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Determination of *cis*- and *trans*-Aconitic Acids in Plant Materials by Chromatography on Anion-Exchange Resins

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A method was devised for the measurement of *cis*- and *trans*-aconitates in plants. This method involves the separation of *cis*- and *trans*-aconitates by elution from a column of a strong anion-exchange resin in the bicarbonate form with an ammonium bicarbonate solution under mild conditions of temperature and pH. *cis*-Aconitate and *trans*-aconitate elute at positions different from those of all the other common naturally occurring organic acids. Quantitative measurement of the aconitates utilizes the formation of color from a mixture of pyridine and acetic anhydride. The chromatographic procedure was shown to avoid the isomerization of *cis*- and *trans*-aconitates. The method was applied to leaf extracts of wheat (*Triticum aestivum* L.) seedlings and gave greater than 98% recovery of added aconitate (5 μmol). Two lots of commercial *cis*-aconitate were contaminated with about 7.0% *trans*-aconitate whereas two batches of commercial *trans*-aconitic acid were over 99% pure.

Many plants can accumulate *trans*-aconitate (TA) to relatively high levels (e.g., up to 6% of the dry weight) (Burau and Stout, 1965; Stout et al., 1967), and this accumulation may be one cause of grass tetany (Mayland and Grunes, 1979). The magnitude of the accumulation can be affected by growing temperature (Stout et al., 1967), root zone temperature (Patterson et al., 1972), and mineral nutrition (Clark, 1968; Barta, 1973). We were interested in understanding the mechanism for aconitate accumulation in plants and required a method for measuring *cis*-aconitate (CA) and TA in plants. A search of the literature revealed that the isomerization of aconitate is facile (Krebs and Eggleston, 1944; Ambler and Roberts, 1948), that most organic acid methods subjected samples to conditions that would cause some isomerization, and that most analysts were more interested in obtaining values for total aconitate rather than accurate values for the individual isomers (Roberts and Ambler, 1947; Poe and Barrentine, 1968; Clark, 1972; Nelson and Rinne, 1977). Molloy (1969) developed a paper chromatographic method that minimizes isomerization. Burau's polarographic method (1969) avoids isomerization conditions except in the last step. He concluded that isomerization was not a serious problem by invoking the results of Ambler and Roberts (1948), whose method of measuring CA and TA is imprecise. Burau (1969) found that his polarographic method caused less isomerization than the silica gel chromatographic method of DeKock and Morrison (1958) and of Coic et al. (1961).

We have devised a procedure for separating CA and TA by salt elution from the bicarbonate form of a strongly basic anion-exchange resin under conditions that avoid isomerization (<0.5%). The separated isomers were quantitated by a colorimetric method.

MATERIALS

CA, TA, *cis*-aconitic acid anhydride (CAA), and *trans*-aconitic acid anhydride (TAA) were purchased from Sigma

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Chemical Co., St. Louis, MO. Analytical grades of pyridine and acetic anhydride were used. Any colored pyridine was decolorized by distillation.

Strongly Basic Anion-Exchange Resin in the Bicarbonate Form. Dowex I-X8 (200–400 mesh, 8% divinylbenzene, fines removed, chloride form) was placed in a column and washed with 1 M $NaHCO_3$ (prepared without heating) until the effluent was free of chloride ions. After the resin had been thoroughly washed with water, it was ready for use. If the resin was to be packed in 50% ethanol, it was resuspended in 50% ethanol several times before packing the column.

Isomerized *trans*-Aconitate for Standard Solution. Dry TA-H^+ was dissolved in sufficient 1 M HCl to give a 10 mM solution. This solution was heated at 100 °C for 4 h and then dried. The residue was dissolved in water and neutralized with NH_4OH to pH 8.0–9.0. The stock solution (0.5 mM) was kept frozen.

NH_4HCO_3 Solution. NH_4HCO_3 solution (1 M) was prepared from analytical-grade crystals in glass-distilled water [avoid heating that converts NH_4HCO_3 to $(NH_4)_2CO_3$]. The stock solution was diluted to 0.49 ± 0.005 M as measured by titration. The concentration giving the best separations may vary with each batch of anion-exchange resin.

