ORGANIC ACIDS OF BARLEY GRAIN[1]

J. S. WALL, L. C. SWANGO, D. TESSARI, AND R. J. DIMLER

ABSTRACT

The identity and amounts of organic acids contained in whole barley grain and in barley grain fractions were investigated, since they may play significant roles in germination and seed metabolism. The relative efficiency of ion-exchange adsorption compared to ether extraction as a means of isolating the acids from alcoholic extracts of the grain was determined. An improved procedure of partition chromatography on silica gel was developed to separate and determine the acids. Paper chromatography was employed to identify individual acids and to make preliminary qualitative studies of the acids in the extract. Most of the organic acids in barley extracts were accounted for as malic, acetic, succinic, fumaric, malonic, alpha-ketoglutaric, lactic, citric, aconitic, and pyrrolidone carboxylic. The reported presence was confirmed of a number of phenolic acids contained in barley, including ferulic, vanillic, *p*-hydroxybenzoic, and *p*-coumaric.

There is evidence that certain organic acids in cereal grains play an important role in determining ease of seed germination. Täufel and Pohloudek-Fabini (21) report that a positive correlation exists between viability and citric acid content of stored seeds of several species, including barley. They attribute this correlation to the stimulation Krebs cycle acids give aerobic respiration. In contrast, an inhibitory action upon barley germination was demonstrated by Cook and Pollach (6) to be due to some aromatic acids in the grain. Only vanillic acid was characterized among these substances.

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There has been little information available pertaining to the nature and amounts of the organic acids in barley grains, including those of metabolic importance. While the present work was in progress, Houston and Kester (11) presented a preliminary report of analyses for various organic acids in barley and in a number of other cereal grains. Van Sumere and his associates (22) recently identified several phenolic acids contained in barley husks.

A study was undertaken to develop improved methods for the isolation, identification, and determination of the various organic acids of barley grain. Pearling was employed to separate the husk fraction of barley to obtain greater concentration of the acids characteristic of this portion of the grain. Distribution of all the barley acids was studied in the fractions obtained by pearling because of the importance of pearled barley as a food.

Materials and Methods

Samples for Analysis. The samples of Tregal variety barley employed in these studies were obtained from the 1957 crop grown at the North Dakota Agricultural Experiment Station. The grain was received shortly after harvesting and stored at 4°C. Some older grain of the same variety and source which had been stored under continuous refrigeration was also submitted to a cursory study to note any effects of this type of storage. Pearling, as carried out on a Strong-Scott laboratory mill [2] for 2 minutes, removed most of the husk and much of the embryo. The pearlings represented 14.5% of the weight of the initial grain.

Ethanol Extraction of Barley. By means of a large Waring Blendor (Model CB3), 300 g. of barley, pearled barley, or pearlings were macerated together with 900 ml. of 80% ethanol for a period of 15 minutes. During this time the blender speed was increased, at intervals, to the maximum; the temperature of the extracting solution rose almost to boiling, thereby favoring extraction and enzyme inactivation. The suspension was then cooled and centrifuged. The insoluble residue was re-extracted twice with 800-ml. volumes of 80% ethanol in the Waring Blendor. The extracts were combined. The pH of the extracts was 5.5; for extracts to be run through ion-exchange columns, sufficient alkali was added to bring the pH to 7.2. The solutions were then concentrated under vacuum to a volume of about 30 ml. Insolubles were removed by centrifugation.

Ion-Exchange Treatment. One procedure investigated for separat-

[2] Mention of firm names or trade products is for identification only and does not imply endorsement by the U.S. Department of Agriculture.

ing the organic acids in the extract of the barley from the carbohydrates and other substances that might interfere with subsequent chromatography was adsorption of the acids on an ion exchanger as described by Resnik *et al.* (17). The extract was passed through a 1.2 by 25-cm. column of Dowex 1 × 8 (50–100 mesh) in the carbonate form. The column was then washed with 200 ml. water and the acids were eluted with 500 ml. of 1.5*N* ammonium carbonate. After vacuum concentration of this effluent almost to dryness to remove ammonium carbonate, the residue was made up to a volume of 1 ml. with water.

