Determination of Organic Acids in Tall Fescue (*Festuca arundinacea*) by Gas–Liquid Chromatography

Richardo L. Boland and George B. Garner*

A quantitative glc method for biologically important organic acids in plant tissue was developed. The acids were extracted from lyophilized plant tissue with 50% ethanol. Interfering substances were removed by ion-exchange chromatography. Derivatives were made by use of *N,O*-bis(trimethylsilyl)acetamide (BSA). The TMS esters were chromatographed on temperature-programmed 3% SE-52 and 3% XE-60 columns and detected by flame ionization. Complete resolution was obtained for 14 organic acids usually present in plant tissues. Multiple peak formation of α-keto acids was avoided by reaction with methoxyamine hydrochloride prior to silylation. Recoveries of added acids varied from 77 to 105%, except for aconitic and α-ketoglutaric acids, which were concentration dependent. The method was applied to study seasonal variations of organic acids in tall fescue and in comparing acid distribution in tall fescue, bluestem, and switchgrass.

The role of the Krebs cycle acids in respiration and protein synthesis in plant cells is well established. These di- and tricarboxylic acids accumulate in grasses in amounts ranging from 2 to 7% D.M. (Jones and Barnes, 1967). Playne and McDonald (1966) found that organic acids are responsible for most of the buffering capacity in herbages and silages. Citric, quinic, and shikimic acids have been related to preference ranking in grazing trials with sheep and cattle (Jones and Barnes, 1967). *trans*-Aconitic and citric acid have been implicated in hypomagnesemia when fed to ruminants (Bohman *et al.*, 1969). These considerations indicate the importance of organic acid composition as a parameter in the selection and evaluation of forages for ruminants. More recently, the discovery of the C_4-dicarboxylic acid pathway of carbon assimilation in tropical grasses (Downton, 1971; Hatch and Slack, 1966) has renewed interest in plant organic acid metabolism. Therefore, a simple, rapid, and accurate method for the routine quantitative analysis of organic acids in plant material is desirable.

Several methods are described in the literature for the enzymic, manometric, colorimetric, fluorimetric, and polarographic determination of organic acids (Bergmeyer, 1965; Burau, 1969; Hummel, 1949; Poe and Barrentine, 1968). However, they are time consuming and one can not simultaneously determine all of the acids. Ion-exchange chromatography, partition chromatography on silica gel, and thin-layer chromatography have been used for multiacid analysis (Busch *et al.*, 1952; Freeman, 1967; Higgins and von Brand, 1966). In recent years, gas–liquid chromatography (glc) has been the technique of choice for the separation and identification of organic acids. However, application to biological materials and quantification have been infrequent. Most of the investigators using glc methods have separated the methyl ester derivatives of organic acids (Harvey *et al.*, 1970; Kellogg *et al.*, 1964; Kuksis and Vishwakarma, 1963; Palmer, 1972; Rumsey and Noller, 1966; Simmonds *et al.*, 1967). Multiple peak formation for α-ketoglutaric, aconitic, and fumaric acids occurred in many instances. Horii *et al.* (1965), using trimethylsilyl derivatives (TMS), obtained single peaks in the qualitative determination of organic acids by glc.

This paper describes a method for the isolation of organic acids from plant materials and their quantitative glc determination as TMS derivatives with application to a study of tall fescue (*Festuca arundinacea* Shreb) organic acid composition.

Department of Agricultural Chemistry, University of Missouri/Columbia, Columbia, Missouri 65201.

EXPERIMENTAL SECTION

Reagents. Reagents of the highest purity available were purchased from various commercial sources: tartaric, *trans*-aconitic, malonic, α-ketoglutaric, shikimic, D(−)quinic, and oxalacetic acids (Nutritional Biochemical Corporation); citric, fumaric, succinic, lactic, glutaric, and oxalic acids (Fisher Scientific Company); malic acid, methoxyamine hydrochloride, and pyridine, spectroscopic grade (Eastman Organic Chemicals); *N,O*-bis(trimethylsilyl)acetamide (Pierce Chemical Company); anthracene and naphthalene, scintillation grade (New England Nuclear); Chromosorb W 80/100 mesh, acid washed, DMCS treated, and silicone nitrile XE-60 (Hewlett-Packard); silicone GE-52 (Applied Science Laboratories); Duolite cation (sodium form) and anion (chloride form) exchange resins, 50–100 mesh (Diamond Shamrock Corporation) were converted to the hydrogen and acetate forms, respectively.

