Tables II and III show the quantitative recovery of [^{3}H]estradiol and [^{3}H]estrone added to bovine plasma and tissue extracts under the column conditions in Table I and the purification scheme in Figure 2. Statistical analysis demonstrated excellent reproducibility (<3% coefficient of variation) and high accuracy (>95% mean recoveries of [^{3}H]estrogens added to plasma and tissue extracts). Water and ether extraction of the HOAc column effluents did not significantly ($P < 0.05$) change the mean recoveries.

To determine the efficiency of the purification columns, the acetone extracts of the tissues were spiked with [^{3}H]estradiol and yielded near-quantitative (95%) recoveries. However, when tissues were spiked with [^{3}H]-estradiol prior to extraction, recoveries ranged only from 65 to 75% after exhaustive extraction (i.e., sonication and centrifugation three times). The loss of 25–35% is apparently due to irreversible binding of the added estrogen with tissue components. This phenomenon was also reported by Metzler (1981) and Gridley et al. (1983). Therefore, adding this 25–35% loss to actual values measured can overestimate results. The amount of estrogen resulting from this extraction technique can only be interpreted as extractable estrogens, and results in suspect samples should be compared to estrogen levels in tissues of untreated animals.

The use of this cleanup procedure has shown that there is no background interference with 17β-estradiol, DES, or zeranol when these anabolic estrogens were fortified in avian muscle tissue extracts and analyzed by normal-phase HPLC equipped with a UV detector (Medina and Sherman, 1986). A multiresidue TLC screening analysis of anabolic estrogens fortified in avian muscle tissue extracts and purified by this cleanup technique also showed no background interference band with estradiol, DES, or zeranol (Medina and Schwartz, 1986). The procedures for HPLC or TLC analysis of anabolic drugs were also developed in our laboratory and are described in detail in these references.

This cleanup technique offers a rapid extraction and isolation procedure allowing the preparation of at least 10 columns in less than 2 h and sample purification in less than 1 h. The materials used are inexpensive (25¢/column), and the simplicity, speed, and accuracy of this technique make it suitable for routine chemical analysis.

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Registry No. Estradiol, 50-28-2; estrone, 53-16-7.

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Monitoring Organic Acids and Carbohydrates in Cotton Leaves by High-Performance Liquid Chromatography

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Extraction methodology, sampling procedures, and separations by high-performance liquid chromatography (HPLC) have been developed for analyzing organic acids and carbohydrates found in cotton leaf tissue. The major components sucrose, glucose, malic, and citric acids have been quantitated with calculations based upon fresh weight, dry weight, and leaf surface area. Automated HPLC analyses allow efficient screening and monitoring of large numbers of plant samples for the compounds of interest. Profiles by HPLC of various cottons are being used to characterize responses of plants to stress environments such as drought.

INTRODUCTION

Our research objective is to determine qualitative and quantitative alterations in chemical compositions of crop plants induced by stress environments (such as drought) that correlate with continuance of agronomic growth under stress conditions. Organic acids and carbohydrates have been reported to provide adaptive behavior in response to water-deficit conditions. Among the many acids found in plants, certain ones, namely malic, citric, and oxalic acids, are frequently found in large amounts in mature tissues in plants (Beevers et al., 1966). In cotton leaves, citric and malic acids comprise 5–10% of dry weight (Ergle and Eaton, 1949) and have been reported to be the only two

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acids present in more than trace quantities (Cutler and Rains, 1978; Radin and Parker, 1979). Leaves typically have greater concentrations of these components than other vegetative parts. The major carbohydrate components in cotton leaves are sucrose and glucose in amounts ranging from 0.2 to 1% of fresh weight (or 4–9% of dry weight) (Eaton and Ergle, 1948) with diurnal levels ranging from 25 to 100 g/cm^2 (Ackerson, 1981). Organic acids have been extracted from dried, ground plant materials under strongly acidic conditions or with 80% ethanol followed by lengthy extraction with ether (Ergle and Eaton, 1949; Beevers et al., 1966). Carbohydrates have been extracted immediately from fresh cotton leaf material with water or ethanol (Eaton and Rigler, 1945). Alternately, cotton leaf samples were quick-frozen on dry ice, extracted in 80% ethanol, and subsequently evaporated to dryness under vacuum (Ackerson, 1981). After extraction with ether, the water-soluble portion was analyzed for glucose and reducing sugars. Purification of the saccharides after extraction has been primarily by column chromatography. For analysis of sugars in wheat, leaves were placed in liquid nitrogen and then freeze-dried and extracted in boiling water (Azcon-Bieto and Osmond, 1983).