Ether Extraction of Barley Acids. Another method tested for recovering the organic acids from the aqueous concentrate of the initial alcohol extract of barley was based on ether extraction. The aqueous solution was adjusted to pH 1 by careful addition of 9*N* sulfuric acid and transferred to a liquid-liquid extractor (Kutscher-Steudel) of 50-ml. capacity. After extraction with 100 ml. of ether for 72 hours, the ether solution was twice extracted with 150 ml. of 2% sodium bicarbonate solution. The combined bicarbonate solutions were neutralized and concentrated to 30 ml., acidified to pH 1, and again extracted for 72 hours with ether. After removal of the ether, the acids were made up to 1 or 2 ml. in water or 50% acetone solution.

Paper Chromatography. Paper chromatography was used to obtain preliminary information about the number, kind, and relative quantities of organic acids in the extracts. Two-dimensional chromatography similar to that described by Cheftel *et al.* (5) separated most of the nonvolatile aliphatic acids. The initial descending irrigation on Whatman No. 1 paper, 46 by 57 cm., in the long direction was with 95% ethanol, concentrated ammonium hydroxide, and water (8:5:15) for 24 hours. The terminal edge of the paper was serrated to allow the solvent to run off uniformly. A solution of n-butanol, formic acid, and water (120:20:20) was employed as the solvent in the second direction for 18 hours, and it also was used extensively in unidirectional chromatograms for characterizing substances eluted from chromatographic columns. After drying, the papers were examined under ultraviolet light with a lamp having emission maximum at 260 mμ, to detect aromatic acids and such unsaturated acids as fumaric and aconitic. After the papers were sprayed with semicarbazide reagent (14), keto acids, such as pyruvic and alpha-ketoglutaric, were detected under the ultraviolet light. The papers were next sprayed with bromcresol green, 40 mg. in 100 ml. 95% ethanol adjusted with alkali to a green color, to develop spots for acids.

The aromatic acids were separated by descending chromatography in a two-dimensional system consisting of 95% ethanol and 2*N*

ammonium hydroxide (9:1) in the initial direction for 18 hours and tertiary amyl alcohol, water, and glacial acetic acid (6:94:0.5) in the second direction for 6 hours (6). Spots were detected by ultraviolet light and characteristic fluorescence or absorbance noted. The papers were also sprayed with diazotized *p*-nitroaniline and colors were noted before and after an additional alkaline spray (20). Volatile organic acids were separated by the paper-chromatographic procedure of Kennedy and Barker (13).

Column Chromatography. Separation of organic acids was based on a combination of many reported modifications of the original Isherwood (12) technique of partition chromatography on silica gel columns. The silica gel was prepared from Mallinckrodt analytical grade 100-mesh silicic acid as described by Bulen, Varner, and Burrell (2). Ten grams of the dried silica gel were ground together with 6 ml. of 0.5*N* sulfuric acid. Chloroform saturated with 0.5*N* sulfuric acid, 30 ml., was added to form a thin slurry after thorough stirring. The slurry was poured into a glass tube, 1.0 by 50.0 cm., fitted near the end with a porous glass disk and terminated with rubber tubing and screw clamp to regulate solvent flow. The silica gel was packed in the tube under 2 lb. pressure of nitrogen. The samples were introduced to the column in aqueous solution by the method of Zbinovsky and Burris (24). A circular pad of filter paper cut from sheets 2-mm. thick was applied to the silica gel surface under a layer of chloroform saturated with 0.5*N* sulfuric acid. The chloroform was then run through the column to the disk surface and the glass tube's inner surface wiped with a cotton swab. Then 0.2 ml. of 9*N* sulfuric acid followed by 0.3 ml. of extract and 0.1 ml. of 9*N* sulfuric acid were added in that order to the surface of the disk. This aqueous solution was barely allowed to enter the column and 3 ml. of chloroform saturated with 0.5*N* sulfuric acid were placed above the disk. The rubber tubing was removed, and the column was transferred to the fraction collector and connected to the solvent reservoir as illustrated in Fig. 1. The solvent reservoir had previously been placed in position and filled as described below.

