	orange	yellow
cinnamic acid	58.40	0.94	0.96	0.64	dark purple	bright yellow
(+)-catechin	17.20	0.11	0.05	0.51	dark purple	grey
dihydroquercetin	38.80	0.33	0.11	0.51	greenish purple	greyish purple
naringenin	66.00	0.79	0.78	0.35	purple	brownish pink
(standard)						
naringenin related compound in <i>ant</i> 17 mutants	66.00	0.89	0.88	0.35	purple	brownish pink

Figure 2. Chromatogram (HPLC) of 16 phenolic standards. 1. gallic acid, 2. protocatechuic acid, 3. (+)-catechin, 4. gentisic acid + p-hydroxybenzoic acid, 5. chlorogenic acid, 6. vanillic acid, 7. caffeic acid, 8. syringic acid, 9. p-coumaric acid, 10. dihydroquercetin, 11. ferulic acid, 12. sinapic acid, 13. o-coumaric acid, 14. cinnamic acid, 15. naringenin. 0.25 μ g of each standard was injected.

bined and the acetone evaporated in vacuo. The water phase was delipidated with 3×50 ml of petroleum benzin. To increase the yield of free phenolic acids, the water phase was subjected to either acidic or alkaline hydrolysis, as unhydrolysed extracts contain only a small amount of phenolic acids compared to hydrolysed ones. Acidic hydrolysis was performed in 2 N-HCl at 100 °C for 20 min. Alkaline hydrolysis was done in 1 M-NaOH with N_2 bubbling at room temperature over night. Afterwards the pH was adjusted to 2.0. The phenolic compounds were extracted from the hydrolysed extracts with 3×40 ml ethylacetate, evaporated to dryness in vacuo and dissolved in 4 ml 75% (v/v) ethanol. After centrifugation at $9000 \times g$ for 15 min, the samples were analysed by HPLC.

2.4. Chromatography

2.4.1. High performance liquid chromatography (HPLC)

Analyses were performed using a Waters Assoc. (Milford, Mass., USA) instrument composed of two Model 6000A solvent delivery systems, a WISP Model 710B sample injector, a

Model 450 variable wavelength detector, a data module and a Model 720 system controller. The separations were carried out on a μ -Bondapak C_{18} Column, 30cm $\times$ 7.9 mm (Waters Assoc.), which was eluted isocratically for 10 min with solvent A (methanol:acetic acid:water, 20:5:75, v/v/v), then with a linear gradient to solvent B (methanol:acetic acid:water, 40:5:55, v/v/v) for 10 min and maintained at solvent B for 20 min. The flow rate was $2 \text{ ml} \cdot \text{min}^{-1}$ and the elution was monitored spectrophotometrically at 280 nm. Elution volumes for the different phenolic acids and flavonoids are given in Table I. A mixture of 16 standard compounds was used to identify the peaks on the chromatograms (Figure 2). In order to quantify the amounts of phenolic compounds present in grains of the mutants and their mother varieties, known amounts of standards were analysed by HPLC. The reproducibility of the peak areas was within $\pm 2\%$. Each standard was injected in 3-4 different concentrations. A linear relationship between peak area and concentration was observed for all standards (Figure 3). The standard curves were used to calculate the amounts of different phenolics on HPLC chromatograms of mutants and wild-types.

Figure 3. Standard curves for HPLC of selected phenolic compounds. 1. cinnamic acid, 2. dihydroquercetin, 3. syringic acid, 4. gallic acid, 5. (+)-catechin, 6. p-hydroxybenzoic acid.

Figure 4. Preparative HPLC analysis of hydrolysed extracts of *ant* 18-102. 11 fractions were collected and tested for their reaction with ferric ions in alkaline solution according to Jerumanis.

2.5. The Jerumanis test

Jerumanis method for measurement of polyphenol content (1, 13), is frequently used for measurement of polyphenols in beer and wort. A microscale version of this procedure has been

2.4.2. Thin layer chromatography (TLC)

TLC was used to confirm the identity of phenolic compounds separated by HPLC. Fractions, eluted from the HPLC column, were evaporated to dryness in vacuo, dissolved in 1 ml 75% ethanol (v/v) and chromatographed together with standard compounds. Separations were performed on Merck (Darmstadt, West Germany) precoated cellulose F_{254} plates (0.1 mm).