Enzymatic determinations of carbohydrates achieve high sensitivity and specificity (Ackerson, 1981), but that specificity places a limitation on the scope of possible components present that may be identified in a complex product mixture in a single determination. Gas chromatographic techniques have been employed because of sensitivity and automation (Heatherbell, 1974). The disadvantages of gas chromatography are in requiring volatile compounds for separation that generally necessitates derivatization requiring additional sample preparation with potential loss (McGinnis and Fang, 1980). Recently, high-performance liquid chromatography (HPLC) has been applied for carbohydrate and organic acid analyses employed in fermentation, food, and plant research because of the ease of sample preparation, automation, and good quantitation (Abeydeera, 1983; Fales et al., 1982; Scobell et al., 1979). This report describes the extraction and methodology for rapid screening of organic acids and carbohydrates in cotton leaf tissue to determine varietal characteristic profiles and changes induced by stress environments.

EXPERIMENTAL SECTION

Reagents. The carbohydrate and organic acid standards were obtained commercially and used without purification. Comparisons were made with solutions (0.2% w/v) prepared for carbohydrates commonly occurring in plants and organic acids of the tricarboxylic acid cycle. Of these, the following standards were used for reference: acetic acid, citric acid, and oxalic acid (Fisher); ascorbic acid, formic acid, and raffinose (Eastman); cellobiose and fumaric, succinic, and isocitric acids (Sigma); fructose (Baker); D-glucose (Mallinckrodt); malic acid (Allied). Three concentrations of each standard were prepared (1.0–0.1% w/v) by serial dilution. The water was deionized (MilliQue, Millipore, pH 5, conductivity 0.016 Ω^{-1}). Sulfuric acid (MCB) and absolute ethanol (U.S. Chemicals) were diluted with deionized water. The polyvinylpolypyrrolidone (PVPP, Sigma) was the insoluble form.

Plant Materials. Cotton seeds were planted in flats (57 cm × 57 cm × 15 cm) at a density of 50 seeds per flat (variety T25). Samples were taken from plants at 30, 60, 75, and 80 days after planting (DAP). Stressed (nonirrigated) plants (T25) that had been field grown to 108 DAP were evaluated for removal of phenolics. Punches (disks) made by #5 cork borer (0.8-cm diameter) were taken from at least eight leaves at the same leaf position from different plants to randomize the distribution. Twelve punches or disks cut between the large veins of leaves were used per sample to maintain the same surfaces area for all samples.

Extraction Procedure. Duplicates for each sample were taken in all cases. The leaf samples were placed in tared 7-mL polyethylene scintillation vials (with cap, Kimble) and weighed for the fresh weight. The leaf tissue was frozen in liquid nitrogen by suspending the vials in liquid nitrogen and then pulverized to a fine powder with a large-diameter glass rod (1-cm diameter × 11-cm length). Phenolics were removed by grinding with PVPP in the amount equivalent to or twice that of the fresh weight of the sample. After the sample was allowed to come to room temperature, addition of 3 mL of solvent was made, followed by homogenization (Tissumizer SDT100EN, Tekmar) for 1 min to effect extraction. A low-cost nonbreakable sample vial permitted in situ grinding in liquid nitrogen and subsequent homogenization without transfer during the processing, thereby reducing sample losses. Vial caps provided sealing during storage. The volume of extraction solvent was determined by trial and error to maximize the amount of material extracted but to minimize the volume required for effective processing through multiple steps.