Elution of the acids with good resolution and recovery was achieved by a modification of the continuous gradient elution procedure of Donaldson *et al.* (8), employing an all-glass apparatus (Fig. 1). The arm of the lower vessel was just below the 350-ml. capacity level. In the lower container were placed 390 ml. of chloroform saturated with 0.5*N* sulfuric acid; in the upper chamber, 350 ml. of 20% tertiary amyl alcohol-80% chloroform saturated with 0.5*N* sulfuric acid. With stopcock *A* closed and clamp *C* and stopcock *B* open, elution with only

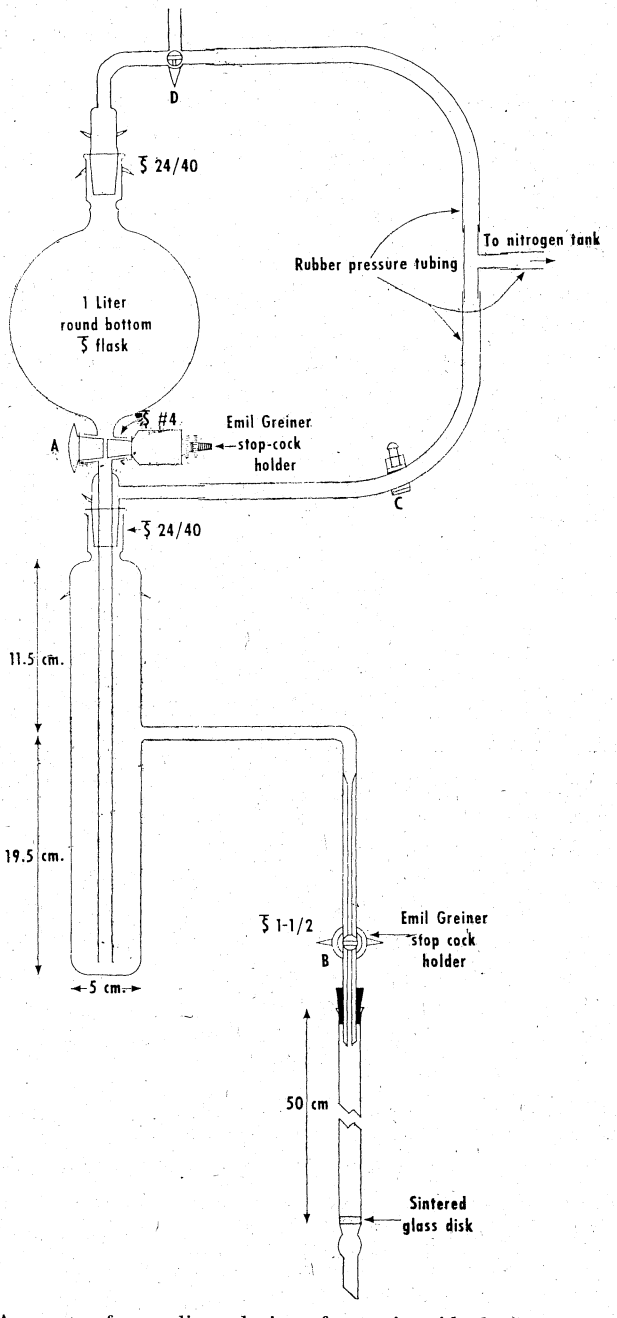

Fig. 1. Apparatus for gradient elution of organic acids during partition chromatography on silica gel.

the chloroform was begun with collection of fractions. The nitrogen pressure was gradually elevated to about 2 lb. so that a flow rate of 0.75 ml. per minute was attained. After 40 ml. were collected, stopcock *A* was opened and clamp *C* closed, permitting gradual mixture of the contents of the upper chamber with that in the lower. When the contents of the upper chamber were exhausted, 350 ml. of a solution of 60% tertiary amyl alcohol-40% chloroform saturated with 0.5*N* sulfuric acid were placed in it. Pressure was maintained in the lower vessel during this operation by closing stopcocks *D* and *A*, which were again opened to permit mixing with the new solvent. Elution was then continued overnight at the previous rate.