Instrumental and Gas Chromatographic Conditions. The instrument used was an F&M Model 402 high efficiency gas chromatograph equipped with a dual flame ionization detector. The chromatograms were recorded on a Honeywell Electronic 16 strip chart recorder equipped with a Model 227 disk chart integrator. Separation of the TMS esters of organic acids was carried out using two U-shaped glass columns packed with 3% SE-52 (6 ft × ⅛ in. i.d.) and 3% XE-60 (4 ft × ⅛ in. i.d.) coated on MDMCS-treated, acid-washed, 80/100 mesh Chromosorb W, respectively. Both columns were conditioned overnight at 200° with a carrier flow rate of 20 ml/min before being used. The operating conditions for each column are given in Table I. Injections were made with a Hamilton 10-μl syringe. The detectors were routinely sprayed with dichlorofluoromethane to remove silylation decomposition products coating the anode.

Isolation of Organic Acids from Tall Fescue. Leaf tissue was frozen with Dry Ice immediately after harvesting and then lyophilized. The freeze-dried tissue was ground to pass a 40-mesh sieve, using a Wiley mill, Laboratory Model for greenhouse samples and a larger model for field-grown samples. One gram of dried leaf tissue was stirred 2 hr with 25 ml of 50% ethanol and filtered with suction using Whatman No. 41 filter paper. The residue was washed twice with 12.5 ml of 50% ethanol. The combined filtered extract was stirred for 1 hr with 5–6 ml of cation exchange resin (hydrogen form). After filtration of the suspension through Whatman No. 1 filter paper, the resin was washed with 5 ml of deionized water three times. The combined filtrate was passed through a 10 × 1 cm anion exchange resin (acetate form) column. The flow rate was 1.3 ml/min. After washing the resin with 35 ml

