Rapid Estimation of Fruit Starch and Soluble Sugar Concentrations in Kiwifruit

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Additional index words. carbohydrates, *Actinidia deliciosa*, analysis

Abstract. Experiments investigating kiwifruit [*Actinidia deliciosa* (A. Chev) C.F. Liang et A.R. Ferguson var. *deliciosa*] maturation were undertaken requiring the determination of total soluble sugar (TSS) and starch concentrations in numerous fruit samples. The phenol-sulfuric acid assay was judged to be a convenient method for determining TSS from tissue extracts and gave results similar to those obtained by high-pressure liquid chromatography. The starch procedure adopted involved gelatinizing fruit tissue using hot water and a thermostable α -amylase (Termamyl); hydrolizing starch using amyloglucosidase; and determining glucose using glucose oxidase. The methods enabled one person to analyze up to 40 kiwifruit samples for TSS and starch concentrations during 9 hours and likely will be applicable to research concerning fruit development and maturation.

The predominant storage carbohydrate in kiwifruit is starch, which is stored in the outer pericarp and core tissue of the fruit. Fruit starch concentrations increase and reach a peak $\approx$ 1 month before harvest and then rapidly decrease with a concomitant rise in free sugars during the latter phases of maturation (Beever and Hopkirk, 1990). The major sugars in kiwifruit at eating ripeness are glucose (2% to 6% of fresh weight), fructose (1.5% to 8%), and sucrose (2%) (Beever and Hopkirk, 1990), with traces of polyols, such as inositol (Kawamata, 1977).

Studies have investigated the changes in carbohydrate partitioning between starch and sugars in response to temperature during kiwifruit maturation (Seager et al., 1991). Mature fruit are regarded as those having reached eating ripeness. This work required analysis for sugars and starch in fruit samples that were collected regularly during fruit development from plants subjected to various temperatures. However, as it was not feasible to analyze the carbohydrates at the time of sampling, it was necessary to preserve the fresh tissue at harvest. The very large number of samples accumulated (6000) required the analytical meth-

ods to have as high a sample throughput as possible.

Sugar and starch concentrations have been determined for a wide range of types of fresh and dried plant tissue. Typically, sugars are quantified using calorimetric (Hodge and Hofreiter, 1962) or chromatographic procedures following extraction of the fresh or dried tissue with alcohol-based solvents. After soluble sugars have been removed, starch usually is hydrolyzed enzymatically, following pretreatment, to render the substrate available to enzyme attack, and the released glucose is measured using specific enzyme systems (Haslemore and Roughan, 1976; MacRae et al., 1974). None of the published methods appear to have been designed or adapted specifically for fresh kiwifruit tissue or other fruit types. Our colleagues have applied the Boehringer Mannheim starchkit (International Division, Mannheim, Germany) based on the methods of Keppler and Decker (1974) for the analysis of starch in fresh kiwifruit tissue (Bowen et al., 1988; MacRae et al., 1989a), but the method was found to be unsuitable for rapid analysis of numerous fruit samples.

The aim of this work was to develop a simple and practical method for determining total soluble sugar (TSS) and starch concentrations in kiwifruit tissue with sufficient throughput to undertake thousands of analyses during each fruit-growing season.

Preparation of samples for storage and analysis. Samples, each $\approx$ 4 to 5 g, were taken from the outer pericarp or core tissue of each 'Hayward' kiwifruit. The tissue was chopped into cubes ($\approx$ 5 mm), weighed, and steeped in 20 ml of 12 methanol : 2 water : 1 formic acid (by volume) (E.A. MacRae, personal communication) in capped vials before storage at 3°C for up to 1 year. For analysis, the extractant was decanted into a beaker, and the tissue was transferred into a preweighed 50-ml centrifuge tube, homogenized with 20 ml of dis-

tilled water using a Polytron homogenizer (Kinematica GmbH, Amlehnhalde, Switzerland), then centrifuged at 2000 $\times$ g for 15 min. The supernatant was combined with the original extractant and used for soluble sugar analysis. The weight of residual fruit tissue was obtained by reweighing the tube plus contents and setting it aside for starch analysis.

Total sugar concentration. Calculations were based on the mean total volume of extractant estimated from at least 10 replicate measurements from outer pericarp or core tissue samples. These volumes varied according to the kiwifruit tissue type being extracted but were consistent among either outer pericarp or core fractions. Aliquots of 1 ml were removed from the extract solutions and diluted with distilled water by a factor between 10 and 120, depending on sugar concentration; then, 1 ml of diluted extract was assayed for total carbohydrate (by comparison with a range of glucose standards) by the phenol-sulfuric acid method of Dubois et al. (1956).

The phenol-sulfuric acid assay was compared with a high-pressure liquid chromatography (HPLC) procedure to evaluate independently the recovery of sugars from the fruit extracts. Subsamples from selected sugar extracts were evaporated to dryness under reduced pressure at 40°C, reconstituted to the original volume with distilled water, and filtered through a 0.2- μ m nylon membrane. Samples were injected into a HPX-87C Bio-Rad (Bio-Rad Laboratories, Richmond, Calif.) column (300 $\times$ 7.8 mm) and eluted with water at 85°C for 90 min. Component peaks were detected by refractive index.