Determination of Eluted Acids. To determine the acids of the column effluent by titration, fractions of 2 ml. each were collected by means of a drop count automatic collector in tubes containing 4 ml. of an aqueous phenol red solution. These were titrated with 0.01*N* sodium hydroxide while the two-phase system was agitated by means of a jet of nitrogen.

An accurate determination of the aromatic acids in the column effluent was not possible by titration because of their small concentration. However, more sensitive ultraviolet absorption measurements could be employed for their detection and analysis as shown by Sondheimer (19). For this purpose 4-ml. fractions were collected and the aqueous indicator solution was omitted. The absorption of these fractions was measured at 260 and 320 mμ in a Beckman DU spectrophotometer against chloroform saturated with 0.5*N* sulfuric acid as a blank. Even after redistillation, the reagent-grade tertiary amyl alcohol exhibited considerable absorbance at 260 mμ which resulted in an increasing background absorbance of the effluent at that wave length. Therefore the absorbance values of effluent fractions from a control column to which no extract sample was added was used as a blank to correct for the background absorbance at 260 and 320 mμ. The absorbance of standard acid solutions at 260 mμ in similar solvents (Table I) was used to calculate the amounts of phenolic acids in the effluent.

Characterization of Eluted Compounds. In addition to the position of elution from the column and paper chromatography, specific chemical reactions and ultraviolet absorption spectra were employed to characterize the separated barley acids.

The ultraviolet absorption spectra of aromatic acids were determined on fractions containing peaks from columns upon which the UV absorption of the effluent was measured. The spectral curves were obtained with a Cary automatic recording spectrophotometer employ-

TABLE I

ULTRAVIOLET ABSORPTION CHARACTERISTICS OF AROMATIC ACIDS IN BARLEY

ACID	$\lambda_{MAX.1}$[a]	$\lambda_{MAX.2}$[a]	$\lambda_{MIN.}$[a]	E $_{MAX.1}$[b] $\times 10^{-4}$	E 260[b] $\times 10^{-4}$	E 320[b] $\times 10^{-4}$
	mμ	mμ	mμ			
Ferulic	320	295	262	1.92	0.25	1.92
Vanillic	260	290	...	1.18	1.18	0.00
p-Coumaric	310	...	257	2.06	0.18	1.65
p-Hydroxybenzoic	255	...	240	1.48	1.40	0.00
Unknown A	280	310	260	....	....	....
Unknown B	260	310	275	....	....	....
Unknown F	272	....	....	....	....	....
Unknown G	260	...	245	....	....	....

[a] Determined upon effluent fractions from column.
[b] Determined upon knowns in chloroform saturated with 0.5*N* H_2SO_4.

ing as blanks analogous fractions from the control column used for UV background correction.

For paper chromatography the fractions from a column containing a peak were combined and dried under vacuum. Nonvolatile acids present as sodium salts after titration were taken up in small volume (0.2–0.5 ml.) of 0.1*N* sulfuric acid before application to the paper. Certain of the free aromatic acids were dissolved in acetone.

Standard Acids. The standard acids employed for both column and paper chromatographic studies were from commercial sources and were recrystallized when necessary. Some of the phenolic acids tested were obtained from A. N. Booth of the Western Utilization Research and Development Division, U. S. Department of Agriculture. Compounds synthesized in our laboratory by established procedures, as indicated, are pyrrolidone carboxylic acid (9) and *p*-coumaric acid (16).

Phosphorus Determination. Phosphorus was determined by a modification of the method of Allen (1).

