LC Determination of Phytic Acid in Food by Postcolumn Colorimetric Detection

Johannes J. L. Cilliers and Pieter J. van Niekerk*

A method is described for the high-performance liquid chromatographic (LC) determination of phytic acid (myoinositol 1,2,3,4,5,6-hexakis(dihydrogen phosphate)) in grain sorghum and soybean samples after extraction with 3% trichloroacetic acid (TCA). The only sample preparation necessary after acid extraction was centrifugation at 50000*g* for 10 min and filtration. Separation and quantitation were achieved within 5–8 min on an anion-exchange column, IC-PAK-A (0.46 cm i.d. × 5 cm) with 0.045 M $NaNO_3$ containing 0.018 M HNO_3 as mobile phase at a flow rate of 1.2 mL/min. The phytic acid was detected at 500 nm by means of an in-line postcolumn reaction (0.015% $FeCl_3$ containing 0.15% 5-sulfosalicylic acid). Inositol mono-, di-, tri-, tetra-, and pentaphosphates can also be detected with this method.

INTRODUCTION

Phytic acid is widely distributed in foods of plant origin. In most seeds phytic acid is found as magnesium phytate in the form of either one or all three of the following salts: Ca_2Mg phytate (phytin), which is soluble, and Na_2Mg_5 and K_2Mg_5 phytate, which are insoluble (Morris and Ellis, 1981; Satterlee and Abdul-Kadir, 1983; Tecklenburg et al., 1984).

Many publications mention the involvement of phytic acid in the bioavailability of minerals in the diet as well as its interaction with protein (Erdman, 1979; Flanagan, 1984; Maga, 1982). Some conclusions made over the years were incorrect or contradictory, mainly because of the complexity of the composition of foods and diets and the interaction of other components such as polyphenols and fiber with minerals (Gillooly et al., 1983; Platt and Clydesdale, 1984; Schweizer et al., 1984).

The importance of phytic acid resulted in the appearance of many articles on its determination. Some of these methods make use of its property of forming insoluble salts with iron in a slightly acidic solution (Jaffe, 1981). Assays relying on analysis of iron from the ferric phytate precipitate are, however, not recommended because of the variable nature of the molar ratio of iron and phosphorus in this precipitate (Thompson and Erdman, 1982). Many of these methods have lengthy sample preparation procedures involving various steps and are thus very costly and time consuming (Oberleas, 1971; Wheeler and Ferrel, 1971).

Phytic acid may also be determined by LC. The main disadvantages of the published methods are lengthy sample preparation procedures and/or the elution of phytic acid on the solvent front (Camire and Clydesdale, 1982; Knuckles et al., 1982; Tangendjaja et al., 1980). The latter may give rise to coelution of other material and result in peak areas that are too large for some methods. Careful manipulation of sample preparation conditions is necessary to avoid the formation of extra peaks or vacancy peaks with ion-pair chromatography (Lee and Abendroth, 1983). The use of strong anion-exchange resins for cleaning or concentrating samples increases sample preparation times (Graf and Dintzis, 1982a,b). As stated by Lee and Abendroth (1983), the detection limit for analysis using these methods is dependent on the efficiency of sample preparation.

There are different ways of removing phytate from plant materials, for example abrasive dehulling (Reichert et al., 1984), fermentation (Fardiaz and Markakis, 1981; Lopez et al., 1983; Nayini and Markakis, 1983; Sudarmadji and Markakis, 1977), and chemically by adding calcium to soymilk products followed by centrifugation or ultrafiltration (De Rham and Jost, 1979; Ford et al., 1978; Omosaiye and Cheryan, 1979). Since we planned to investigate the various ways of removing phytate during the development of food products, the need arose for a method with a short and simple sample preparation procedure and would be well suited to the handling of many samples. We found that the method presented in this paper met with those requirements.

MATERIALS AND METHODS

Materials. Phytic acid from Sigma was used as standard. It was found to contain 15.6% moisture, and this was taken into account when making standard solutions. Trichloroacetic acid (TCA), sodium nitrate, nitric acid, ammonium ferric sulfate dodecahydrate, 2,2′-bipyridine, and thioglycollic acid are guaranteed reagents obtained from Merck (Darmstadt). Phytase (crude) from wheat was obtained from Sigma.