METHODS

Preparation of Extracts of Wheat Leaves. Wheat seeds (*Triticum aestivum* L., cv. Tascosa) were germinated and grown on 0.5 mM $CaSO_4$ plus 5 mM K_2SO_4 for 7 days in continuous light (500 ft-c). The leaves were excised, cut into 1-cm segments, weighed, and covered with liquid nitrogen in a chilled mortar. The frozen leaves were pulverized, covered with 50% ethanol (0 °C) (about 5 mL/g fresh weight), and then thoroughly ground. The insoluble material was removed by centrifugation at −10 °C and reextracted four times in the same way. Three additional extractions resulted in less than 0.1% additional aconitate (data not shown).

Colorimetric Methods for the Measurement of Aconitate. We have devised a method for the measurement of aconitate by optimizing the Molloy procedure (1969) wherein a color is formed from a 9:1 (v/v) mixture of pyridine and acetic anhydride (PA).

Aconitate in 50 μL of aqueous solution was placed in a test tube along with 1 mL of absolute ethanol. Alternatively, a dry sample was shaken with 50 μL of water, and then 1 mL of absolute ethanol was added. After the mixture was cooled to 0 °C, 4 mL of cold (0 °C) PA was added to each tube and the solution mixed. Samples were kept at 0 °C in the dark until absorbance was measured at 387 nm (Figure 1) at 40 ± 2 min after the addition of PA. The color increased gradually up to about 2 h and then faded. Absorbance measurements could be made at other times as long as the time was the same for samples and standards. The standard curve followed Beer's law up to an absorbance of 1 (data not shown).

By comparison with Molloy's published data (1969), this method was about 20 times more sensitive for CA and 2.5 times more sensitive for TA (Table I).

Colorimetric Method for the Measurement of Fumarate and Malate. Fumarate and malate were determined by a modification of the aconitate method, which involved mixing a dry sample with 5 mL of PA and measuring absorbance at 387 nm after 24 h at room temperature in the dark.

Chromatography and Measurement of *cis*- and *trans*-Aconitates and Fumarate. *cis*- and *trans*-aconitates and fumarate were readily separated by chromatography on a strongly basic anion-exchange resin in the bicarbonate form with a solution of NH_4HCO_3 (Figure 2). A 50 × 0.4 cm column of Dowex I-X8 (HCO_3 form, 200–400 mesh) was packed at room temperature. The column was chilled to 0–4 °C, a neutral (pH 6–8.5) aconitate solution (containing 2–20 μmol of each acid) was applied, and the resin was washed with one column volume of water. The acids were eluted from the resin with cold 0.49 ± 0.01 M NH_4HCO_3 (see Discussion concerning critical concentrations). The use of mild pressure (up to 0.05 bar) hastened elution without affecting separations.

Two procedures were used to isolate CA and TA. In the first procedure, the column eluate was collected as 80–100 fractions of about 1 mL. Aliquots (50–400 μL) of each fraction or every other fraction were removed to test tubes (13 × 100 mm) and dried in vacuo in a desiccator containing solid NaOH and concentrated H_2SO_4. The dry

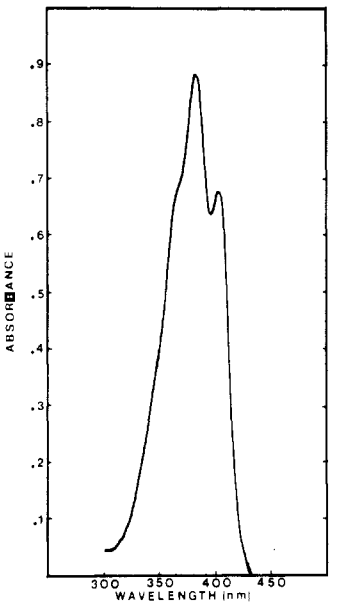

Figure 1. Spectral curve of the reaction product from ammonium *cis*-aconitate (40 μM) and pyridine–acetic anhydride. The curve for *trans*-aconitate was similar to that of *cis*-aconitate.