Results

Ion-Exchange Adsorption. The passage of the alcoholic extract of barley or a known mixture through the column of Dowex 1 resin resulted in almost quantitative removal of acidic substances (Table II). Only 3.7% additional acids were recovered by a passage of the washings from the column through a second anion exchanger of similar dimensions. The passage of additional ammonium carbonate solution through the column after elution resulted in only 2.6% additional recovery of acids. However, the rapidity of elution of individual acids with ammonium carbonate varies, depending upon their structure, so that certain ones were preferentially retained. As evidenced from the paper-chromatographic studies, phenolic acids were absent from the

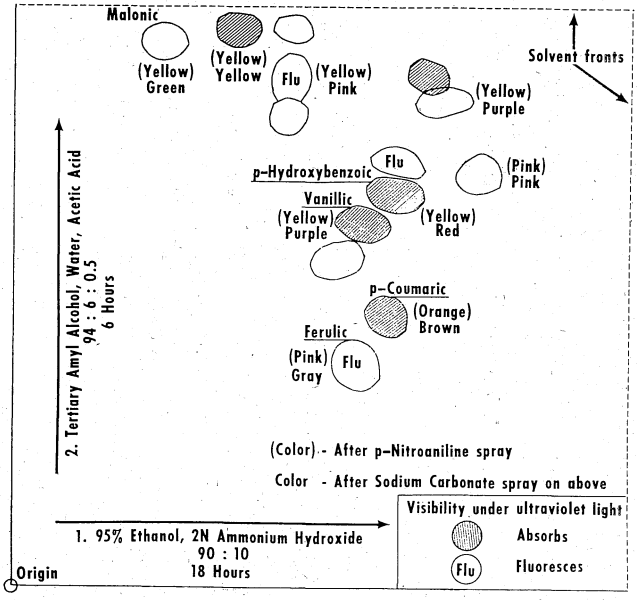

Fig. 3. Two-dimensional paper-chromatographic separation of phenolic acids of barley. Approximately 60 μl. of concentrate from ether extract of whole barley applied.

Some of the acid peaks found in ether extracts have not been positively characterized and are designated by letter. The UV absorption characteristics of certain of these substances are tabulated in Table I.

Aconitic, malonic, and pyrrolidone carboxylic acids were eluted in close proximity (Fig. 4). Vanillic and ferulic acids were not well resolved, as is also the case for *p*-hydroxybenzoic and acetic (Figs. 4 and 5). However, each of the phenolic compounds can be detected and analyzed independently by utilizing their characteristic UV absorptions as listed in Table I. The cinnamic acid derivatives, ferulic and *p*-coumaric acids, were determined by absorption at 320 mμ; vanillic and *p*-hydroxybenzoic acids were determined at 260 mμ.

Recoveries of individual acids applied in known quantities to the columns were quantitative (greater than 95%). This recovery indicated that little esterification of the acids occurred during their exposure to the eluting solvent.

Amounts of Acids in Extracts. The data in Table III, which summarizes the amounts of each of the identified acids contained in the various extracts, represent the averages of duplicate analyses of at least two extractions.