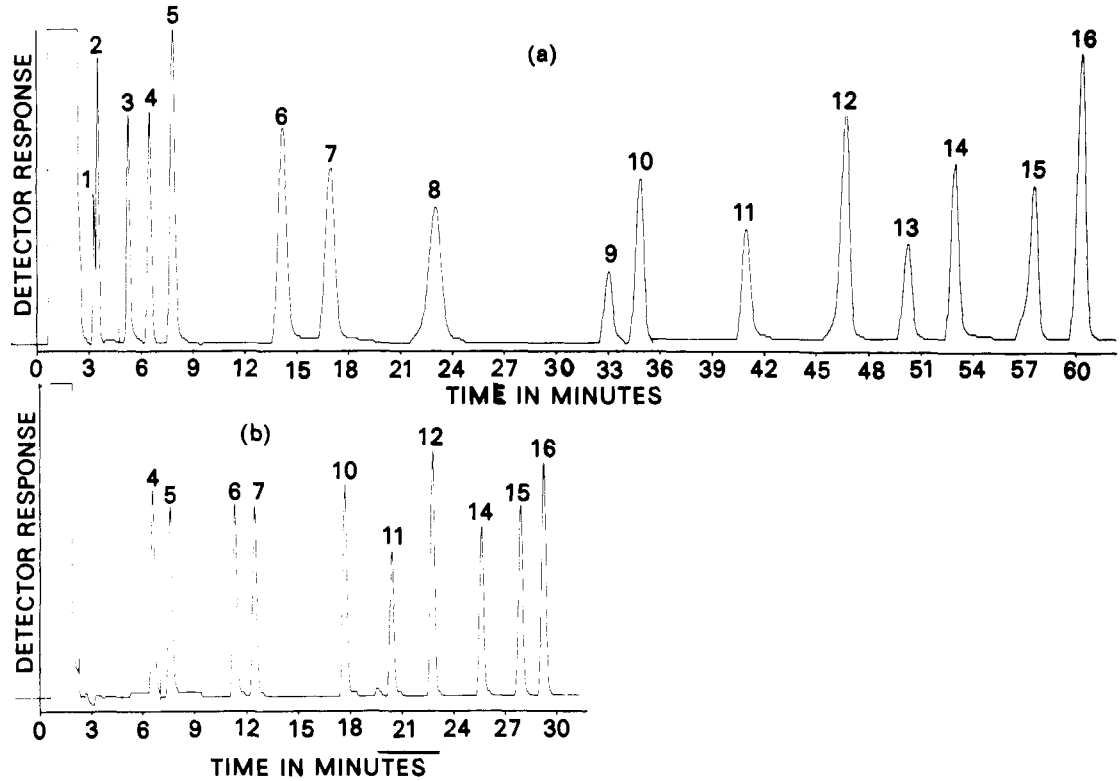

Figure 1. Chromatogram of a standard mixture of organic acids as TMS derivatives on 3% SE-52. Peak identification: (1) impurity from lactic acid source; (2) lactic acid; (3) oxalic acid; (4) naphthalene (IS); (5) malonic acid; (6) succinic acid; (7) fumaric acid; (8) glutaric acid; (9) oxaloacetic acid; (10) malic acid; (11) α-ketoglutaric acid; (12) tartaric acid; (13) anthracene (IS); (14) aconitic acid; (15) citric acid (shikimic acid gives a peak with the same retention time); (16) quinic acid. Temperature programmed (a) 2°/min and (b) 4°/min.

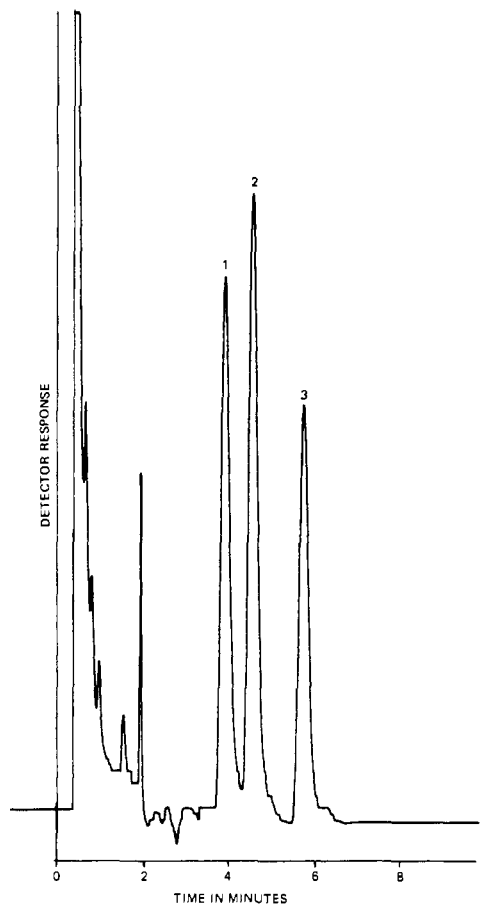

Figure 2. Chromatogram of citric and shikimic acid on 3% X E-60. Peak identification: (1) shikimic acid; (2) citric acid; and (3) anthracene (IS).

Table I. Operating Conditions for Gas Chromatography

Type of column	3% SE-52	3% XE-60
Gas flows		
Carrier (nitrogen)	55 ml/min	37 ml/min
Hydrogen	35 ml/min	28 ml/min
Air	500 ml/min	500 ml/min
Temperature		
Flash heater	200°	200°
Detector	270°	270°
Column (temperature program)	90 to 190° at 2°/min or 4°/min	140 to 180° at 5°/min

of deionized water, the organic acids were eluted with 50 ml of 6 N formic acid at a flow rate of 1.3 ml/min. A 25-ml aliquot was placed in a 125-ml flat-bottomed flask and evaporated in a Virtis Sublimator with the shelf temperature at 3°. Following lyophilization, the flask was placed in a vacuum desiccator over NaOH and $CaCl_2$ to remove traces of moisture and formic acid.