The LC equipment consisted of a Varian 5000 pump equipped with a Valco inlet valve fitted with a 10-μL sample loop, a Vari-Chrom UV/vis detector (500 nm), and a Hewlett-Packard 3390 A integrator/plotter. The anion-exchange column (0.46 cm i.d. × 5 cm) used was an IC-PAK-A (Millipore S.A.). A Milton Roy minipump (LDC) was used for the postcolumn reagent. The reaction coil was 20 cm long, from the connection at which the column effluent and postcolumn reagent were mixed to the detector. It was kept at room temperature. A refractive index (RI) detector (Waters R401) was also used. Membrane filters with 0.45-μm pore size (HVLP 04700) were supplied by Millipore.

Methods. Samples (2.0 g) were weighed in Erlenmeyer flasks (100 mL), and 3% TCA (20.0 mL) was added (Camire and Clydesdale, 1982; Davis, 1981; Fardiaz and Markakis, 1981; Tangendjaja et al., 1980). These were shaken mechanically for 2 h, and the extract was then centrifuged at 50000*g* for 10 min. It was not necessary to defat the samples. After filtering through a 0.45-μm pore size membrane filter, the extract was ready for injection onto the column.

For LC determination the mobile phase was made by mixing equal volumes of 0.09 M $NaNO_3$ and 0.036 M HNO_3. The flow rate was 1.2 mL/min. Postcolumn reagent was pumped at 0.72 mL/min and was made up as a 0.015% $FeCl_3$ solution containing 0.15% sulfosalicylic

National Food Research Institute, Council for Scientific and Industrial Research, Pretoria 0001, South Africa.

0021-8561/86/1434-0680$01.50/0

acid (Latta and Eskin, 1980; Wade and Morgan, 1955). This reagent reacts with phosphates. The iron in the reagent is bound by the phosphate and thus removed, resulting in a decrease in the intensely purple color. The decrease in color is measured at 500 nm. When the polarity of the detector is changed, the "negative" peak is registered and integrated as a positive, normal peak. A calibration curve was obtained by injecting 10 μL each of 10 standards containing different concentrations of phytic acid (0.2–6.0 g of phytic acid/L). Recovery experiments were done by adding 0.044 g of phytic acid to 2 g of maize germ containing 1.53 g/100 g of phytic acid and by adding 0.032 g of phytic acid to 2 g of a soybean sample containing 1.7 g/100 g of phytic acid.

A calibration curve using refractive index detection was also obtained by injecting seven standards containing different concentrations of phytic acid (1.0–10.0 g of phytic acid/L).

The influence of phytase on a phytic acid standard was followed with LC by adding phytase to reaction mixtures each containing 2 mL of 0.1 M malic acid–Tris buffer (pH 5.0) and 20 mg of phytic acid. After mixing well, the test tubes were incubated at 55 °C for 3 h. One test tube was taken every hour and put into a boiling water bath to inactivate the phytase. After centrifugation and filtration the hydrolysate was analyzed. In order to detect inositol mono-, di-, tri-, tetra-, and pentaphosphates, their k' values were increased by diluting the mobile phase to 0.03 M $NaNO_3$ containing 0.012 M HNO_3.

Samples were also analyzed for phytic acid by the colorimetric method of Haug and Lantzsch (1983) using their varient (b). When the LC and colorimetric methods were compared, the same TCA extracts were used for both methods. A calibration curve for the colorimetric method was obtained by using 10 phytic acid standards in the range 0.002–0.35 g/L. A recovery experiment was done with this method by adding 0.032 g of phytic acid to 2 g of a soybean sample containing 1.7 g/100 g of phytic acid.

RESULTS AND DISCUSSION

Various systems for separating phytic acid from inorganic phosphate and other possible interfering peaks were tried. An amino silica gel column gave a symmetrical peak for phytic acid with detection at 200 nm and 0.3 M phosphate buffer (pH 4.5) as the mobile phase. This column was, however, easily deactivated (probably by the formation of Schiff bases). Strong anion-exchange columns (5-μm SAX Spherisorb 0.46 cm i.d. × 25 cm) were also used initially with success, but as the high molarity of the phosphate buffers (1 M, pH 4.5) necessary for elution caused problems by dissolving the silica-based packing material, this type of column was discarded.

