phytate in corn germ, for example, could not possibly exist there as phytin, Ca_5Mg phytate, as is frequently stated. The chief cations associated with phytate in corn germ are potassium and magnesium. The analyses suggest that the ratios of phytate:Mg:K are approximately 1:3:5. From the point of view of human and livestock nutrition, it appears that high lysine corn is as valuable a source of nutrients, other than lysine, as a commercial hybrid. In fact it contains a higher concentration of potassium, a positive asset. Furthermore, the concentration of phytate was not different.

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Rapid Method for Determining Aconitic Acid and Its Adaptability for Assaying Aconitate in Plant Extracts

Ralph B. Clark

Color formation in the acetic anhydride–pyridine method for aconitic acid was complete upon the addition of pyridine to the reaction and the absorbance could be measured immediately at 425 nm. Reaction mixtures were prepared and maintained at 0°C. The absorbance curve for aconitic acid concentration was linear to 80 μg. Absorbance losses were insignificant within 20 min after color formation and were 12% after 2 hr. Of the organic acids tested only *trans*- and *cis*-aconitic acids yielded significant absorbance under these conditions and the molar extinction coefficient of *cis*-aconitic acid was two-thirds that of *trans*-aconitic acid. Amino acids and sugars did not interfere with the assay. Crude extracts of plant material could be assayed directly.

Aconitic acid (1,2,3-prop-1-enetricarboxylic acid) accumulates at relatively high concentrations in some plants (Yoder, 1911; Nelson and Mottern, 1931; McCalip and Seibert, 1941; Buch, 1960; Beevers *et al.*, 1966; Stout *et al.*, 1967; Clark, 1969), especially in species from Gramineae and Ranunculaceae (Buch, 1960; Stout *et al.*, 1967). The importance of *cis*-aconitic acid in the tricarboxylic acid cycle is well documented; however, the importance of *trans*-aconitic acid is generally unknown. *Trans*-aconitic acid appears to be closely associated with the mineral status of plants (Coic *et al.*, 1961; Torri and Laties, 1966; Brown, 1968; Clark, 1968; Grunes *et al.*, 1970) and has been suggested as a causative factor to higher incidence of hypomagnesenia (grass tetany) in cattle (Grunes *et al.*, 1970). *Trans*-aconitic acid has also been known to inhibit aconitase (Dickman, 1961). Aconitic acid has been recognized as a complicating factor in the clarification and crystallization of refined sugar and molasses (McCalip and Seibert, 1941; Balch *et al.*, 1946; Ambler and Roberts, 1947). The presence of *trans*-aconitic acid in certain plant species has led scientists to seek more rapid and accurate methods for its determination.

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Several methods have been used for the determination of aconitate. Some of the disadvantages and problems involved in many of these methods are discussed by Poe and Barrentine (1968). These authors also described a colorimetric method for the determination of aconitate in sorgo juice (Poe and Barrentine, 1968) and in oats (Poe and Barrentine, 1970). In determining citrate, Spencer and Lowenstein (1967) noted that if the temperature was maintained at 0°C, aconitate yielded color while citrate yielded no color. No further description of the reaction was given. The object of this study was to determine if this difference in behavior of citrate and aconitate at 0°C could be used as a method for measuring the amount of aconitate in plant tissue.

EXPERIMENTAL

Materials. The chemicals used in the procedures were of purified (ACS) grade and the organic acids, sugars, and amino acids were of high purity (obtained from Sigma Chemical Co., St. Louis). Standard solutions of compounds to be assayed were prepared in water and frozen until used for assay. Fully-expanded maize leaves about 3 weeks after emergence were the source of plant material used in this study.

Methods. PREPARATION OF PLANT MATERIAL FOR ACONITATE ASSAY. One-gram samples of dried leaf material were

Table I. Effect of *trans*-Aconitic Acid Concentration on Absorbance at 425 nm

μg acid	Absorbance Mean		Absorbance Std dev
10	0.181	±	0.008
20	0.343	±	0.014
25	0.436	±	0.016
37.5	0.634	±	0.028
50	0.821	±	0.024

Table II. Changes in Absorbance for *trans*-Aconitic Acid Reaction Mixtures with Time (25 μg of Acid)

Time, min	Absorbance 425 nm[a]
5	0.416 a
10	0.421 a
15	0.409 ab
20	0.407 abc
25	0.404 bc
30	0.404 bc
40	0.398 c
50	0.394 c
60	0.382 d
75	0.380 d
90	0.378 d
120	0.366 e

[a] Numbers not followed by the same letter are significantly different at 0.01 level.

Table III. Aconitate Contents of Corn Leaf Extracts Assayed Before and After Being Passed Through Ion Exchange Resins

Extract no.	Crude extract	Crude extract purified with resins
	Percent aconitate[a]	
1	1.38	1.37
2	1.41	1.54
3	1.15	1.18
4	1.37	1.37
Average	1.33	1.36
Std dev	0.12	

[a] Average of four determinations for each value.

vacuum infiltrated with 80% ethanol for several minutes The samples were transferred to homogenizing flasks and ground at high speed in 80% ethanol for 3 min using a VirTis Model 45 homogenizer. The homogenates were filtered through Buchner funnels under vacuum and the residue was thoroughly rinsed with boiling 80% ethanol. The filtrates were dried at 50–55°C on a water bath under forced air and suspended in 25.0 ml of water before being centrifuged 10 min at 10,000 × *g*. If the solid material in the centrifuge tubes broke loose, the solutions were filtered. These crude extracts were stored at −15°C until assayed for aconitate.