Preparation for HPLC analysis requires only filtration if no halide ions are present and the sample is dissolved in compatible medium (Snyder and Kirkland, 1982). To filter single samples, solid plant parts were separated by centrifugation in the vials with the supernatant and then filtered through a Swinney (Millipore) fitted with a fiber glass prefilter (AP 25, Millipore) and a 0.5-μm filter (type FH, Millipore). More efficiently, we filtered multiple samples simultaneously by outfitting a commercially available vacuum extraction apparatus (Model 10, Baker) with disposable 0.45-μm filters (Acrodisc, Gelman) onto which 5-cm^3 disposable syringes (BD) with prefilter (AP24, Millipore) were attached. Sample vials (1 mL, Wheaton) were placed inside the extraction system rack to receive the filtered sample. Up to 10 samples could be handled simultaneously. The homogenized sample were transferred directly from the plastic vial into the syringe/filters and filtered successfully into sample vials ready for the HPLC autosampler, eliminating the need for centrifugation. The expense of the disposable filters is offset by the increased speed with which to handle large numbers of samples and the lack of possible contamination from poor cleaning of the Swinney filter apparatus. Standards were processed through the procedures to verify that no losses or alterations had occurred.

For those samples that were refluxed, heating was carried out on a magnetic stirrer/heater with heating block with thermistor control (Combimag, IKA). Samples were stored in −80 °C freezer (So-Low, Environmental Equipment). To obtain the dry weight of sample, the material was dried to constant weight in a microwave oven (Model 564.8878420, Sears).

Chromatographic Procedure. A Beckman HPLC system (Model 322) was used: a dual-piston pump (Model 100A); 20-μL injection loop in the autosampler (Model 500; refractive index detector (Model 155); variable-wavelength-scanning UV–visible detector (Model 165) with microprocessor-based, programmable system controller (Model 421). The mobile phase was 0.014 N sulfuric acid (pH 2.1). The acid was filtered twice through 0.45-μm filters (type HA, Millipore) and degassed by sonicating and drawing a vacuum on the flask simultaneously. The "organic acid" column (Aminex HPX-87H, BioRad) was

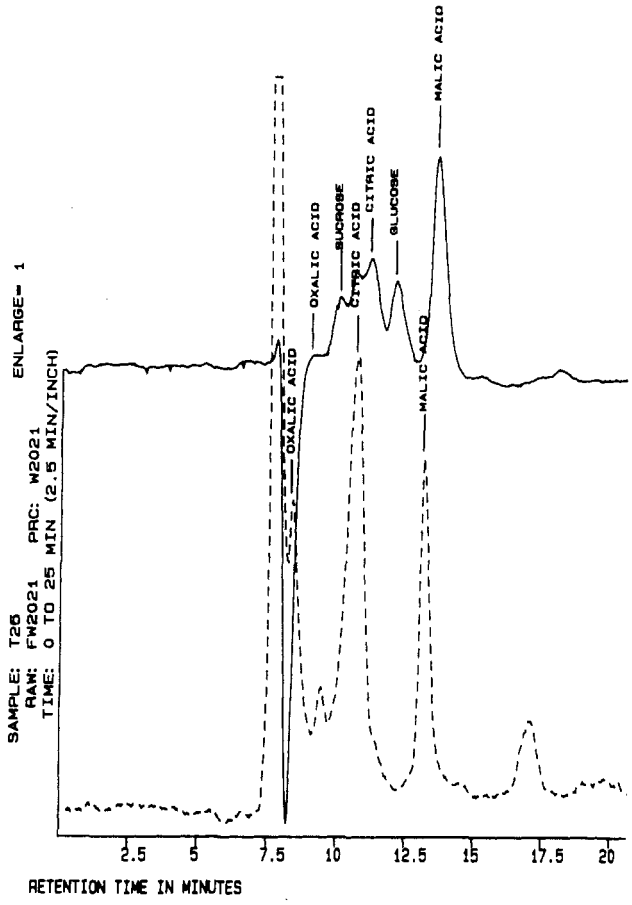

Figure 1. HPLC analysis of aqueous extract of cotton leaves, 7th node, 75 DAP: Simultaneous outputs from RI (—) and UV at 210 nm (---).

