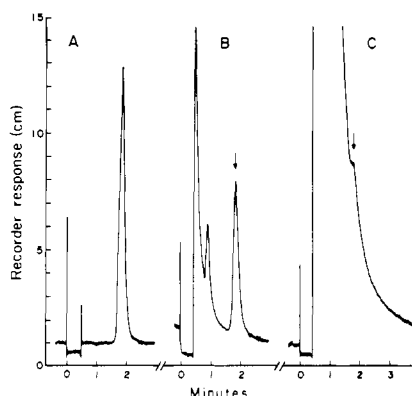

Figure 2. Flame photometric detector response to 20 ng of *N*-thiomethylcarbaryl standard (A) and to string beans fortified with 10 ppm carbaryl with (B) and without (C) treatment with $HgCl_2$–$CaCO_3$ solution after derivatization.

Table I. Recovery of Carbaryl Added to Various Crops and Quantitated as Its *N*-Thiomethyl Derivative

Crop	Fortification, ppm	Recovery, % Replicate A	B	C	Mean
Beans	10	118	96	68	94
	5	56	105	100	87
Carrots	10	118	105	90	104
	5	115	96	92	101
Tomatoes	10	118	78	78	91
	5	63	63	57	61
Lettuce	10	90	83	86	86

Attempts to analyze for the *N*-thio-*p*-tolyl derivative by gas chromatography were unsuccessful due to interfering peaks which coeluted with the derivative peak. Florisil column chromatography and treatment of the final solution with $HgCl_2$–$CaCO_3$ to remove interfering sulfur compounds were unsuccessful. Use of methylsulfenyl chloride as the derivatizing reagent also produced many interfering peaks, but they could be reduced by treatment with $HgCl_2$–$CaCO_3$ to the point where crop samples fortified at 5 to 10 ppm could be analyzed. Figure 2 shows a sample chromatogram for string beans fortified at 10 ppm carbaryl and analyzed by the *N*-thiomethyl derivatization technique. The figure demonstrates that much interference was produced that needed to be eliminated before quantitation could be accomplished. The chemical and physical properties of the interferences produced from the *p*-tolylsulfenyl chloride were probably too similar to that of *N*-thio-*p*-tolylcarbaryl to be readily separated.

Table I gives the recovery data for carbaryl added to beans, carrots, tomatoes, and lettuce prior to extraction and cleanup. Recoveries for a 10 ppm fortification were above 85%. Thus, direct derivatization of carbaryl residues with a sulfur-containing moiety to utilize the selective sulfur response of a flame photometric detector has been demonstrated. Unpleasant odors are produced by the reagent or its by-products, but can be largely contained by good ventilation. Due to interferences formed, the procedure cannot be applied to crops containing less than 5 ppm carbaryl without further refinement; however, most agricultural crops have a food tolerance of 10 ppm. The overall length of the procedure, though excessive for routine samples, is approximately equal to the 2,4-dinitrophenyl ether formation procedure.

LITERATURE CITED

Black, A. L., Chiu, Y., Fahmy, M. A. H., Fukuto, T. R., *J. Agric. Food Chem.* **21**, 747 (1973).
Epstein, A. J., Gaskill, D. R., Lucchesi, C. A., *Anal. Chem.* **39**, 721 (1967).
Fishbein, K., Zielinski, W. L., *J. Chromatogr.* **20**, 9 (1965).
Hankock, K. G., Wylie, P. L., Lau, J. T., *J. Am. Chem. Soc.* **99**, 1149 (1977).
Holden, E. R., *J. Assoc. Off. Anal. Chem.* **56**, 713 (1973).
Kühle, E., *Synthesis* 11, 561 (1970).
Lau, S. C., Marxmiller, R. L., *J. Agric. Food Chem.* **18**, 413 (1970).
Magallona, E. D., Gunther, F. A., *Philipp. Agric.* **58**, 265 (1974).
Magallona, E. D., Gunther, F. A., *Arch. Environ. Contam. Toxicol.* **5**, 185 (1977).
Moye, H. A., *J. Agric. Food Chem.* **23**, 415 (1975).
Seiber, J. N., *J. Agric. Food Chem.* **20**, 443 (1972).
Sullivan, L. J., Eldridge, J. M., Knaak, J. B., *J. Agric. Food Chem.* **15**, 927 (1967).
Tracor Instruments, *Retent. Times* **2**(1), 2 (1975).
Westlake, W. E., Ittig, M., Gunther, F. A., *Bull. Environ. Contam. Toxicol.* **8**, 109 (1972).
Zehner, J. M., Simonaitis, R. A., *J. Chromatogr. Sci.* **14**, 348 (1976).