Malic acid is the preponderant organic acid present in barley. It

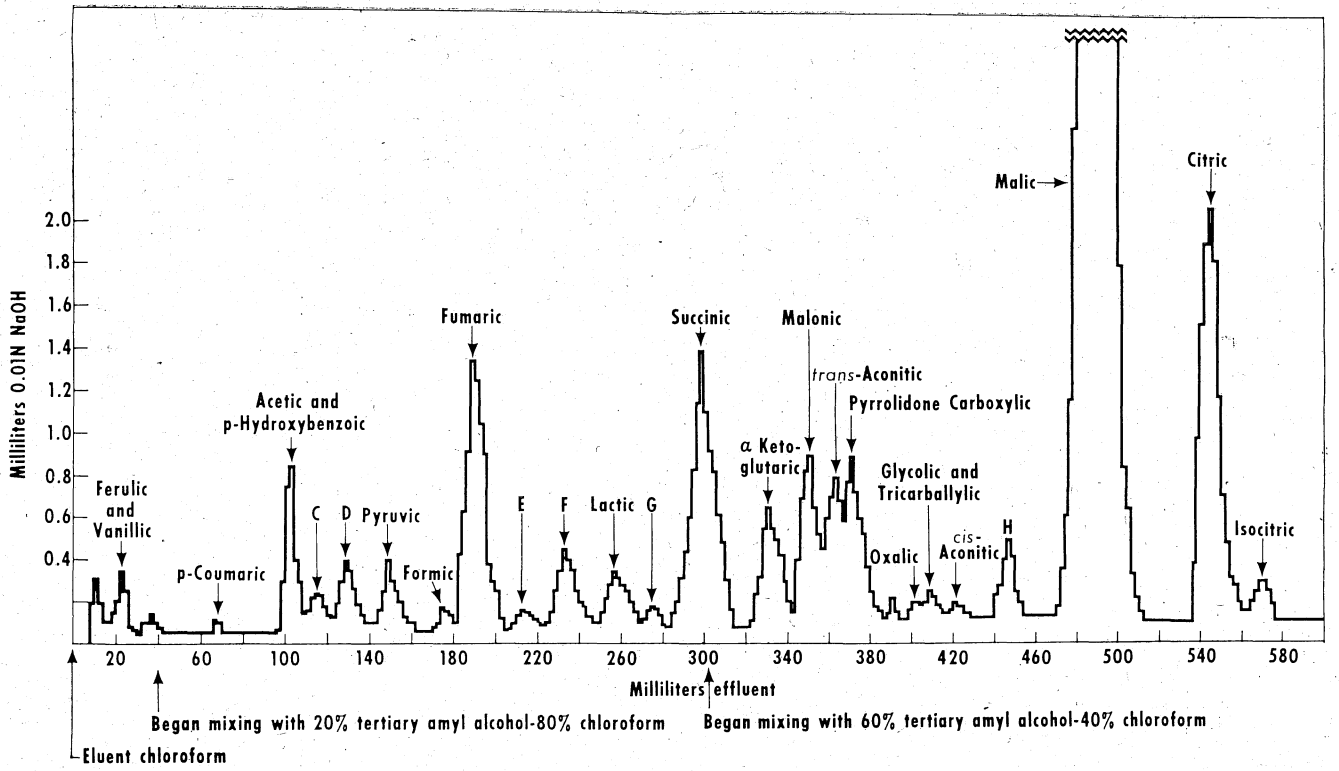

Fig. 4. Determination by titration of organic acids separated from an ether extract equivalent to 90 g. of whole barley upon partition chromatography on a silica gel column. All eluents were saturated with 0.5*N* sulfuric acid.