If the low pK_a values of phytic acid ($pK_{a_{1-6}}$ 1.84 and $pK_{a_{7,8}}$ 6.3; Cosgrove, 1980) are taken into account, then it is clear that the retention of phytic acid can be reduced by lowering the pH of the mobile phase. This, together with the use of low-capacity ion-exchange material, resulted in a reduction in the concentration of the buffer required for elution of phytic acid. Low-capacity pellicular material (Zipax, Du Pont) with a particle size distribution of 25–37 μm was used with 0.05 M HNO_3 containing 0.125 M $NaNO_3$ (pH 1.8) as the mobile phase at 1.2 mL/min with colorimetric detection. Three of these columns (each 0.46 cm i.d. × 25 cm) had to be used in series because of the low efficiency of a single column, and a retention time of 18 min for phytic acid was obtained. The columns were finally replaced by the Waters IC-PAK-A column, which is packed with low-capacity 10-μm resin-based anion-exchange material. This 0.46 cm i.d. × 5 cm column resulted in extremely short separation times with good resolution between inorganic phosphate and phytic acid as shown in Figure 1.

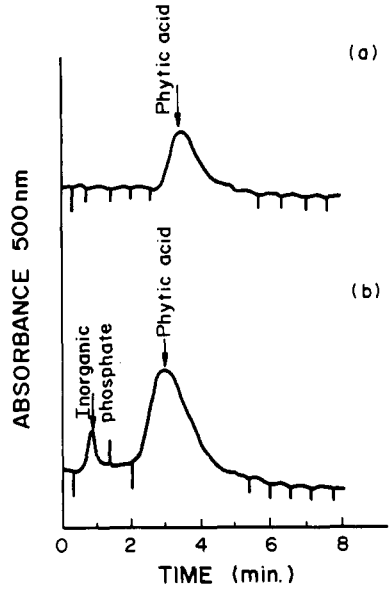

Figure 1. LC separation: (a) 1.0% standard phytic acid using an anion-exchange IC-PAK-A (0.46 cm i.d. × 5 cm) column with 0.045 M $NaNO_3$ containing 0.018 M HNO_3 at a flow rate of 1.2 mL/min; (b) grain sorghum sample containing 1.5% phytic acid.

Table I. Phytic Acid Content of Samples Determined by the Colorimetric Method Using 3% TCA and 0.2 M Hydrochloric Acid for Extraction (Mean of Duplicate Determinations)

sample	phytic acid, g/100 g	
	3% TCA	0.2 M HCl
sunflower seed	4.48	4.25
tofu (Prima)	2.95	3.29
tofu (Hutton)	2.49	2.91
tofu (Edgar)	2.26	2.65
high-protein barley flour	2.88	2.41
high-bran barley flour	3.21	3.48

With LC and post-column colorimetric detection, a linear calibration curve of $y = (3.9x + 23)10^5$ and a correlation coefficient of $r = 0.9971$, $N = 10$, was obtained for the peak area against phytic acid concentration (g/L) injected. Recovery of phytic acid from maize germ was 104% (coefficient of variation 4%, $N = 5$) and from a soybean sample it was 100% (coefficient of variation 9%, $N = 5$).

With refractive index detection a linear calibration curve of $y = (21.6x + 2.6)10^3$, correlation coefficient $r = 0.9999$, $N = 7$, was obtained for the peak area against phytic acid concentration (g/L). The refractive index detector was found to be less specific than colorimetric detection, and unknown peaks were found with grain sorghum samples that could interfere more with the determination of lower inositol phosphates than with phytic acid. The sensitivity for refractive index detection at an attenuator setting of 1× was of the same order of magnitude as for the colorimetric detector. When more sensitive settings were used, base-line noise became excessive.

The colorimetric method of Haug and Lantzsch (1983) requires the use of a 0.2 M hydrochloric acid extract of the sample. Since we wished to compare the colorimetric and LC methods by using the same extract, it was necessary to determine whether the TCA and hydrochloric acid extracts would give equivalent results with the colorimetric method. For this purpose six samples (soya and barley products) were extracted by 0.2 M hydrochloric acid and