Preparation of TMS Esters. The silylation reaction was carried out in the same flask and the exposure of reagents to air was minimized. One milliliter of pyridine (dried over KOH pellets) containing 2 mg of naphthalene as internal standard was added to the isolated acids prepared above. Methoxyamine hydrochloride (5 mg per mg of α-keto acid present) was added, mixed, and allowed to react for 8 min at room temperature. Then, 1 ml of N,O-bis(trimethylsilyl)acetamide was added and the flask was shaken until a clear solution was obtained. The flask, tightly stoppered, was heated in an oil bath at 70° for 30 min. Three microliters of this solution were chromatographed in the SE-52 column. For the determination of citric acid in samples containing shikimic acid, the remaining solution was diluted with 2 ml of pyridine con-

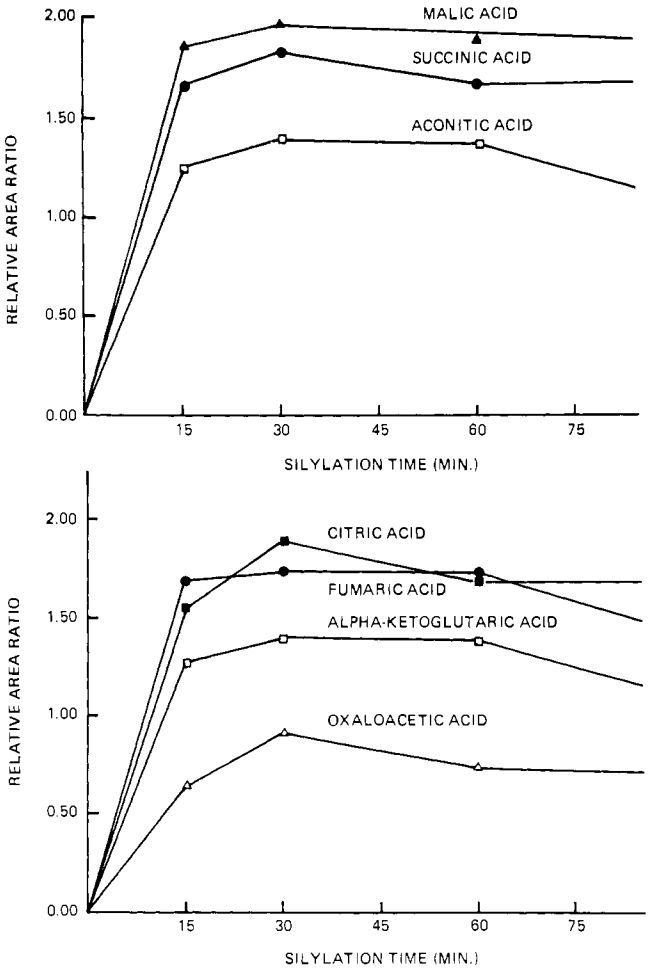

Figure 3. Effect of reaction time on the silylation of organic acids with BSA at 70°. Each point is an average of two separate derivatizations.

taining 2 mg of anthracene/ml. Three microliters of diluted sample were then chromatographed on the XE-60 column.

Glc Calibration Curves. One milliliter of a standard solution of organic acids (0.5, 1.0, 2.5, and 5.0 mg for each acid), containing naphthalene (2 mg) in pyridine, was silylated and chromatographed as described above. The ratios between the areas of each TMS organic acid peak and of the internal standard peak were calculated and plotted against concentration.

Thin-Layer Chromatography of Organic Acids. Organic acids were separated by the two-dimensional thin-layer chromatography procedure described by Higgins and von Brand (1966).