Shyam B. Mathur[1]
Yutaka Iwata*[2]
Francis A. Gunther[2]

[1]Departamento de Quimica
Universidad de Oriente
Cumana, Venezuela
[2]Department of Entomology
University of California
Riverside, California 92521

Received for review September 2, 1977. Accepted December 9, 1977. This work was supported in part by Western Regional Research Project W-45.

A Rapid Technique for Total Nonstructural Carbohydrate Determination of Plant Tissue

A simple and inexpensive technique was developed based on the digestion of total nonstructural carbohydrates by a MYLASE enzyme system followed by colorimetry at 250 nm using the Teles' reagent. The technique is very precise with a standard deviation of less than 0.3%. An average of 96 samples were analyzed easily in 24-h elapsed time by one technician.

The energy readily available to a plant is determined by total nonstructural carbohydrate (TNC) analyses. This total is sometimes termed total available carbohydrate (TAC) by plant scientistis, rather than TNC (Smith, 1969). There are various techniques published for TNC determination (Burris et al., 1967; Greub and Wedin, 1969; Heinze and Murneek, 1940; Smith et al., 1964; Smith, 1969; Weinmann, 1947; and Yemm and Willis, 1954). All of these require extensive time, reagents, and glassware, in addition to considerable skill and practice by the analyst.

 © 1978 American Chemical Society

The most frequently used technique is the one introduced by Smith (1969) although it takes 4 days for total completion and uses a considerable amount of glassware. The technique described in this paper was developed in answer to the disadvantages described above.

EXPERIMENTAL SECTION

In order that the technique may be followed in a more standardized way, considerable detail in the various steps is presented.

Materials. The following equipment was used (unspecified sizes and numbers based on the number of samples to be analyzed): (1) two volumetric flasks; one for buffer and one for sodium bisulfite; (2) one graduated cylinder for preparation of Teles' (1977) reagent; (3) culture tube, screw cap, 16 × 100 mm (Kimble 45066); (4) blood sugar tube, Folin, graduated at 12.5 and 25 mL; (5) three glass beakers for dispensing buffer, enzyme, and Teles' reagent; (6) wash bottle, conventional polyethylene; (7) S & S, No. B-2 weighing papers, size 5 cm × 5 cm; (8) rubber stoppers, solid, No. 1; (9) one transfer pipetting system, MACRO-SET, Oxford, adjustable from 1 to 5 mL with slim tips; (10) serological test tube racks, Kolmer no. 69518, holes 16 mm o.d., or equivalent; (11) test tube support, holes 20 mm o.d. for Folin blood sugar tubes.

It is assumed that the laboratory would have normal basic facilities such an an autoclave, analytical balance, centrifuge, water bath, etc. The use of a flow-through, 10-mm cuvette for the colorimeter improves precision and rapidity.

Chemicals. Glacial acetic acid, phenol (loose crystals), picric acid, sodium bisulfite, sodium hydroxide (all analytical grade).

Enzyme. MYLASE 100 (Wallerstein Co., Deerfield, Ill.), a pharmaceutical grade fungal α-amylase derived from *Aspergillus oryzae*. A test for cellulose activity was negative. Prepare a 0.3% (w/v) enzyme solution in distilled water just before the enzyme is needed. Keep under constant mixing while dispensing to the tubes.

Buffer. *0.2 M Acetic Acid.* Transfer 12.0 mL of glacial acetic acid to a 1000-mL volumetric flask and dilute to volume with distilled water.

0.2 M Sodium Acetate. Dissolve 16.408 g of anhydrous sodium acetate in 700 to 800 mL of distilled water, transfer to a 1000-mL volumetric flask, and dilute to volume.

Stock Buffer Solution. Mix three volumes of 0.2 M sodium acetate with two volumes of 0.2 M acetic acid. The pH should be 4.9 (theoretically 4.92). Store in a well-stoppered, dark-colored bottle under refrigeration.

Working Buffer Solution (0.02 M acetate buffer). To one volume of stock buffer solution, add nine volumes of distilled water and shake to mix. This dilution should be carried out with a pipet and volumetric flask. Prepare a new working buffer solution immediately before each analysis.

Teles' Reagent. Prepare the following stock solutions in distilled water: solution A, 1% (w/v) phenol; solution B, 5% (w/v) sodium hydroxide; solution C, 1% (w/v) picric acid; solution D, 1% (w/v) sodium bisulfite (high-quality analytical grade).