Table I. Molar Extinction Coefficients of the Product of Reaction of Organic Acids with Pyridine–Acetic Anhydride at Different Times[a]

		molar extinction coeff at time after PA addn				
acid	form	40 min	2 h	4 h	24 h	Molloy[b]
CA	H^+	25600	25600	25200	11300	900
CA	NH_4^+	24500	24300	22100	10800	
CA	Na^+	26350	26250	23800	10850	
CA	Ba^{2+}	1840				
TA	H^+	20000	20100	18300	9400	8300
TA	NH_4^+	20100	20150	18500	9400	
TA	Na^+	19250	19400	18100	8700	
IA	H^+	19700	19800	17900	9100	
IA	NH_4^+	20100	20200	18300	9400	
IA	Na^+	19150	19300	17700	8500	
fumaric	H^+	229	224	212	124	
fumaric	NH_4^+	217	214	204	115	
citric	H^+	9730	11700	11180	4670	
citric	NH_4^+	9580	11520	11170	4800	
isocitric	H^+	3630	4550	4290	1770	
isocitric	NH_4^+	4250	5220	4930	2120	
itaconic	H^+	15050	14780	13750	6450	
itaconic	NH_4^+	14450	14120	13200	6380	
maleic	H^+	12	11	12	5	
maleic	NH_4^+	10	8	8	4	

[a] Glycolic, oxalic, succinic, tartaric, malonic, malic, and quinic acids gave no color. [b] Data taken from Molloy (1969).

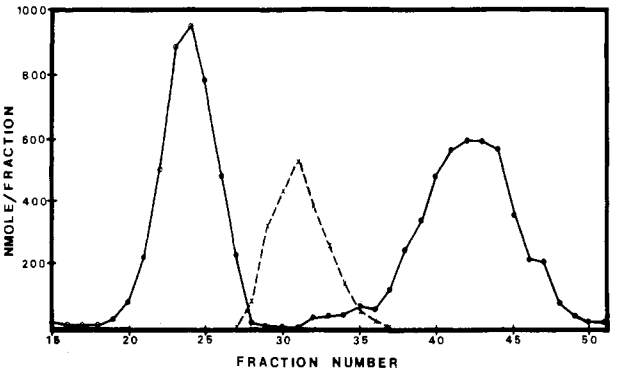

Figure 2. Elution profile of CA, TA, and fumarate from a column of Dowex I (HCO_3 form) eluted with NH_4HCO_3. Five micromoles of CA, TA, and fumarate were chromatographed according to the procedures of this paper. The acids were eluted with 0.495 M NH_4HCO_3, and 1.05-mL fractions were collected. Two-fifths of each fraction was assayed by the method specified for the determination of fumarate and malate. The elution profiles for CA (first peak) and TA (third peak) are represented as solid lines. The dashed line (with each data point designated as an ×) presents the elution pattern for fumarate.

residue was dissolved in 100–200 μL of 25% ethanol and redried as before. The latter operation was repeated until there was no visible NH_4HCO_3 residue (usually once or twice more). If there was more than 5 μmol of CA or TA present in the sample, 50-μL aliquots could be tested directly without drying. The aconitate in each fraction was measured to locate the CA and TA peaks. The remainder of the solution in each fraction containing the CA or TA was combined. These combined fractions were dried and redried, after the residue was dissolved in 50% ethanol, until the NH_4HCO_3 had evaporated.

In the second procedure, four fractions were collected. One fraction was collected before the CA peak and contained most naturally occuring organic acids (except fumarate, CA, and TA). A second fraction contained the CA while a third fraction contained fumarate and a fourth fraction contained TA. The volumes for the four fractions were determined on a comparable column tested by the first procedure. The CA and TA fractions were dried and redried (with 50% ethanol) and treated as below.