RESULTS AND DISCUSSION

Gas Chromatography. Complete resolution and a single peak were obtained for the TMS esters of lactic, oxalic, malonic, succinic, fumaric, glutaric, oxaloacetic, malic, α-ketoglutaric, tartaric, aconitic, citric, and quinic acids on the SE-52 column using a temperature programming of 2°/min. In addition, well-separated peaks were also obtained for two internal standards, naphthalene and anthracene. This is illustrated in Figure 1. A temperature programming of 4°/min was found to be satisfactory for the separation of organic acids usually present in grasses, thus reducing the analysis time to 30 min. TMS derivatives of citric acid and shikimic acid could not be resolved on the SE-52 column at either temperature programming. Both acids were separated on the XE-60 column in only 7 min. However, if quinic acid was present, it had a retention time identical to shikimic. The other organic acids and naphthalene gave peaks masked by the solvent peak. Since plant material contained all three acids, each could be resolved by use of two columns. Anthracene was employed as the internal standard in this column. A typical chromatogram is shown in Figure 2.

Table II. Slope Factors from TMS Organic Acid Calibration Curves

Organic acid	Molecular weight	Number of derivatizable groups	Slope factor
Malonic	104.06	2	0.390
Fumaric	116.07	2	0.458
Succinic	118.09	2	0.478
α-Ketoglutaric	146.10	2	0.402
Malic	134.09	3	0.603
Aconitic	174.11	3	0.644
Shikimic	174.15	4	0.940
Citric	192.12	4	0.913
Quinic	192.17	5	1.095

Reaction time studies established that silylation was complete after a heating period of 30 min at 70°. The results are shown in Figure 3. Prolonged heating after 30 min resulted in decreased responses, with the exception of the TMS derivative of malic acid, which remained stable up to 90 min of silylation time.

The response of the flame ionization detector was linear in the concentration range studied, 0.75 to 7.5 μg of acid. The response to malic acid was studied at greater concentrations and found to be linear to 19.5 μg of acid injected. Slope factors calculated from the calibration curves are given in Table II. These factors have been defined as the relative detector response of 10 μmol of organic acid to 2 mg of naphthalene. In general, a higher detector response was obtained with organic acids of higher molecular weight and a higher number of derivatizable groups.

Isolation of Organic Acids. A single extraction of organic acids from tall fescue with 50% ethanol was found to be adequate. This was shown by the absence of peaks in gas–liquid chromatograms obtained from samples subjected to a second extraction with 50% ethanol and similar isolation procedures. The single extraction proved superior to procedures described previously (Boland, 1971).

A typical chromatogram of organic acids isolated from tall fescue is shown in Figure 4. The peaks were identified by comparison of their retention times to the retention times of known TMS esters. Additional identification was obtained by tlc. An aliquot from the same purified extracts used for glc analysis was chromatographed on cellulose thin-layer plates. The R_f values of the spots obtained were related to the R_f values of standard acids. The number of acids identified by tlc agreed with those identified by glc. There was also a relationship between spot intensity and peak area.

Recoveries and Precision. The mean recoveries for the organic acids are shown in Table III. Each acid was added to a 50% ethanol solution and carried through the method described for organic acid determination in tall fescue. The recoveries for succinic, fumaric, malic, tartaric, citric, and quinic acids were above 70% at all the concentrations studied. Malonic acid gave recoveries less than 50% at low concentrations. Lowest recoveries were obtained for α-ketoglutaric and aconitic acids. This was particularly noticeable when less than 2.5 mg of each acid was added.

The precision of the method (standard error for individual acids) was estimated from duplicate analyses carried out on fescue genotypes which were part of a varietal study of tall fescue organic acid composition (Boland, 1971). The data in Table IV show that the standard error