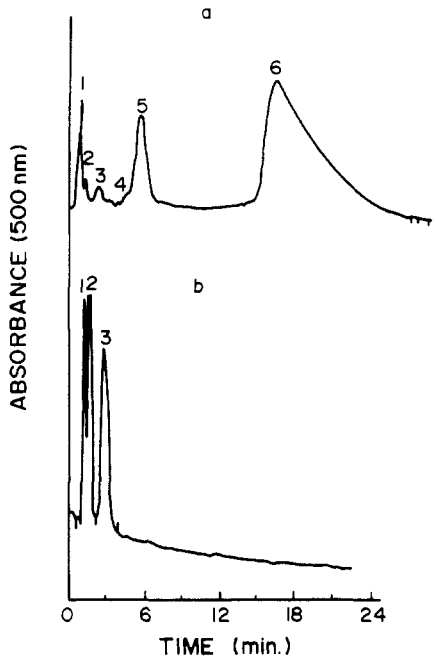

Figure 2. Separation of inositol phosphates after treatment of phytic acid with phytase: (a) inositol phosphate profile after 1 h of incubation; (b) inositol phosphate profile after 15 h of incubation. Separation was accomplished with an anion-exchange IC-PAK-A (0.46 cm i.d. × 5 cm) column using 0.03 M $NaNO_3$ containing 0.012 M HNO_3 as the mobile phase at a flow rate of 1.2 mL/min. Peaks: 1, inorganic phosphate and inositol monophosphate; 2, inositol diphosphate; 3, inositol triphosphate; 4, inositol tetraphosphate; 5, inositol pentaphosphate, 6, inositol hexaphosphate (phytic acid).

by 3% TCA, and the phytic acid was determined colorimetrically. The results are given in Table I. A pairwise T-test on the results for the two extractions indicated no difference at a probability $P \leq 0.4$ (Winkler and Hays, 1975).

The LC and colorimetric methods were compared by analyzing 21 samples, and the results are given in Table II. A T-test on the difference between the results of the two methods indicated that it did not differ from zero at $P \leq 0.6$ (mean difference 0.0143, $t = 0.42$, $N = 21$). A correlation coefficient of $r = 0.982$ was found between the LC and colorimetric methods. A linear regression line $y = 1.063x - 0.081$ was calculated by the method of Deming (Cornbleet and Gochman, 1979) for the relationship between the results obtained by the LC method vs. the results obtained by the colorimetric method. The slope does not differ statistically ($P \leq 0.05$) from unity ($t = 1.743$).

Addition of a phytase to a phytic acid standard resulted in the formation and disappearance of what is believed to be inositol mono-, di-, tri-, tetra-, and pentaphosphates. A chromatogram of the reaction mixture after 1 h is shown in Figure 2a. Phytic acid was still present, but the formation of lower inositol phosphates had taken place. After 16 h phytic acid inositol penta- and tetraphosphate had disappeared, but increased concentrations of the lower inositol phosphates were observed (Figure 2b).

This method for determining phytic acid is sensitive, specific, reproducible, and very fast and requires only a simple sample preparation procedure. No interferences from other material were found because of the specific reaction of the postcolumn reagent with phosphates. The method was successfully applied to a variety of samples. Values obtained for phytic acid in these samples compared favorably with an established colorimetric method and were also similar to those given in the literature (Davis, 1981; Oberleas, 1983; Omosaiye and Cheryan, 1979; Sudarmadji and Markakis, 1977).

Table II. Phytic Acid Content of Various Samples Determined with the LC and Colorimetric Methods (Mean of Duplicate Determinations)

sample	phytic acid, g/100 g	
	colorimetric	LC
soybeans (cultivar Prima)	1.1	1.1
soybeans (cultivar Hutton)	1.4	1.5
soybeans (cultivar Edgar)	1.7	1.5
soybeans (cultivar Highveld Top)	1.4	1.7
tofu (from Prima)	2.7	2.9
tofu (from Hutton)	2.4	2.4
tofu (from Edgar)	2.0	2.2
soy-based meat substitute (TG)	1.2	1.1
soy-based meat substitute (TB)	1.3	1.3
soy-based meat substitute (SS)	0.01	0.01
soy-based meat substitute (BR)	0.09	0.09
soy-based meat substitute (BC)	0.13	0.13
milk substitute (IS)	0.5	0.3
milk substitute (IM)	0.4	0.2
slimming preparation	0.5	0.5
grain sorghum pearlings (12)	1.3	1.1
grain sorghum pearlings (27)	1.3	1.2
pearled grain sorghum (BR12)	0.6	0.8
pearled grain sorghum (BR27)	0.2	0.2
pearled grain sorghum (HO12)	1.3	1.0
pearled grain sorghum (HO27)	0.7	0.7

ACKNOWLEDGMENT

We thank A. Fourie for her technical assistance and W. B. van der Riet for the soybean and tofu samples.

Registry No. Phytic acid, 83-86-3.