The following solvent systems were used:

- (i) CAW (chloroform:acetic acid:water, 10:9:1, v/v/v).
- (ii) BzAW (benzene:acetic acid:water, 125:72:3, v/v/v).
- (iii) 15% acetic acid (v/v).

The phenolic compounds were visualized under UV light before and after treatment with NH_3 fumes, or by spraying with ethanolic FeCl_2 (21), which produces different colours with the different phenolic acids. R_f values, UV fluorescence and colours with FeCl_2 are shown in Table I.

Figure 5. Standard curves of selected phenolic compounds, tested for their reaction with ferric ions according to Jerumanis. 1. gallic acid, 2. protocatechuic acid, 3. chlorogenic acid, 4. (+)-catechin, 5. dihydroquercetin.

Figures 6-13. HPLC analyses of acetone extractable phenolic compounds in hydrolysed extracts of proanthocyanidin-free mutants in different genes and the variety Triumph. Numbered peaks are: 4. gentisic + p-hydroxybenzoic acids, 5. chlorogenic acid, 6. vanillic acid, 7. caffeic acid, 8. syringic acid, 9. p-coumaric acid, 10. dihydroquercetin, 11. ferulic acid, 12. sinapic acid, 15. naringenin related compound.

used to test barley extract fractions eluted from the HPLC column. As the method of Jerumanis is very insensitive compared to HPLC analysis, it was necessary to use combined fractions from several separations of the same barley sample. Usually eleven fractions (cf. Figure 4) of around 3 – 8 ml were collected from six independent HPLC separations, and the corresponding fractions were combined and concentrated in vacuo, and the volume was adjusted to 1.6 ml with 75% (v/v) ethanol.

1.6 ml of barley sample was mixed in a cuvette with 1.3 ml of 1% (w/v) sodium carboxymethyl-cellulose with the addition of 0.2% (w/v) EDTA (CMC/EDTA). 0.1 ml of green ammonium ferric citrate (3.5%, w/v) and 0.1 ml of NH_3 ($\text{NH}_3\cdot\text{H}_2\text{O}$, 1:2, v/v) were added. The cuvette was shaken, and after 10 min the absorbance was measured on a Zeiss PMQ 3 spectrophotometer at 525 nm. A mixture of 1.6 ml 75% ethanol, 1.3 ml CMC/EDTA, 0.1 ml ammonium ferric citrate and 0.1 ml NH_3 was used as a blank.

All phenolic standards were tested with the procedure of Jerumanis and standard curves were made of the o-diphenols. The optical density (OD) was proportional to the amount of all standards containing adjacent hydroxyl groups, but there was a large difference in absorption at 525 nm among the different phenolic acids and flavonoids (Figure 5).

3. RESULTS

3.1. Phenolic acids in barley grains

Phenolic acids were identified by comparison of HPLC retention times with those of authentic compounds, and by TLC co-chromatography in three different solvent systems of standards and concentrated fractions eluted from the HPLC column. (Table I). There are no qualitative differences in the array of identified phenolic acids present in the grains of wild-type barley and proanthocyanidin-free mutants in the 6 different genes (Figures 6 – 13).

The following phenolic acids have been identified in acetone extracts after acidic hydrolysis: gentisic acid, p-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, syringic

acid, p-coumaric acid, ferulic acid, sinapic acid and cinnamic acid.

In barley extracts after alkaline hydrolysis the following phenolic acids were found: gallic acid, protocatechuic acid, gentisic acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid and sinapic acid.

Gallic, gentisic and cinnamic acids occur only in trace amounts. The others are more abundant. Gallic acid and protocatechuic acid are the most polar ones of the phenolic acids and are therefore eluted shortly after the void volume on a reversed phase HPLC column. The acid hydrolysed extracts contain contaminating material, which is eluted at void volume, and good separation of the more polar phenolic acids cannot be obtained. However, TLC analyses of these first fractions did not identify gallic and protocatechuic acid either. Caffeic acid and chlorogenic acid are probably broken down in alkaline solution. In some extracts after alkaline hydrolysis trace amounts of caffeic acid are found, but chlorogenic acid has not been identified.