The dried CA and TA from either procedure were transferred with 1 M HCl to a test tube and were isomerized by heating 2 h at 100 °C. This solution was adjusted to a convenient volume; proper aliquots were removed to 13 × 100 mm test tubes and dried. The amount of IA (isomerized aconitate) present was determined by comparison to a standard curve prepared concomitantly with 0–300 nmol of IA-NH_4^+.

If the samples had appreciable color, a blank correction was made by treating a duplicate aliquot as described above except that 4 mL of pyridine was substituted for 4 mL of PA.

Analysis of CAA and TAA. Commercial CAA and TAA were dissolved in cold water and neutralized immediately to pH 7–8.5 with NH_4OH. The resultant solutions were analyzed by the above chromatographic procedures. The CAA and TAA peaks, which elute prior to the CA peak, were analyzed separately. After removal of NH_4HCO_3 by drying, the anhydrides were hydrolyzed and isomerized by heating for 2 h at 100 °C in 1 N HCl. The HCl was removed by evaporation and aconitate measured colorimetrically.

Determination of *cis*- and *trans*-Aconitates in Plant Material. Plant material was frozen, pulverized, and extracted as presented above. A column of Dowex I (HCO_3 form, 50 × 0.4 cm) was packed in 50% ethanol and cooled to 0 °C. The 50% ethanol extract was passed through the resin at 0–4 °C as rapidly as conveniently possible (using up to 0.20-bar pressure), preferably within 16 h. The resin was washed with one column volume of 50% ethanol, one column volume of 25% ethanol, and two column volumes of water.

The organic acids were eluted from the resin by the procedures outlined above. The fractions or portions of fractions containing CA or TA were combined. The NH_4HCO_3 was removed by repeated drying. TA and CA peaks were isomerized and measured as in the previous section.

Most plant organic acids elute ahead of CA, but fumarate elutes between the CA and TA peaks (Figure 2). Since fumarate reacts poorly (in comparison to CA and TA) with PA (Table I), and because separation from CA and TA is essentially complete, it does not interfere with the measurement of CA and TA.

Table II. Recovery (%) of *cis*- and *trans*-Aconitates

treatment	*cis*-aconitate	*trans*-aconitate
none	99.5 ± 1.0 (6)[a]	98.2 ± 1.0 (6)
added to wheat leaf extr	97.8 ± 1.7 (5)	99.5 ± 3.2 (4)

[a] Average recovery ± standard error. The number in parentheses is the number of samples analyzed. Samples of CA (5.2 μmol) or TA (5.5 μmol) were analyzed by the procedures of this paper. The same quantitites of CA and TA were added to a wheat leaf extract having about 1.2 μmol of CA and 5.5 μmol of TA.

RESULTS

Evaluation of This Method. The validity of these procedures was tested by measuring the recovery of CA and TA alone and when added to a wheat leaf extract. The results in Table II show that recovery of CA and TA either alone or added to a leaf extract was better than 98% when about 5 μmol of CA or TA was analyzed. We found that accuracy was increased when the procedure was scaled up and, conversely, accuracy decreased with less CA or TA.

Another important consideration is the amount of isomerization that occurs during the chromatographic procedure. We have prepared two batches of CA-Ba^{2+} by unpublished procedures and found that they contained 99.9 and 99.8% CA. Since isomerized aconitate is predominantly in the TA form (Ambler and Roberts, 1948), this result indicated that these procedures cause little, if any, isomerization of CA. The lack of isomerization is undoubtedly due to the fact that procedures keep the pH near neutrality and at low temperatures (ca. 0 °C).

Analysis of Commercial Samples of Aconitate. We examined two commercial samples each of CA and TA (Sigma) and found TA to be 99.1 and 99.9% pure while CA was 93.0 and 93.5% pure. One unexpected result of this study was that neutralization of the pure anhydrides to pH 8.0–8.5 did not hydrolyze all the anhydride. Both CAA and TAA gave a separate peak that eluted ahead of CA in these procedures. The identity of the peak was confirmed by its isolation and hydrolysis to CA and TA as demonstrated by chromatography. Hydrolysis of the CA peak showed that 57.2% remained as CAA after neutralization and 98.0% of the hydrolyzed material was CA. When a similar test was performed on TAA, 29.3% remained as TAA and 83.4% of the hydrolyzed material was TA.