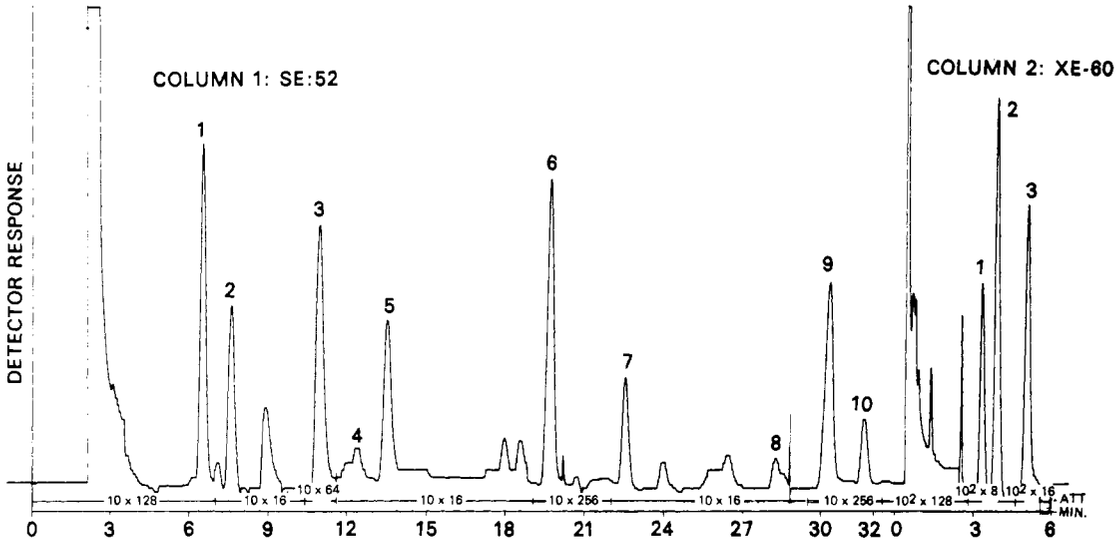

Figure 4. Chromatogram of organic acids isolated from tall fescue. Peak identification—Column 1: (1) naphthalene (IS); (2) malonic acid; (3) succinic acid; (4) fumaric acid; (5) glyceric acid; (6) malic acid; (7) α-ketoglutaric acid; (8) aconitic acid; (9) citric and shikimic acids; (10) quinic acid. Column 2: (1) quinic and shikimic acids; (2) citric acid; (3) anthracene (IS).

Table III. Combined Isolation and Chromatographic Recoveries (as % w/w) for Individual Organic Acids

	Acid added, mg							
	2.5		1.25		0.5		0.25	
	Mean[a]	Range	Mean	Range	Mean	Range	Mean	Range
Malonic	78.6	77.2–80.0	71.8	67.8–75.8	49.6	44.7–54.4	38.3	36.19–40.5
Succinic	105.6	104.4–106.8	104.4	100.9–107.9	103.8	101.5–106.0	105.6	103.6–107.5
Fumaric	89.4	88.8–90.0	85.7	79.3–92.1	81.7	79.6–83.7	73.7	71.7–75.6
Malic	100.6	99.6–101.5	100.3	98.4–102.2	99.6	98.6–100.6	122.3	110.2–135.4
α-Ketoglutaric	34.3	29.5–39.0	8.8	8.0–9.6				
Tartaric	101.1	100.5–101.6	98.5	94.5–102.4	97.1	94.2–100.0	100.0	92.3–107.7
Aconitic	37.6	35.8–39.4	25.5	23.9–27.1	10.9	10.2–11.6	14.5	14.5
Citric	105.4	103.4–107.4	96.8	92.8–100.8	107.8	96.0–119.6	99.9	98.0–101.9
Quinic	84.0	82.8–85.2	86.0	84.8–87.2	86.0	84.0–88.0	92.0	84.0–100.0

[a] Mean of two determinations from isolation step through glc analysis.

Table IV. Standard Error of Mean for Individual Organic Acids[a]

Acids	Mean, mg/g DM	Std error[b] ± mg/g DM
Malic	7.98	0.216
Citric	4.24	0.436
Succinic	3.64	0.278
Shikimic	2.29	0.283
Quinic	1.95	0.123
Malonic	0.29	0.024
α-Keto	0.25	0.086
Aconitic	0.11	0.027

[a] Analysis includes purification, derivatization, and gas chromatography. [b] From analysis of variance data calculated on duplicate analysis of four genotypes.