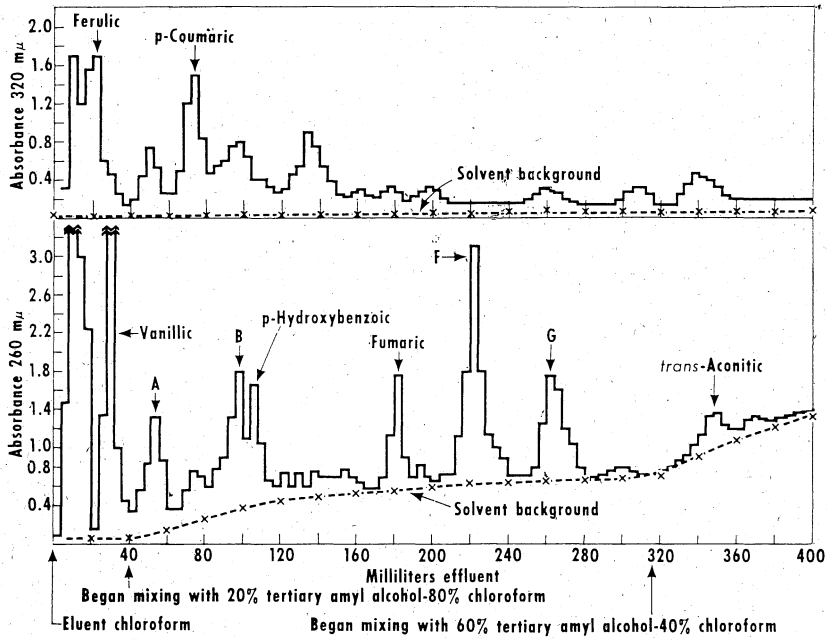

Fig. 5. Determination by ultraviolet absorbance of organic acids separated from ether extract equivalent to 90 g. whole barley upon partition chromatography on silica gel column. All eluents were saturated with 0.5*N* sulfuric acid.

constitutes 60% of the total organic acid of the whole-barley extracts. Other acids of the Krebs or "tricarboxylic acid" cycle including citric, succinic, fumaric, alpha-ketoglutaric, isocitric, and *cis*-aconitic acids were present in lesser amounts. The *trans*-aconitic acid was probably produced from the less stable biologically active *cis*-isomer during the isolation procedure. Only small amounts of the *cis* compound were recovered. Other acids of metabolic importance, including acetic, pyruvic, and lactic, were also present in the barley extracts. Malonic acid and compound F were present in significant quantities. Only traces of formic, oxalic, glycolic, and tricarballylic acid were detected. Pyrrolidone carboxylic acid is probably derived from glutamine during extraction.

Some differences occur between the amounts of a single acid found in the ion-exchange extract, as compared to those obtained by ether extraction. The quantities of citric acid were slightly greater in the ion-exchange extract than in the ether. This difference is probably due to poorer recovery of the polar acids in the ether process. In contrast, the ether-extraction procedure yields more fumaric acid than does the ion exchange.

TABLE III

ORGANIC ACIDS OF BARLEY AND BARLEY PEARLING FRACTIONS

METHOD USED TO DETERMINE ACID	WHOLE BARLEY		PEARLED BARLEY ETHER EXTRACT	BARLEY PEARLINGS ETHER EXTRACT
	Ion-Exchange Extract	Ether Extract		
	μeq/100 g	*μeq/100 g*	*μeq/100 g*	*μeq/100 g*
UV Absorption				
Vanillic	....	2.4	0.6	16.3
Ferulic	....	0.9	0.4	5.4
p-Coumaric	....	1.0	0.1	6.8
p-Hydroxybenzoic	....	0.9	0.2	5.1
Titration				
Acetic	62.5	29.2	21.2	35.2
Pyruvic	7.1	8.1	....	....
Formic	4.0	3.6	....	....
Fumaric	40.5	58.3	49.3	115.0
Compound F	....	19.2	....	....
Lactic	14.9	16.7	12.0	42.7
Succinic	78.0	82.2	63.7	181.5
Alpha-ketoglutaric	30.8	40.0	32.5	75.0
Malonic	22.0	25.5	18.6	48.3
trans-Aconitic	16.0	17.9	14.2	20.1
Pyrrolidone carboxylic	25.4	30.5	20.3	40.4
Oxalic	2.1	2.0	....	....
Glycolic	3.5	2.5	....	....
Tricarballylic	3.5	2.5	....	....
cis-Aconitic	4.0	3.5	....	....
Malic	875.3	822.0	533.0	1400.0
Citric	100.5	80.7	30.4	170.0
Isocitric	10.3	8.7	....	30.8

Data of Table III establish that the phenolic acids of barley are primarily concentrated in pearlings. Studies by Van Sumere *et al.* (22) indicate that they are associated with the husk. Barley pearlings contained appreciably greater amounts of all the organic acids on a weight basis than did the whole grain. However, the pearled barley, which is primarily endosperm, contained a greater total amount of organic acid.