Separation of Other Organic Acids. We chose NH_4HCO_3 as the eluting salt in the chromatography because it is readily removed by volatilization. In the course of these investigations, we observed that nonvolatile organic acids could be detected by measuring the ammonium

ion left after evaporation of NH_4HCO_3 by the phenol–hypochlorite method of Weatherburn (1967). The NH_4HCO_3 was sufficiently volatile that fractions without organic acid had the same color as those without NH_4HCO_3. The ammonium assay provided a simple qualitative assay for organic acids but was unsuitable as a quantitative measurement because some ammonium volatilized from the organic acids.

Since the ammonium assay provided a general method for locating organic acids, it was convenient to try the above chromatographic procedure for the separation of other naturally occurring organic acids. This procedure separated citric acid and malic acid plus succinic acid, as well as CA, fumaric acid, and TA. The various acids in standard mixtures and in wheat leaf extracts were readily detected by measurement of residual NH_4^+. After the acids had been located, the remainder of the fractions from each peak were combined and dried until the NH_4HCO_3 was removed. Acids were identified by position. Citric acid was determined as specified for aconitate determination except that color was measured 2 h after the addition of PA. Fumarate and malate were measured as described above. Other acids were measured by converting ammonium salts to free acids by passage through a column of Dowex 50 (H^+ form), concentrating by drying, and measuring the color formed with an indicator (Prior et al., 1973).

DISCUSSION

The procedures presented in this paper were designed to obtain accurate values for CA and TA, which have been difficult because of the ease of isomerization (Krebs and Eggleston, 1944; Ambler and Roberts, 1948). The procedures do not provide quick results, but they are relatively simple and do not require unusual apparatus (e.g., a polarograph). In the chromatographic procedure, we suggested collecting many (80–100) small fractions or four relatively large ones. Although the latter is the preferred method, we found it somewhat irreproducible even though conditions were kept as similar as possible.

We have specified that 0.49 ± 0.01 M was an appropriate concentration for the eluting NH_4HCO_3. This concentration was chosen as a compromise between separation and sharpness of peaks. The appropriate concentration varied among batches of Dowex-1, due to differences in exchange capacity. Once a suitable concentration has been chosen, it needs to be verified periodically to maintain the concentration within ±0.005 M of the desired concentration. The critical nature of the NH_4HCO_3 concentration for separation is illustrated by the fact that we found the top of the CA peak at 33.0 mL with 0.495 M and at 38.9 mL with 0.480 M NH_4HCO_3.

Proper testing of methods for measuring CA required pure samples of CA that have been unavailable commercially. As a result of this study, we have been able to devise a method of preparing nearly pure CA (unpublished results). The availability of pure CA enabled us to show that our procedures gave accurate analyses for CA and TA in plant samples as measured by recovery tests (Table II).

The specificity of these procedures depends on the fact that *trans*-aconitate is retained more tightly on strong anion-exchange resins than other naturally occurring organic acids. The stronger binding of TA as compared to CA is presumably the result of the lower pK_3 for TA than CA (pH 4 vs 6.5) (Pratt and Smith, 1966; Klinman and Rose, 1971). The stronger retention of CA than most naturally occurring acids other than fumarate is probably due to the greater number of carboxyl groups in CA than in succinic and malic acids and its lower polarity and higher acidity than acids like citric and isocitric. Of the usual organic acids found in plants (oxalic, malonic, malic, succinic, itaconic, glycolic, tartaric, citric, quinic, shikimic, isocitric), none interfered with the determination of CA and TA.

When mild conditions are utilized for separation, isomerization of aconitate is virtually eliminated. Once the isomers are separated, the possibility of subsequent isomerization is not a concern.