Table V. Organic Acid Composition of Three Grass Species

	Species[a]			
			Fescue genotype	
Acid	Bluestem grass	Switch-grass	Kennmont	Alta V-2
	mg/g of dry matter			
Malonic	0.12			0.01
Succinic	1.02	1.76	0.06	0.46
Fumaric	0.36	0.18	0.11	0.07
Malic	7.50	3.04	22.72	10.27
α-Ketoglutaric	0.28	0.31	0.32	0.25
Aconitic	0.24	0.39	0.23	0.17
Quinic	1.25	0.88	1.07	1.89
Shikimic	2.98	1.49	1.86	2.06
Citric	1.29	1.62	4.03	4.17
Total	15.23	10.20	30.38	19.35

[a] Greenhouse samples represent 3–6 weeks regrowth.

of the mean was lowest for malic acid, 2.73%. This may be related to high malic acid concentrations in tall fescue and to the greater stability of its TMS derivative (Figure 3). High standard errors of means for aconitic (25%) and α-ketoglutaric acids (34%) are probably due to the very small concentrations in these samples.

Application of the glc Method. The method was used in a study designed to follow seasonal changes in organic acid composition of tall fescue (Ky-31). Analyses were carried out on field-grown samples harvested periodically from August 19, 1970, to January 4, 1971. The plant tissue analyzed represented regrowth following a July harvest of all vegetation and fertilization with 168 kg of NH_4NO_3/ha. The results are presented in Figure 5. Malic acid was again the predominant acid, accounting for seasonal change in total organic acid content.

In another study with greenhouse-grown plants (Table V), a comparative study of organic acid composition was

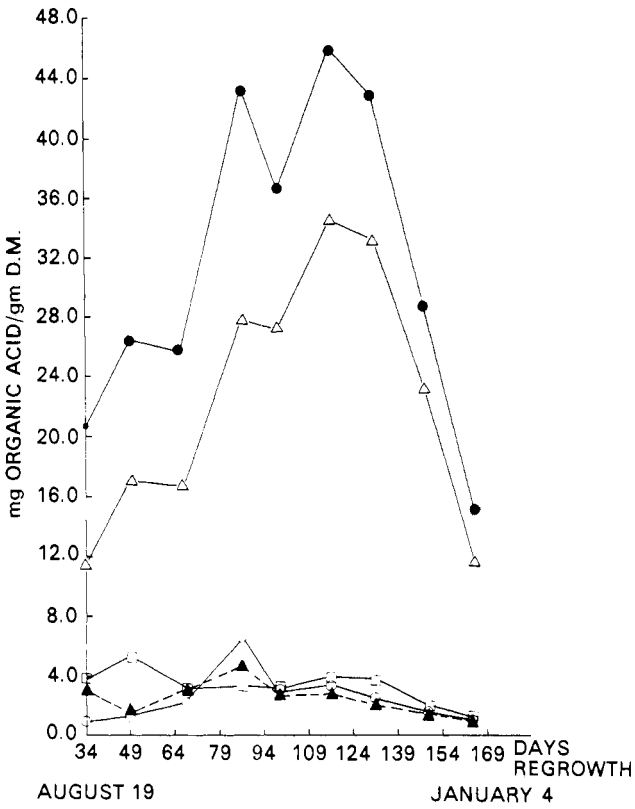

Figure 5. Seasonal variation of organic acids in tall fescue. (△——△) malic; (□——□) citric; (○——○) quinic; (▲——▲) shikimic; (●——●) total.

desirable. Malic and citric acid concentrations were different between bluestem (*Andropogon caucasica*) and switchgrass (*Panicum virigatum*) and two genotypes of tall fescue (*Festuca arundinacea*). The method has been applied to a more complete genotypical study of tall fescue to be reported elsewhere.

ACKNOWLEDGMENT

The technical assistance of Matt Pross and the supply of tall fescue samples from Kay Asay, Department of Agronomy, are gratefully acknowledged.