Unhydrolysed extracts contained only small amounts of phenolic acids, indicating that they usually occur in bound form as glucosides or esters. These compounds are not very easily extracted into ethylacetate but remain in the water phase. After hydrolysis the phenolic acids are extracted into the ethylacetate, but the most polar ones are not transferred quantitatively.

In order to investigate the efficiency of the extraction procedure, barley flour samples of *ant* 17-148 (Galant) were extracted four times with 100 ml 75% acetone. Each extraction step was analysed by HPLC, and the three first extractions were found to contain an average of 81, 14 and 4% of the acetone extractable phenolic acids. After four times extraction the barley flour samples were hydrolysed with 100 ml of 2 N-HCl at 100 °C for 30 min, and the hydrolysate reextracted. HPLC analyses of the latter extracts revealed that a large fraction of some of the phenolic acids present in barley grains cannot be extracted with acetone. Only about 10-15% of ferulic and p-coumaric acids are acetone extractable. The rest is probably bound to lignin and cellulose in the cell walls. Direct acidic hydrolysis of barley flour of Triumph and *ant* 17-148

yielded about the same amounts of vanillic, caffeic, syringic and sinapic acids as acetone extraction, but the amounts of p-coumaric and ferulic acids were ten times higher. A considerable amount of gallic acid was also obtained with this method.

3.2. Flavonoids in barley grains

Proanthocyanidins and (+)-catechin are the most abundant phenolic compounds in wild-type barley grains. Acidic hydrolysis will degrade the proanthocyanidins with the formation of anthocyanidins and catechin. The conversion is not quantitative, and it appears that catechin is further degraded during hydrolysis, so the amounts of the compound can be greatly underestimated. A sample of unhydrolysed extract of the barley variety Nortal contained 26.9 mg (+)-catechin $\cdot$ 100 g⁻¹ dry weight according to HPLC analysis. The amount of (+)-catechin in hydrolysed extracts could not be determined by HPLC due to the large UV-absorbing peak at the void volume, but analysis by TLC revealed that only trace amounts of (+)-catechin were present, indicating that the acidic hydrolysis had destroyed a large part of the compound. Dihydroquercetin is found in mutants in the gene *ant* 18, but only in extracts subjected to acidic hydrolysis. All mutants in the gene *ant* 17 accumulate a compound, which seems to be related to naringenin. The HPLC retention time is identical to standard naringenin, but TLC analysis of the fraction containing the peak reveals three spots. The R_f value of the main spot differs from the standard in two of the three solvent systems used (Table I). λ -max. of this compound in 96% ethanol is 289 nm, which is identical to standard naringenin. The same compound is found in a mutant in the gene *ant* 22.

The late eluting part of the HPLC chromatograms of barley grain extracts contain a number of small, still unidentified peaks. TLC analyses show, that fractions corresponding to these peaks have R_f values and UV fluorescence properties characteristic of flavones. The amounts of these compounds are lower in *ant* 13 mutants than in mutants in the genes *ant* 17 and *ant* 18 and in the mother varieties.

3.3. Quantitative estimations of phenolic compounds in barley grains

The amounts of the most abundant phenolic acids, and of dihydroquercetin and the naringenin related compound are presented in Table II for wild-type barley and proanthocyanidin-free mutants in 6 different genes. p-Hydroxybenzoic acid is also one of the more important phenolic acids in barley grains, but it is not separated from gentisic acid by HPLC with the methanol:acetic acid:water solvent system used in the present study. When p-hydroxybenzoic acid and gentisic acid are injected into the HPLC column as single standards their retention times differ by about 10 sec, but when injected together in a standard mixture or a barley sample, they elute as one peak (Figure 2). Change of methanol concentration and gradient did not improve the separation.