The analysis of commercial CA and TA showed that TA is essentially pure (>99%) but that CA is substantially (≃7%) contaminated with TA. This difference may well derive from the fact that, at equilibrium, TA is the predominant isomer (Ambler and Roberts, 1948) and that recrystallization from water can result in pure crystals. Recrystallization of CA may not result in pure crystals due to isomerization during heating (Thomson et al., 1966). Thomson et al. (1966) found one sample of CAA contaminated with 6% TAA.

The analysis of CAA and TAA gave two interesting results: First, a substantial percentage of the anhydrides remained intact at pH 8.5 even through lengthy (24-h) chromatography. Presumably the maintenance of the solution at 0–4 °C reduced hydrolysis. Second, the apparent purity of CAA was substantially better than that of TAA. However, this result may be misleading if there is a differential rate of hydrolysis of CAA and TAA.

It was noteworthy that the TAA did not appear to be as pure as CAA, since commercial TA is purer than CA. However, this could be due to lot to lot variation or to an artifact of hydrolysis.

These procedures were suitable for separating fumarate from other organic acids and can be used as a sensitive, specific method for fumarate. However, as shown in Table I, fumarate did not yield nearly as much color as CA and TA in the procedure designed for CA and TA measurement. However, a modified procedure for fumarate (and malate) (see Methods) yielded considerably more color (≃5-fold).

There has been much interest in measuring TA because it is found in sugar cane and sorgo juice and interferes with crystallization of sucrose. In this situation where TA is by far the most abundant organic acid, separation of aconitate from other organic acids is unnecessary and analytical methods can be relatively simple (Roberts and Ambler, 1947; Poe and Barrentine, 1968).

CA and TA can be relatively sensitively and specifically measured with a polarograph (Burau, 1969), but this method requires careful preparation of sample and attention to assay conditions as well as special equipment.

Interest in metabolism and accumulation of organic acids has resulted in a number of chromatographic methods. These methods depend on chromatography on silica gel (DeKock and Morrison, 1958; Coic et al., 1961; Prior et al., 1973; Bulen et al., 1952), chromatography on an anion-exchange resin using a volatile acid as eluant (Busch et al., 1952; Palmer, 1955; Von Korff, 1969), or gas–liquid chromatography (Barta and Osmond, 1973; Sasson et al., 1976; Burke et al., 1985). Since these methods were not designed specifically to measure CA and TA, they either expose aconitate to isomerizing conditions of pH and temperature, are slow, or require derivatization. In addition, none of these methods was tested for the amount of isomerization or recovery of aconitate isomers.

We tested the methods reported in this paper with a wide range of naturally occurring organic acids and have been able to separate citrate, succinate plus malate, CA, fumarate, and TA. It is likely that separations could be

improved by use of longer resin beds, a finer resin, and a salt gradient for elution. Modest attempts to use HPLC to measure CA and TA have been unsuccessful.

The original method of Molloy (1969) for measuring aconitate was optimized to make it more sensitive (see Table I) and less time consuming. The colorimetric procedure presented in this paper is simple, reproducible, and sensitive. It is not specific for CA and TA because other acids give appreciable color.

ABBREVIATIONS USED

TA = *trans*-aconitic acid or its salt (e.g., TA-H^+ = *trans*-aconitic acid, TA-Na^+ = sodium *trans*-aconitate, etc.); CA = *cis*-aconitic acid or its salt (other forms analogous to *trans*-aconitate; e.g., CA-H^+, Ca-Na^+, Ca-NH_4^+, etc.); IA = isomerized aconitic acid or its salts; CAA = *cis*-aconitic acid anhydride; TAA = *trans*-aconitic acid anhydride; PA = pyridine–acetic anhydride (9:1, v/v).

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Stephen Schaefer provided valuable assistance.

Registry No. *cis*-Aconitic acid, 585-84-2; *trans*-aconitic acid, 4023-65-8; pyridine, 110-86-1; acetic anhydride, 108-24-7; fumaric acid, 110-17-8; citric acid, 77-92-9; isocitric acid, 320-77-4; itaconic acid, 97-65-4.

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