LITERATURE CITED

Camire, A. L.; Clydesdale, F. M. *J. Food Sci.* **1982**, *47*, 575.
Cornbleet, P. J.; Gochman, N. *Clin. Chem.* **1979**, *25*, 432.
Cosgrove, D. J. *Inositol Phosphates. Their Chemistry, Biochemistry and Physiology*; Elsevier Scientific Publishing: Amsterdam, 1980.
Davis, K. R. *Cereal Chem.* **1981**, *58*(4), 347.
De Rham, O.; Jost, T. *J. Food Sci.* **1979**, *44*(2), 596.
Erdman, J. W. *JAOCS* **1979**, *56*, 736.
Fardiaz. D.; Markakis, P. *J. Food Sci.* **1981**, *46*, 523.
Flanagan, P. R. *J. Nutr.* **1984**, *114*, 493.
Ford, J. R.; Mustakas, G. C.; Schmutz, R. D. *JAOCS* **1978**, *55*, 371.
Gillooly, M.; Bothwell, T. H.; Torrance, J. D. *Br. J. Nutr.* **1983**, *49*, 331.
Graf, E.; Dintzis, F. R. *Anal. Biochem.* **1982a**, *119*, 413.
Graf, E.; Dintzis, F. R. *J. Agric. Food Chem.* **1982b**, *30*, 1094.
Haug, H.; Lantzsch, H. *J. Sci. Food Agric.* **1983**, *34*, 1423.
Jaffe, G. *JAOCS* **1981**, March, 493.
Knuckles, B. E.; Kuzmicky, D. D.; Betschart, A. A. *J. Food Sci.* **1982**, *47*, 1257–1258, 1262.
Latta, M.; Eskin, M. *J. Agric. Food Chem.* **1980**, *28* 1313.
Lee, K.; Abendroth, J. A. *J. Food Sci.* **1983**, *48*, 1344.
Lopez, Y.; Gordon, D. T.; Fields, M. L. *J. Food Sci.* **1983**, *48*, 953.
Maga, J. A. *J. Agric. Food Chem.* **1982**, *30*, 1.
Morris, E. R.; Ellis, R. *Cereal Chem.* **1981**, *58*(5), 363.
Nayini, N. R.; Markakis, P. *J. Food Sci.* **1983**, *48*, 262.
Oberleas, D. *Methods Biochem. Anal.* **1971**, *20*, 87.
Oberleas, D. *Cereal Food World* **1983**, *28*(6), 352.
Omosaiye, O.; Cheryan, M. *Cereal Chem.* **1979**, *56*(2), 58.
Platt, S. R.; Clydesdale, F. M. *J. Food Sci.* **1984**, *49*, 531.
Reichert, R. D.; Oomah, B. D.; Youngs, C. G. *J. Food Sci.* **1984**, *49*, 267.
Satterlee, L. D.; Abdul-Kadir, R. *Lebensm.-Wiss. + Technol.* **1983**, *16*, 8.
Schweizer, T. F.; Frølich, W.; Del Vedovo, S.; Besson, R. *Cereal Chem.* **1984**, *61*(2), 116.
Sudarmadji, S.; Markakis, P. *J. Sci. Food Agric.* **1977**, *28*, 381.
Tangendjaja, B.; Buckle, K. A.; Wootton, M. *J. Chromatogr.* **1980**, *197*, 274.

Tecklenburg, E.; Zabik, M. E.; Uebersax, M. A.; Dietz, J. C.; Lusas, E. W. *J. Food Sci.* **1984**, *49*, 569.
Thompson, D. B.; Erdman, J. W. *Cereal Chem.* **1982**, *59*(6), 525.
Wade, H. E.; Morgan, D. M. *Biochem. J* **1955**, *60*, 264.
Wheeler, E. L.; Ferrel, R. E. *Cereal Chem.* **1971**, *48*, 312.
Winkler, R. L.; Hays, W. L. *Statistics: Probability, Inference and Decision*; Holt, Rinehart and Winston: New York, 1975; p 450.

Received for review December 10, 1984. Revised manuscript received March 25, 1986. Accepted April 28, 1986.

Water Stress and Seasonal Effects on Rubber Quality in Irrigated Guayule

William W. Schloman, Jr.,* Donald J. Garrot, Jr., and Dennis T. Ray

Guayule (line N396) was harvested monthly from January through July, 1984, from irrigated plots grown in Tucson, AZ. Irrigations were scheduled according to the crop water stress index (CWSI) to maintain three irrigation regimes. Plots were irrigated when their respective CWSI values reached or exceeded 0.3 (wet), 0.6 (medium), and 0.9 (dry). Individual plants from four replicated plots of each regime were analyzed for rubber molecular weight by gel permeation chromatography. The molecular weight ($\bar{M}_w$) values for all three irrigation regimes increased from January through March during the plants' winter quiescent period. Molecular weight decreased between March and April for all regimes when active growth resumed. By May, $\bar{M}_w$ had increased above March values. Between irrigation regimes, significant differences in $\bar{M}_w$ were observed only in January and June, with the dry plots yielding the highest molecular weight in both instances.