The sum of the six common phenolic acids in barley grains varied between 5-12mg $\cdot$ 100g⁻¹ of barley (dry weight). The mother varieties contained similar quantities of phenolic acids as most of the mutants. Mutants in the gene *ant* 18 have a somewhat higher amount of phenolic acids than mutants in the other genes and the mother varieties. With the exception of the *ant* 18 mutants, the variation in phenolic acid content among mutants in the same gene seems to be as large as that among mutants in different genes, and there is a similar variation in phenolic acid content in the same mutant from year to year.

Mutants in the gene *ant* 21 accumulate a variable but significantly higher quantity of caffeic acid than mutants in the other genes and wild-types. A sample of *ant* 21-194, analysed in the spring of 1984 contained 5.2mg caffeic acid $\cdot$ 100g⁻¹ of barley flour (24), whereas another sample of the same mutant analysed in the autumn 1984 contained a much lower amount of caffeic acid, namely 1.77mg $\cdot$ 100g⁻¹ barley flour. Another mutant in the same gene, *ant* 21-1507, analysed at the same time, contained 1.89mg caffeic acid $\cdot$ 100g⁻¹ (Table II). A similar big variation is also recorded in the amount of the naringenin related compound accumulated in samples of *ant* 22-1508 from different years.

The quantities of different phenolic compounds vary also according to the method of

Table II

Major phenolic compounds in acetone extracts of wild-type barley grains and proanthocyanidin-free mutants in different genes as mg per 100 g dry weight. 1 = Extract subjected to acidic hydrolysis. 2 = Extract subjected to alkaline hydrolysis. DHQ = dihydroquercetin. The amount of the naringenin related compound was calculated as naringenin.

		Vanillic acid	Caffeic acid	Syringic acid	p-Coumaric acid	Ferulic acid	Sinapic acid	DHQ	Naringenin related compound	Total sum of 6 acids
Triumph	1	1.76	0.91	1.53	0.72	1.69	1.53	-	-	8.14
Triumph	2	1.90	-	0.58	1.28	2.64	2.62	-	-	9.02
Nordal	1	1.93	0.72	1.54	0.68	1.60	1.50	-	-	7.43
Nordal	2	2.03	-	1.06	1.31	2.33	2.62	-	-	9.35
ant 13-152	1	2.15	0.89	1.29	0.57	1.38	1.29	-	-	7.57
ant 13-152	2	1.26	0.05	0.64	0.57	1.62	0.82	-	-	4.96
ant 13-101	1	1.64	0.68	1.18	0.76	1.37	1.28	-	-	6.91
ant 13-101	2	1.08	-	0.34	0.95	1.36	2.17	-	-	5.90
ant 17-148	1	1.16	0.75	1.36	0.98	1.99	2.50	-	4.42	8.80
ant 17-148	2	1.35	0.08	0.33	0.90	2.61	1.69	-	2.21	6.96
ant 17-142	1	1.18	0.75	1.52	0.63	1.47	1.40	-	1.17	6.95
ant 17-142	2	0.96	-	0.34	1.05	1.84	1.92	-	0.69	6.11
ant 18-186	1	1.31	1.18	1.35	0.57	2.62	3.02	0.57	-	10.62
ant 18-186	2	1.75	-	0.83	1.14	3.33	5.04	-	-	12.10
ant 18-141	1	1.39	1.22	1.80	0.82	2.47	2.35	0.74	-	10.05
ant 18-141	2	1.66	-	0.92	1.06	2.95	4.72	-	-	11.31
ant 19-109	1	1.08	0.94	1.17	0.83	1.81	2.00	-	-	7.83
ant 19-109	2	1.41	-	0.28	0.89	2.50	2.88	-	-	7.95
ant 21-194	1	0.48	1.77	0.88	0.48	1.39	1.62	-	-	6.62
ant 21-194	2	1.05	-	0.28	0.70	1.76	2.55	-	-	6.34
ant 21-1507	1	0.80	1.89	0.96	0.86	1.28	1.41	-	-	7.20
ant 22-1508	1	1.47	0.70	1.48	0.54	1.70	1.26	-	3.47	7.15
ant 22-1508	2	1.27	-	0.32	0.80	1.92	1.66	-	1.80	5.97

Table III

HPLC fractions according to Figure 4 of extracts from *ant* 13-152, *ant* 17-148, *ant* 18-102 and Nordal. The extracts were tested according to Jerumanis for reaction with ferric ions in alkaline solution. The values are optical densities (OD) at 525 nm per 10 g of barley grain.