LITERATURE CITED

Bergmeyer, H. V., Ed., "Methods of enzymatic analysis," Williamson, D. H., trans., Verlag-Chemie, Gmbt, Weinheim/Bergstr., 1965, 313-344.
Bohman, V. R., Lesperance, A. L., Harding, G. D., Grunes, D. L., *J. Anim. Sci.* **29** (1), 99 (1969).
Boland, R. L., Ph.D. thesis, University of Missouri, Columbia, Mo., Nov 1971.
Burau, R. G., *J. Agr. Food Chem.* **17,** 1332 (1969).
Busch, H., Hulbert, R. B., Potter, van R., *J. Biol. Chem.* **196,** 717 (1952).
Downton, W. J. S., *Can. J. Bot.* **49,** 1439 (1971).
Freeman, G. G., *J. Chromatogr.* **28,** 338 (1967).
Harvey, W. R., Hall, R. W., Ikeda, R. M., *Tobacco Sci.* **14,** 141 (1970).
Hatch, M. D., Slack, C. R., *Biochem. J.* **101,** 103 (1966).
Higgins, H., von Brand, T., *Anal. Biochem.* **15,** 122 (1966).
Horii, Z., Makita, M., Tamura, Y., *Chem. Ind.* **34,** 1494 (1965).
Hummel, J. P., *J. Biol. Chem.* **180,** 1225 (1949).
Jones, E. C., Barnes, R. J., *J. Sci. Food Agr.* **18,** 321 (1967).
Kellogg, H. M., Hanssen, E., Svendsen, A. B., *J. Pharm. Sci.* **53,** 420 (1964).
Kuksis, A., Vishwakarma, P., *Can. J. Biochem. Physiol.* **41,** 2353 (1963).
Palmer, A. M., *Tobacco Sci.* **16,** 83 (1972).
Playne, M. J., McDonald, P., *J. Sci. Food Agr.* **17,** 264 (1966).
Poe, W. E., Barrentine, B. F., *J. Agr. Food Chem.* **16,** 983 (1968).
Rumsey, T. S., Noller, C. H., *J. Chromatogr.* **24,** 325 (1966).
Simmonds, P. G., Pettit, B. C., Zlatkis, A., *Anal. Chem.* **39,** 163 (1967).

Received for review July 3, 1972. Accepted March 12, 1973. Contribution from the Missouri Agricultural Experiment Station. Journal Series Number 6432.

Free Fatty Acid Content of Cacao Beans Infested with Storage Fungi

Arthur P. Hansen,* Ronald E. Welty, and Rong-sen Shen[1]

Cacao beans from four sources allowed to mold with surface-contaminating fungi and cacao beans (Bahia source) inoculated with *Aspergillus amstelodami, A. flavus, A. niger, A. repens, A. ruber,* a species of *Penicillium,* and a species of *Paecilomyces* had increases in free fatty acid (FFA) content and changes in the FFA composition of the lipids. In beans molded by contaminating fungi (naturally molded), the FFA content of the lipid increased from 1.0–1.5 to 7.4–41.3%. The FFA content of lipid from beans molded by single species of storage fungi (pure culture-molded) increased from 1.4 to 28.0–62.1%. The major FFA present were palmitic, stearic, and oleic and these three fatty acids comprised 36.7–62.5, 49.2–92.4, and 92.0–95.4% of the total FFA for nonmoldy, naturally molded, and pure culture-molded cacao beans, respectively. Linoleic acid comprised 13.8–17.4% of the total FFA in nonmoldy and naturally molded Bahia beans. The fungi increased the acidity of the cocoa lipid through liberation of fatty acids from glycerides.

Cacao beans are produced in tropical regions where storage fungi can be troublesome. Following natural fermentation the moisture content (MC) of the whole bean is reduced from about 60 to 8% or below for sale or storage. Beans are dried in the sun or artificially (Rohan, 1963). If drying is too slow or stopped above 8% MC, storage fungi will invade and grow in the bean. Dry cacao beans are hygroscopic and readily absorb moisture from the humid atmosphere in the tropics. Scott (1963) investigated molds associated with stored cacao beans and concluded that conditions above 75% relative humidity (RH) and 10° fa-

Department of Food Science, North Carolina State University, Raleigh, North Carolina 27607.
[1] Present address: National Institute of Environmental Health Sciences, Public Health Service, Department of Health, Education, and Welfare, Research Triangle Park, North Carolina 27709.