The processing behavior of natural rubber (*cis*-polyisoprene) reflects the physical properties of the raw polymer. Properties such as bulk viscosity can be correlated with the structure of the rubber molecule itself, including its molecular weight distribution (Bristow, 1982; Ong and Lim, 1983; Subramaniam, 1972). Natural rubber produced from wild stands of guayule (*Parthenium argentatum* Gray) has a processing profile that compares favorably with that of natural rubber from *Hevea brasiliensis* Muell. Arg. (Winkler et al., 1977; Ponce and Ramirez, 1981). Although the chemical structure of rubber from guayule and *Hevea* are quite similar (Ramos-de Valle and Aramburo, 1981; Swanson et al., 1979), Hager et al. (1979) reported that juvenile guayule, estimated to be 6 months old, has a significantly lower weight-average molecular weight ($\bar{M}_w$) and broader molecular weight distribution (MWD: $\bar{M}_w$ divided by the number-average molecular weight, $\bar{M}_n$). Rubber from these juvenile plants had a $\bar{M}_w$ of (6.4–7.8) $\times 10^5$ and MWD of 6.2–10.4. Mature plants yielded rubber with a $\bar{M}_w$ of (9.8–14.6) $\times 10^5$ and MWD of 1.7–2.4. Meeks et al. (1950) reported that the yield of acetone-soluble, low molecular weight guayule rubber was seasonally dependent. These observations raise the question of whether the quality of guayule rubber, and ultimately its commercial acceptability, is dependent on shrub age and harvest date. One method of determining rubber yield (Smith, 1985) is based on the assumption that the molecular weight of guayule rubber is constant.

Rubber accumulation in guayule is cyclical. Spence and McCallum (1935) reported that there is relatively little accumulation during periods of vegetative growth, with accumulation being greatest in the fall and winter. Environmental factors that affect the production and accumulation of rubber in guayule include temperature, light intensity, nutrient availability, and water stress (Hammond and Polhamus, 1965). When plants were subjected to temperatures above 7 °C, the rate of rubber accumulation dropped significantly (Bonner, 1943). Rubber precursor incorporation is higher in cold-treated plant tissue (Goss et al., 1984). Light intensity has an effect on both plant growth and rubber production: a reduction in incident sunlight produced a proportional reduction in dry weight and rubber accumulation (Bonner and Galston, 1947). These effects are related to rubber precursor availability. Spence and McCallum (1935) reported that shrub defoliated in the fall, a period of rapid rubber accumulation, halts almost all rubber production in the remaining woody tissue. Carbohydrate reserves in the wood were slowly metabolized, but not used for rubber production. Normally, rubber precursors are synthesized in the leaves and transported to the bark for rubber formation.

Water stress management has been studied as a means of controlling rubber production. Benedict et al. (1947) grew plants under alternating low- and high-stress periods. Plants were forced to divert isoprenoid metabolism toward rubber production during short, nonwinter stress periods. Maximum growth rates were resumed by reducing moisture stress. In this way, rubber accumulation was optimized. If adequate nutrition is maintained, water is the only independent variable by which guayule rubber production can be controlled. The impact of the resulting stress-induced shifts in rubber synthesis on rubber quality has not been addressed to date.

We report here the characterization of rubber in a single guayule line from irrigated plots sampled over a 7-month period beginning 17 months from seed germination. Three irrigation regimes were maintained by the crop water stress index (CWSI) (Idso et al., 1981; Jackson et al., 1981). The sample interval includes the period of cold-weather stress associated with rubber formation (Bonner, 1943) and the period of renewed vegetative growth.

EXPERIMENTAL SECTION

Shrub Origin. Seeds of guayule line N396 growing within increase plots at Mesa, AZ, were harvested in October 1981 from single plants exhibiting common phen-

Central Research Laboratories, The Firestone Tire & Rubber Company, Akron, Ohio 44317 (W.W.S.), and Department of Plant Sciences, University of Arizona, Tucson, Arizona 85721 (D.J.G., D.T.R.).

0021-8561/86/1434-0683$01.50/0