Fraction no.	Compounds reacting with Fe ³⁺ in alkaline solution	<i>ant</i> 13-152	<i>ant</i> 17-148	<i>ant</i> 18-102	Nordal
1	gallic acid	0.124	0.809	0.416	2.476
	protocatechuic acid				
	(+)-catechin				
2	(protocatechuic acid)	0.091	0.327	0.336	0.305
	(+)-catechin				
3	chlorogenic acid	-	0.150	0.228	0.145
4	caffeic acid	0.040	0.106	0.178	0.212
5	-	-	0.100	0.182	0.149
6	-	0.040	0.027	0.240	0.022
7	dihydroquercetin	-	0.080	0.255	0.372
8	-	-	0.117	0.286	0.172
9	flavone related compounds	-	0.417	0.357	0.838
10	flavone related compounds	0.293	1.706	1.168	2.242
11	flavone related compounds	-	0.553	0.587	1.467

hydrolysis used in the preparation of the extracts. Usually the amounts of vanillic, p-coumaric, ferulic and sinapic acids are increased in extracts subjected to alkaline hydrolysis, compared to corresponding extracts after acidic hydrolysis. On the other hand, dihydroquercetin and caffeic acid are absent or occur only in trace amounts, and the quantity of syringic acid is much reduced compared to extracts after acidic hydrolysis. The amount of the naringenin related compound observed in *ant* 17 mutants after acidic hydrolysis is about twice the amount obtained in the corresponding extracts after alkaline hydrolysis. In solution, naringenin and other flavanones exist in an equilibrium with the corresponding chalcone. In acidic media, the formation of the flavanone is favoured and in alkaline media the chalcone (20). In extracts of standard naringenin, treated in the same way as the *ant* 17 extracts, part of the naringenin is converted to naringenin chalcone during alkaline hydrolysis.

3.4. Reactions with ferric ions in alkaline solution

In the test according to Jerumanis the following equation is used to calculate the amount of polyphenols in beer and wort samples (13): $E_{525} \times 630 = \text{polyphenols given as mg} \cdot \text{l}^{-1}$.

The equation is based on a standard curve made up with undefined polyphenols isolated from malt, beer and hops, and it cannot be used to calculate the amounts of phenolic acids and catechin present in individual fractions of barley grain extracts, as it gives polyphenol values about 2-5 times higher than the real value, when applied to known amounts of standards. As some of the 11 fractions collected from hydrolysed barley extracts (Figure 4) could contain more than one o-diphenol, no attempt was made to calculate the quantity of each one. Instead the optical densities (OD) of the different fractions from the wild-type and three mutants were compared (Table III).

In all barley extracts the first and the last three fractions had the highest OD. The first fraction

of the hydrolysed wild-type extracts contains some (+)-catechin and shows a higher OD than that of the *ant* mutants. In addition to (+)-catechin, gallic and protocatechuic acids are eluted in this fraction. There might be some protocatechuic acid and (+)-catechin eluted in fraction 2 as well. In unhydrolysed wild-type extracts the proanthocyanidins are eluted in the first fraction. The last fractions, 9-11, have the highest OD in extracts from the proanthocyanidin-free *ant* 17 and *ant* 18 mutants as well as in the hydrolysed extracts from the mother varieties. When fractions 9-11 of *ant* 13-152 were tested, their OD was significantly lower than in corresponding fractions of *ant* 17 and *ant* 18 mutants and the wild-types. In fractions 9-11 cinnamic acid is the only compound fully identified so far, and it does not react with ferric ions in alkaline solution. Fractions 9-11 contain the flavone related compounds mentioned earlier, and flavones with two adjacent hydroxyl groups in the aromatic rings do react with ferric ions under these conditions. These compounds might be responsible for the high OD obtained in the latter fractions.