a cross-linked polystyrene ion-exchange resin in the hydrogen form optimized for organic acid and/or carbohydrate, fitted with a guard column of similar material (MicroGuard, ion exchange, BioRad). The column was maintained at 30 °C by a column heater (Model LC22, Bioanalytical Services). Detection with the UV–vis 165 detector was conducted at wavelengths of 210 and 254 nm accompanied by automatic rapid scan of peaks detected on the 210-nm channel over the range from 190 to 700 nm at a rate of 20 nm/s. Analog/digital (A/D, Model 18652A, Hewlett-Packard) conversion of detector outputs facilitated on-line data collection by computer (Laboratory Automation System, Model 3357, Hewlett-Packard). Integration was by areas under peaks, and retention times were measured from the time of injection. Levels of sample components were quantitated from response curves for the appropriate external standard.

RESULTS AND DISCUSSION

The reproducibility of the fresh weights of the sample punches was good. For 12 leaf disks as used throughout the study, the average fresh weight ranged from 0.1 to 0.15 g for cotton leaves at 30, 60, 75, and 80 DAP with coefficients of variation (CV) of 2–4% and standard deviation ranging from 0.002 to 0.004 for each data set depending upon the age of the plants. The corresponding dry weights of the samples were from 0.024 to 0.029 g with standard deviation of 0.0001–0.0005 with CV = 1–2%.

Standard mixtures of the five major organic acid and carbohydrate components in plants (malic, citric, and oxalic acids, sucrose, and glucose) were analyzed by HPLC to evaluate the effectiveness of separations at different column temperatures and flow rates. Samples from mature

Table I. Organic Acid/Carbohydrate Analyses by HPLC of Leaves (7th Node) from T25 Cotton, 80 DAP, Extracted with 3 mL of Water (Fresh Weight 0.1077 g, Dry Weight 0.0269 g, Surface Area 6.04 g)

component	CV,[a] % (area)	mg/mL	mg/sample	mg/g fresh wt	mg/g dry wt	μg/cm²
sucrose	5.2	0.21	0.64	6.0	23.9	106
glucose	9.1	0.18	0.54	5.1	20.3	89
citric acid	3.4	0.55	1.65	15.3	61.2	273
malic acid	5.9	0.38	1.15	10.6	42.6	191

[a] Coefficient of variation.

Table II. Comparison of Effectiveness of Extraction Conditions

solvent	temp	amt matl extr	solvent	temp	amt matl extr
water	ambient	1.0	alcohol	ambient[b]	0.5
water	reflux[a]	0.35	alcohol	reflux	0.25

[a] After either grinding in liquid nitrogen and homogenizing or refluxing directly. [b] Either single or multiple extractions.

cotton leaves extracted with both water and alcohol were also employed in judging the separations. Conditions at column temperatures 25, 30, 40, 60, and 85 °C and flow rates 0.5, 0.6, and 0.8 mL/min were examined. A column temperature of 30 °C and flow rate of 0.5 mL/min were deemed most acceptable. In every case, certain trade-offs were necessary because there was insufficient resolution of peaks for either standard or sample usually manifested as overlap of two major peaks monitored by the RI detector. Because of the linearity of response for a wide range of carbohydrates (McGinnis and Fang, 1980), i.e. direct correlation of concentration with peak area, it is desirable to quantitate based on the RI. If the overlapping peaks are comprised of an organic acid and a carbohydrate, the UV peak can be used for quantitation of the organic acid with calculation of the carbohydrate from the total RI peak by difference using the appropriate standard response curves for the individual components.

The HPLC profile of the organic acid/carbohydrate components obtained from the aqueous extraction of cotton leaves is shown in Figure 1. A time of 15–20 min is adequate for sample analysis. The outputs from the RI and UV (210 nm) detectors are superimposed. The major components of sucrose, glucose, and citric and malic acids in the chromatographic separation are labeled. The slight offset in retention times (RT) between the two detectors comes from the tandem configuration. The first large peak detected by UV (rT = 7.5 min) corresponds to excluded material that has not been identified but is under continued study. Trace amounts (0.1% of fresh weight) of oxalic acid could be detected for this sample. Succinic acid is estimated to be present in small amounts as evidenced by the UV peak at RT 17.2 min.