Working Solution of Teles' Reagent. (To be prepared a maximum of 2 days before use). Add in the following exact order, mixing between additions: one volume of phenol (solution A), two volumes of NaOH (solution B), two volumes of picric acid (solution C), and one volume of fresh sodium bisulfite (solution D). Solutions A, B, and C, prepared as stated above, are stable for at least 1 month. The sodium bisulfite solution has a shelf-life of only 2 days. Keep the working reagent solution in a well-stoppered, colored bottle. Prepare the working solution only in amounts according to the volume needed.

Digestion and Extraction. Place a 100-mg sample (ground to pass a 60-mesh sieve), standard or blank, in a culture tube. Size and/or dilute the sample according to carbohydrate concentration and colorimeter capabilities; 100 mg is a good weight for 2 to 30% TNC samples, using a 10-mm cuvette in a Spectronic-20, B & L spectrophotometer. Add 2 mL of 0.02 M acetate buffer, cap the tubes loosely, and immediately autoclave for 15 to 20 min at 1.05 kg/cm^2. This treatment serves to inactivate microorganisms and plant enzymes, as well as to facilitate future enzyme–substrate contact. Cool to room temperature by immersing in tap water and add exactly 4 mL of 0.3% enzyme solution. Cap tightly and incubate for 20 to 24 h at 38 to 40 °C in a shaking water bath or in a fixed unit by swirling several times during incubation. When using a shaking water bath, the results were constant after 8 h of incubation in the case of materials analyzed in this report. This time may vary slightly for different plant materials and will increase with increasing starch concentrations. Remove from incubation, add exactly 4 mL of distilled water, shake the tube vigorously 25 to 30 times to remove reducing sugars, and centrifuge for 5 min at 2500 to 3000 rpm (900 to 1300 RCF). RCF (relative centrifuge force) = $1.2 \times 10^{-5}\, rn^2$, where r = radius in cm and n = rpm. Let tubes stand for a minimum of 5 min to a maximum of 60 min after centrifugation. The final liquid volume should be 10 mL.

Colorimetry. Transfer a 1.0-mL aliquot of the supernatant liquid to a Folin blood sugar tube. Add 2.5 mL of Teles' reagent and stopper tightly with a *dry* rubber stopper. Immerse the bottom of the tube 4 to 6 cm in a violently boiling water bath for exactly 6 min. Cool immediately in cold tap water for a minimum of 5 min. Up to this point there is no need to remove the tubes from the rack. Complete the volume to the 12.5 or 25 mL mark depending on the TNC concentration in tissue. Read the percent transmittance spectrophotometrically at 520 nm in a 10-mm cuvette (Abs = log T; example: 25% T, Abs = log 1/.25 − log 4 = 0.602). This should be done in case the colorimeter in use does not have a linear log scale. The color is stable at room temperature for at least 12 h. This colorimetric reaction may also be used in the determination of lactose in milk and whey (Teles et al., 1978).

Standards. Weigh the desirable amount of dry, pure glucose (or sucrose), 5 to 25 mg, or take 1 mL of freshly prepared standard solution of equivalent concentration. If the solution is used, add only 3 mL of distilled water after incubation. The parallel use of sucrose enables a check for the invertase activity of the enzyme system. If the enzyme preparation is pure, the photometric reading should be the same as that for glucose.

Calculations.

$$\%\ \text{TNC} = \frac{\text{absorbance of sample} \times \text{wt of standard glucose}}{\text{absorbance of glucose} \times \text{wt of sample}} \times 100$$

Use glucose standard whose absorbance is closer to that of the sample, or prepare a standard curve.

RESULTS AND DISCUSSION

In direct comparison and by use of internal standards, the results were comparable with Smith's (1969) technique (Table I). Two uniform samples of cotton stems from green plants with different TNC concentrations analyzed

Table I. Comparison of % TNC in Cotton Stems by the Authors' and Smith's Methods

Sample no.		Methods	
		Authors'	Smith's
1		11.3	12.1
2		8.2	8.2
3		11.5	12.1
4		8.4	8.6
5		11.3	12.0
6		8.0	8.2
7		11.8	12.5
8		8.5	9.4
9		12.0	12.1
10		8.6	8.5
	Av[a]	9.96	10.37

[a] A standard statistical comparison of the averages based on the "t" test showed no significant differences ($P < 0.01$).