4. DISCUSSION

In 1963 URION and co-workers (21) used TLC to separate and identify phenolic acids in hydrolysed extracts of grains of the barley variety *Violette de Guimalaye*. The following acids were found: protocatechuic acid, gentisic acid, p-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, ferulic acid, isoferulic acid and sinapic acid (21). Later studies on the phenolic acid composition of barley, malt, wort, beer and beer hazes, employing the HPLC technique, have given similar results (4, 17, 19, 22). Gallic acid, gentisic acid and cinnamic acid are not reported by all workers, however. In addition, kojic acid has been found in wort and beer by QURESHI et al. (19) and in wort by WACKERBAUER et al. (22).

While different workers agree on the qualitative composition of phenolic acids in brewing materials, the quantitative determinations of phenolic compounds have given very different results. For instance, according to WACKERBAUER et al. (22), gentisic acid is the

most important phenolic acid in barley grains, and an amount of $11.6 \text{ mg} \cdot 100 \text{ g}^{-1}$ dry weight is reported. MCMURROUGH et al. (17) failed to report it, and in the present investigation only trace amounts were found. MCMURROUGH et al. measured the phenolic acid content in grains of *ant* 13-13 and the variety Emma. The amounts of major phenolic acids were $6.5 \text{ mg} \cdot 100 \text{ g}^{-1}$ and $1.5 \text{ mg} \cdot 100 \text{ g}^{-1}$, respectively. In the present study the same amount of phenolic acids was obtained in *ant* 13 mutants, but the wild-type barley varieties also contained between 7-10 mg of major phenolic acids $\cdot 100 \text{ g}^{-1}$ dry matter (Table II). Emma barley might be especially low in phenolic acid content.

The amount of phenolic acids in barley grains is low compared to the amount of proanthocyanidins and catechin. Typically wild-type barley varieties contain around 130-200 mg proanthocyanidin and catechin $\cdot 100 \text{ g}^{-1}$ dry weight as measured by Harris and Ricketts' method and by HPLC (11, 23), and the total amount of acetone extractable phenolic acids in wild-type barley is around 10-20 mg $\cdot 100 \text{ g}^{-1}$, i.e. ca. 10% of the amount of the flavanoids. This result is in agreement with other investigations, which have concluded that simple phenolic acids cannot be considered as significant flavour constituents of beer or major components of beer haze (4, 17).

Only o-diphenols give rise to a coloured complex with ferric ions under the conditions used in the Jerumanis method for measurement of total polyphenols in beer and wort (13). This means that only a few of the phenolic acids present in barley grains, namely gallic acid, protocatechuic acid, chlorogenic acid and caffeic acid can contribute to the total polyphenol content as measured according to this procedure. As the amount of these acids is small compared to the total amount of proanthocyanidin and catechin in wild-type barley, their presence in *ant* 17 and *ant* 18 mutants cannot explain the high amount of polyphenols estimated in beers made from these mutants. Dihydroquercetin reacts with ferric ions in alkaline solution, but the dihydroquercetin content in the *ant* 18 mutants, while significant, is also very low compared to the amount of proanthocyanidin in wild-type barley.

FRÖST, HARBORNE and KING have identified a great number of flavoneglucosides and C-glucosyl flavones in leaves of different barley varieties (9). These compounds react with ferric ions. Also quercetin, which is present in beer and hops, gives a coloured complex with ferric ions in alkaline solution. None of these compounds have so far been identified in barley grains, but it is likely that they occur, and that the flavone related compounds found in the late parts of the HPLC chromatograms indeed are flavones or C-glucosylflavones. The amount of these compounds is lower in *ant* 13 mutants than in *ant* 17 and *ant* 18 mutants and wild-types, and so is the polyphenol content as measured according to the procedure of Jerumanis. Thus, the high polyphenol values obtained in beers brewed from proanthocyanidin-free lines with mutations in the genes *ant* 17 and *ant* 18, are probably due to the presence in the grains of other flavonoids than proanthocyanidins and catechin.

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