Discussion

Silica Gel Chromatography. The column-chromatography procedure described affords numerous advantages over earlier methods in simplicity, resolving power, and rapidity. A system for the separation of organic acids on partition columns employing gradient elution with increased resolving power also has been described recently by Dajani and Orten (7). With an initial 350-ml. volume of chloroform in the mixing flask, satisfactory resolution of lactic and succinic acids or of certain aromatic acids was not achieved with an initial tertiary amyl alcohol concentration of greater than 20% in the upper flask. The use of a second gradient approaching 60% tertiary amyl alcohol was introduced to obtain more rapid and compact elution of the more

polar acids. The volume of 20% tertiary amyl alcohol-80% chloroform employed prior to changing to the 60% tertiary amyl alcohol-40% chloroform is critical with respect to the elution sequence and resolution of alpha-ketoglutaric, aconitic, malonic, and pyrrolidone carboxylic acids. Variations in position of elution of these acids resulting from changes in solvent schedule may be used to advantage to obtain better resolution of certain of the acids.

Extraction. In some experiments 80% acetone was used instead of 80% ethanol to extract barley. The greater volatility of the acetone proved undesirable. The data obtained with the acetone extracts were similar to those with the alcohol extracts, indicating that little esterification of organic acids occurred during extraction with the ethyl alcohol solution. Oxalic acid which might be present as the insoluble calcium salt at the pH of the barley macerate may not be completely extracted by the 80% ethanol.

The two isolation procedures, ion-exchange adsorption and ether extraction, served as a check on the efficiency of each. Ease of recovery of individual acids by either procedure depends upon their relative polarity. Ion-exchange chromatographic separation of organic acids developed by Busch *et al.* (3) gives evidence that malic acid is more readily eluted from the anion exchanger Dowex 1 than fumaric acid is with polar solvents, and that polarity of the acid is important in determining the elution rate. Data in Table II indicate that little additional acids are recovered by doubling the elution volume of ammonium carbonate. However, the retained acids are predominantly the less polar acids which are more highly bound by adsorption to the aromatic resin. Carrol (4) has shown that elution with organic solvents speeds the recovery of fumaric and aromatic acids from ion-exchange resins relative to more polar acids.

The time of liquid-liquid extraction of the aqueous extract with ether should be governed by the time required for removal of the most polar acid component, such as citric. The efficiency of removal of citric acid or similar polar acids should be determined for each Kutscher-Steudel apparatus and the time of extraction of organic acids established accordingly.

Significance of Acid Concentrations. The large quantity of malic acid suggests that the further metabolism of this acid to oxalacetate is rate-limiting during the development of the seed. The accumulation of malic acid may serve as a reservoir for four-carbon units essential to the oxidation of carbohydrate and lipid via the Krebs cycle during seed germination. The other Krebs-cycle acids in barley may serve similarly. The positive correlation observed by Täufel and Pohloudek-Fabini

(21) between citric acid concentration and seed viability may not be specific, because they give no indication as to the existence or absence of any relationship between viability and the presence of other organic acids in the seed.

Elliot (10) also found significant quantities of malonic acid in germinating barley. Only recently, the widespread occurrence of malonate in plant tissues has been demonstrated, and the pathways for its enzymatic utilization and synthesis have been described by Shannon *et al.* (18). Possibly this organic acid plays an important role in plant tissue metabolism.

The phenolic acids were found primarily in the husk-containing fraction. Some have been related to lignin formation (15). Vanillic acid has been shown to delay barley germination, but the other phenolic acids in barley have not been studied relative to their role in this phenomenon. The aromatic acids appear to have wide influences on metabolism and have been shown to be active in combating the development of fungi involved in certain diseases in grain (23).

Fractionation of barley by pearling the grain proved advantageous for attaining the greatest concentration of phenolic acids. The higher concentration of other organic acids in the pearling was probably related to the fact that most of the embryo was present. The pearling process did not dissociate the barley into discrete morphological fractions, so that conclusions as to the exact anatomical distributions of organic acids cannot be deduced.

Only minor differences were found in organic acid distributions in barley of the same variety and from the same source but harvested in different years, or in the barley that had been stored in the cold for several years. No studies were made of changes that might occur in the organic acids of grain stored commercially. The techniques described in this report should facilitate the study of changes of organic acids in barley and other grains during storage and germination.

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