with ten replications showed a high degree of accuracy. Ten replicate analyses of cotton plant samples with 10% to 15% TNC gave standard deviations of 0.28 and 0.14, respectively. The same degree of accuracy was obtained when analyzing different parts of cotton plants and lyophilized cactus pads (*Opuntia*, s.p.) harvested at different seasons of the year. These materials ranged from 10% to 70% TNC. The authors' technique can be conducted for about one-tenth the cost of other methods in use. A complete analysis can be performed in 24 h elapsed time, while other techniques require 3 to 4 days. It should also be noted that a minimum of glassware is required. This represents a saving of initial cost and routine glassware-washing time. It is especially noteworthy that the detergent action of the Teles' reagent allows effective cleansing of the Folin tubes by simple rinsing four times with distilled water. In addition, it also allows the use of a flow-through curvette. The reduced number of operations make the technique more accurate and less tedious. The reagents used, in addition to being less expensive, can be prepared with distilled water with no need for deionizers or other water retreatment. The substitution of dinitrosalicylic acid (Summer and Somer, 1949) for Teles' reagent gave similar results. One technician easily analyzed an average of 96 samples in 24-h elapsed time.

In case the laboratory is equipped with only an ordinary centrifuge (maximum speed ca. 1500 rpm), the use of 4 mL of a 2% solution of neutral lead acetate (only the neutral salt) in water instead of 4 mL of distilled water before centrifugation also gives a clear supernatant. This amount of lead salt does not interfere with the analysis as was demonstrated in the course of the experiment with comparative analyses. The lead precipitation also would allow aliquot filtration, but the authors avoided this operation since it increases time and cost.

ACKNOWLEDGMENT

The authors acknowledge the assistance of Robert E. Briggs, Albert K. Dobrenz, Dwayne R. Buxton, and Ralph L. Price. They also thank Michael L. Rogers, Joseph E. Stone, and Ralph R. Taylor for their valuable help and the Wallerstein Company for supplying the enzyme used in the analyses.

LITERATURE CITED

Burris, J. S., Brown, R. H., Blaser, R. E., *Crop Sci.* **7**, 22 (1967).
Greub, L. J., Wedin, W. F., *Crop Sci.* **9**, 595 (1969).
Heinze, P. H., Murneek, A. E., *Mo., Agric. Exp. Stn. Res. Bull.* **314** (1940).
Smith, D., *Wis., Agric. Exp. Stn. Res. Rep.* **41** (1969).
Smith, D., Paulsen, G. M., Raguse, C. A., *Plant Physiol.* **39**, 960 (1964).
Sumner, J. B., Somers, G. F., "Laboratory Experiments in Biological Chemistry", Academic Press, New York, N.Y., 1949, p 38.
Teles, F. F. F., Ph.D. Dissertation, The University of Arizona, Tucson, 1977.
Teles, F. F. F., Young, C. K., Stull, J. W., *J. Dairy Sci.* **61**, in press (1978).
Weinman, H., *Plant Physiol.* **22**, 279 (1947).
Yemm, E. W., Willis, A. J., *Biochem. J.* **57**, 508 (1954).

Americo J. daSilveira[1]
F. F. Feitosa Teles[2]
J. Warren Stull*[2]

[1]Department of Plant Sciences
[2]Department of Nutrition and Food Science
The University of Arizona
Tucson, Arizona 85721

Received for review September 12, 1977. Accepted January 26, 1978. Arizona Agricultural Experiment Station Technical Paper No. 2763 Supported in part by Grant AID/1a-145 (Brazil Contract). Reference to a firm or trade names does not imply endorsement of firms or products mentioned.

Anise-Like Flavor of *Croton* aff. *zehntneri* Pax et Hoffm.

Estragole, the major constituent of the essential oil of *Croton* aff. *zehntneri*, family Euphorbiaceae, occurs together with minor quantities of camphor, anethole, isoborneol, caryophyllene, γ-elemene, safrol, methylisoeugenol, n-heptadecane, and n-eicosane. These compounds are being reported for the first time in the genus *Croton*. Fifteen predominant volatile constituents of the oil were analyzed by GC–MS and identified. The oil has a typical pleasant odor reminiscent of anise (*Pimpinella anisum* L.)

The genus *Croton* is largely disperse in the semiarid lands of Brazilian Northeast and includes many species that produce essential oils with pleasant odors.

This report deals with the examination of the volatile constituents of *Croton* aff. *zehntneri* (canelinha) family Euphorbiaceae collected in the state of Ceará. The infusion of the leaves is used by local people as a beverage in place of coffee and as a remedy for stomachache and insomnia.

EXPERIMENTAL SECTION

Analytical GLC was performed on a FID instrument