The calculated composition of the aqueous extract from cotton leaves at 75 DAP from HPLC analyses is given in Table I. These values are the average of two different aqueous extractions of the same batch of cotton leaves with two HPLC runs per extraction. The quantities of the various components fall within the ranges quoted earlier. The sucrose values were precise, but their accuracy must be verified by other methods. The presence of fructose was investigated since it would be likely to be present if sucrose is detected. Fructose and malic acid coelute under these chromatographic conditions. However, calculation of the amount of malic acid from both UV and RI peaks tends to support the supposition the only trace amounts of fructose are present.

Comparison of the effectiveness of the extraction procedures and solvents for extraction made by HPLC analyses is given in Table II. Summarizing the extraction studies, the ambient aqueous extraction was the simplest and proved to be most effective in both amount of individual components solubilized and overall profile of components. Another reason for preference for aqueous extractions over using organic solvents would be the potential denaturing of some proteins by organics during the operation, thereby exposing polar sites with possible affinity for free carbohydrates and/or organic acids or possible degradations releasing free carbohydrates into solution (Ap Rees, 1980). Both cases would give inaccurate assessment of the true soluble carbohydrate and organic acid compositions found in the plant.

In any operation, establishing nondegradative storage conditions for handling at various stages of these analyses was vital, particularly for dealing with field-supplied samples. Leaf samples stored at −80 °C were significantly more stable than samples that were stored at −4 °C or those that were stored at room temperature after extraction and filtration.

Large amounts of phenolics may be present in cotton leaves and can interfere with analyses particularly enzymatic assays (Todd, 1972). Removal of phenolics was addressed at several points in the preparation (Loomis and Battaile, 1966; Walker, 1980). The presence of the phenolic components is manifested in the size of the excluded material peak in the HPLC profile. We used PVPP in grinding at the earliest stages as recommended in enzymatic preparations but also evaluated use after homogenization of PVPP in minicolumns for the vacuum extraction device concurrent with the filtration step. The excluded peak in UV was evidently not reduced as much as with incorporation at the grind so the incorporation at the earliest stages was determined to be best. The amount of phenolics was significantly less with the young plants so that the procedures were checked with field-grown stressed samples with large amounts of phenolics. For those crops or samples such as wheat that have less phenolics, use of PVPP minicolumns prepared in the laboratory or commercially available minicolumns for extraction of phenolics should be satisfactory.

Variations in organic acid and carbohydrate composition can be expected to occur for differences in the variables such as variety, age of the plants, growing conditions, and time of sampling. In this study, we have noted differences in these compositions with age of the plants but confined our comparisons within samples at the same age. Profiles by HPLC of the organic acids and carbohydrates found in leaf tissue from cotton are being used to characterize and compare varieties grown under various conditions in the field as well as in the greenhouse in order to ascertain overall similarities in addition to compositional differences and will be the subjects of future reports. These procedures have been developed for organic acid/carbohydrate analyses of cotton leaves, but it is not unreasonable to assume the validity of the methodology for other plants and plant parts.

CONCLUSION

Sampling procedures and methodology to obtain the major accumulated organic acids and carbohydrates from cotton leaves have been achieved, minimizing handling and transfer. Comparison with alcoholic and refluxing conditions indicated the advantages of ambient aqueous extraction. Chromatographic conditions for separation profiles have been determined with simultaneous detection of organic acids and carbohydrates using refractive index and variable-UV channels under automated conditions. Analyses by HPLC of leaf tissue from cotton demonstrated the presence of the major components of glucose, sucrose, and malic and citric acids that have been quantitated. calculations based upon fresh weight, dry weight, and leaf surface area are given for comparison. These procedures provide means for efficient screening and monitoring of large numbers of plant samples for the compounds of interest to characterize and compare cotton plants grown under variable conditions.

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Registry No. Sucrose, 57-50-1; D-glucose, 50-99-7; citric acid, 77-92-9; malic acid, 6915-15-7; oxalic acid, 144-62-7.

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