Some samples were purified further by passing them through glass columns of water-washed cation exchange resin (AG-50-X4, 200–400 mesh, hydrogen form). The effluent dripped into columns containing water-washed anion exchange resin (AG-1-X4, 200–400 mesh, formate form). Both exchange resin beds were 8–10 cm in height and 1 cm in diameter. The anion exchange resin was eluted with 25 ml of 20% formic acid followed by 25 ml of 98% formic acid and washed with water. This eluent was dried at 50–55°C on a water bath and suspended in 25.0 ml of water. The anion fractions were stored at −15°C until they were assayed for aconitate.

Aconitic Acid Assay. Aconitic acid standard solutions, standard solutions of other compounds to be tested, or plant extracts were pipetted at volumes of up to 0.2 ml into test tubes. If less than 0.2 ml of sample was used, water was added to bring the volume to 0.2 ml. The tubes were placed in an ice bath and kept cold through each procedure. To each tube 1.6 ml of acetic anhydride was added and the reactions were shaken, allowed to cool if necessary, followed by the addition of 0.2 ml of pyridine. Upon the addition of pyridine a yellow color appeared instantly and after the reactions were mixed thoroughly the absorbance was measured within a few minutes. Reactions were kept cold until the absorbance was measured at 425 nm. Absorbance measurements were made using a Gilford Model 240 spectrophotometer with a rapid sampler attachment (10 mm pathway length). Reaction mixtures were introduced into the cuvette and allowed to stand 30 sec before readings were taken.

RESULTS AND DISCUSSION

The effect of *trans*-aconitic acid concentration on the absorbance up to 50 μg is shown in Table I. Because most samples assayed contained less than 50 μg in 0.1 or 0.2 ml, the standard used each time contained a maximum of 50 μg. These data represent numerous assay times using new and old preparations of standard solutions. Absorbance was linear with concentration. Additional studies showed linearity to 80 μg of aconitic acid.

The color that formed in the reaction mixtures when pyridine was added was stable up to 20 min if the temperature was kept at 0°C. Reactions read 20 min after color development showed significant decreases (Table II) and losses of 12% were noted after 2 hr. If reactions were not maintained at 0°C, the absorbance decreased within a short time and the reaction mixtures turned yellowish-green and then brown. Upon adding pyridine an exothermic reaction occurs and, if large volumes are used in reaction mixtures, the solution may turn brown rapidly. No problem was encountered in this method using the 2.0 ml total volume suggested by Spencer and Lowenstein (1967).

When assaying for aconitate with a rapid sampler, convection currents in cold solutions occur when solutions are introduced into the spectrophotometer. This stabilizes after several seconds, however, and if a constant time between sample introduction and reading is chosen, the problem can be overcome. A 30-sec lag time between sample introduction and reading the absorbance was used in these experiments. A serious problem that may be encountered with this method is the volatilization of acetic anhydride if reactions warm and the burning effect that acetic anhydride has on the eyes. The use of a rapid sampler or closed system overcomes this, since solutions can be kept cold and contained within a closed system.

This method was essentially specific for aconitic acid. Except for *trans*- and *cis*-aconitic acids, the color formation and absorbance for the other organic acids assayed by this method were very low or nil. The molar extinction coefficients at 425 nm in 0.025 g/l. solutions of *trans*-aconitic, *cis*-aconitic, citric, itaconic, and fumaric acids were 5040, 3350, 60, 60, and 45, respectively. *Cis*-aconitic acid yielded an extinction coefficient two-thirds that of *trans*-aconitic acid. The absorbance due to citric acid was about 1% that of *trans*-aconitic acid on a molar basis. Other acids tested were citraconic, galacturonic, gluconic, glucuronic, glutaric, gulonic, isocitric, malic, pyruvic, quinic, shikimic, succinic, tartaric, and tricarballylic acids. Molar extinction coeffi-

cients for these latter acids under the same conditions as above were 25 or less for each acid.

Assays were also made using as high as 50 μg of sucrose, maltose, lactose, glucose, fructose, galactose and ribose. No absorbance was observed in reaction mixtures using any of these sugars. No absorbance was obtained for 0.2 μmol each of alanine, arginine, aspartic acid, asparagine, α-aminobutyric acid, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine, and ammonium chloride. Neither ethanol or formic acid yielded any absorbance in reaction mixtures when assayed by this method.

Aconitate assayed in crude extracts of maize leaves was no different from aconitate assayed in extracts of the same material purified by passing through exchange resins (Table III). This indicated that the compounds found in crude extracts did not interfere with the aconitate assay. This might be predicted from the results obtained for the various organic acids, sugars, and amino acids that were assayed separately. This method was adaptable to plant extracts and could be used to assay crude extracts directly.

Analyses made on 80% ethanol extracts of leaves were not comparable to extracts that had been dried and suspended in water. The ethanol extracts were light green and high readings were obtained. This interference most likely came from the chlorophyll in the solutions. Chlorophyll was eliminated from the extracts by drying the samples and suspending them in water. A direct assay of aconitate in ethanol extracts of nonchlorophyllous tissue was possible. Filtered extracts of root material contained a considerable amount of colloidal material upon drying (reducing volume) and this interfered with the assay unless the suspensions were centrifuged. No differences were noted between root samples suspended in water or ethanol.

Trans-aconitate in corn represents about 95% of the total aconitate (MacLennan and Beevers, 1964; Clark, 1969). Any absorbance due to the cis isomer in corn tissue represents such a small amount of the total aconitate that this method is essentially a measure of *trans*-aconitate. This may be the case in other plants. In those plants where *cis*-aconitate represents a large portion of the aconitate level, *cis*-aconitate should be the standard for assays. Ratios of *cis*- to *trans*-aconitate may be required before quantitative amounts of the acid can be determined. If this is needed, this ratio may be determined by separating these isomers by gas chromatography (Clark, 1969) or by other chromatographic procedures (Molloy, 1969; Otter and Mehltretter, 1970).

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