Starch concentration. Subsamples of the residual tissue (300 to 900 mg depending on the fruit sample maturity) and three starch standards of 10, 35, and 65 $\pm$ 0.1 mg were weighed into screw-capped Kimax 45066-A (Owens-Illinois, Toledo, Ohio) culture tubes (16 $\times$ 125 mm). The starch standard used was a purified wheat preparation (Sigma S-2760), and weights were corrected for moisture content (10.6% of dry matter determined at 105°C). A 10-ml aliquot of distilled water and 50 μ l of a thermostable α -amylase (Termamyl, activity 120 kNu/g; Novo Industri, A/S Copenhagen, Denmark) were added to each sample, mixed, and the tubes were then heated in a boiling water bath for 90 min. The tubes were capped firmly after heating 5 min, and re-mixed after 45 min. After cooling, the tube contents were poured into a 130-ml container, the tubes rinsed with 10 ml 0.25 M sodium acetate buffer (pH 4.5), and the buffer plus 80 ml distilled water was added to the container with thorough mixing to give a 100 $\pm$ 5-ml final solution.

About 1 ml from each sample was centrifuged in a 1.5-ml microcentrifuge tube at 8000 $\times$ g for 2 min. Omitting the centrifuging step had no effect on starch recovery but gave rise to blocked pipette tips when subsampling for further analysis. A 200- μ l aliquot of supernatant from each sample was added to a culture tube (16 $\times$ 125 mm), followed by 400 μ l sodium citrate buffer (pH 4.6), 350 μ l distilled water, and 50 μ l amyloglucosidase (Boehringer

Received for publication 17 Nov. 1992. Accepted for publication 31 Mar. 1993. N.G.S. thanks HortResearch and the New Zealand Kiwifruit Marketing Board for financial support. We thank Chemcolour Industries for providing the Termamyl and Colin Tod for the high-pressure liquid chromatography analyses. The use of trade names in this publication does not imply endorsement of their use, nor criticism of similar ones not mentioned. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

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Mannheim 737160, 14 U/mg). The contents were mixed and then hydrolyzed by incubating at 60C for 20 min. Released glucose was determined by a glucose oxidase method similar to that of Kilburn and Taylor (1969).

Calculations. Total soluble sugar and starch concentrations were calculated as milligrams per gram of fresh tissue and corrected for the appropriate dilutions. Starch concentration also was corrected for the amount of residual tissue subsampled for analysis and multiplied by 0.9; this factor accounted for the mass of glucose theoretically hydrolyzed from a unit mass of starch.

Total sugar analysis. The major requirement for this procedure was to obtain a reliable estimate of TSS in fruit samples harvested sequentially and preserved in an acidic alcoholic extractant. The number of samples generated meant that the method chosen needed to balance analytical accuracy with sample throughput. Examination of numerous samples, particularly mature fruit high in sugar levels, indicated that the original steeping extraction procedure removed nearly 80% of all soluble sugars and accounted for 96% of all sugars when combined with a cold water wash. Washing the residual tissue a second and third time released the last 3% and 1% of the remaining soluble sugars, respectively. The additional time needed for these steps was not justified for our work, but might be required in other situations. Also, to save time, combined extract volumes were not measured individually but were based on mean volumes estimated from replicated measurements.

Examination of mature fruit extracts using HPLC identified the major components as fructose (48%), glucose (39%), and sucrose (13%). These mean proportions are similar to those reported elsewhere (MacRae et al., 1989b). None of the extracts contained other di- or oligosaccharides. The presence of sugar alcohols such as inositol was not detected using our system. The phenol-sulfuric acid assay proved ideal for the calorimetric determination of TSS in kiwifruit tissue extracts. This procedure gave a similar response to glucose (100%), fructose (114%), and sucrose (96%) when normalized to glucose on a mass basis. It is a quick, convenient, and considerably more cost-effective method than that using HPLC. Furthermore, the results obtained were similar to those using HPLC for measuring TSS in extracts. For example, the analysis of a typical mature kiwifruit sample by the calorimetric and HPLC procedures gave 44.4 ± 1.0 and 42.8 ± 2.2 mg sugar/g fresh weight, respectively (mean and SD for three replicated tissue analyses within a fruit sample).

Starch analysis. Initially, fruit starch assays were carried out using the method of Boehringer Mannheim (1979) with the aim of modifying some of the steps to increase throughput above the 15 samples/day achieved by our colleagues. This analytical procedure involves solubilizing starch using hot acidic dimethyl sulfoxide (DMSO), neutralizing pH 4.5 (monitored by a pH meter), hydrolyzing the solubilized substrate using amyloglucosidase, and determining specifically the released

glucose using the ultraviolet/ hexokinase method. Most of these steps are time-consuming and unsuited to reductions in scale to improve efficiency and throughput.

Solubilizing starch by steeping fruit tissue at 60C in HCl-DMSO in conical flasks (Boehringer Mannheim, 1979) did not permit rapid analysis. The recovery of hydrolyzed glucose from starch standards was $\approx 5\%$ higher when flasks were swirled constantly in a shaking water bath than when they were not. However, the sample processing efficiency was improved substantially when the solubilization step was carried out in screw-capped tubes. Here, preliminary results with fruit samples indicated that starch concentrations were similar to those obtained with flasks, shaken or otherwise. Following steeping in HCl-DMSO, the neutralization procedure recommended by Boehringer Mannheim (1979) used dropwise addition of 5 M NaOH to achieve finally a 4.5 pH. Attempts were made to neutralize these acidic solutions by adding constant volumes of sodium acetate buffer (pH 4.5), but these were not completely successful.

Therefore, we gelatinized starch by treating fruit tissue in 100C water as an alternative to the solubilization-neutralization step. Gelatinization is commonly used to render starch accessible to enzymic hydrolysis (e.g., Haslemore and Roughan, 1976) and, in recent years, this procedure has been extended by boiling tissue in the presence of a thermostable α -amylase preparation such as Termamyl (Aman and Hesselman, 1984; Bengtsson and Larsson, 1990), which converts the substrate to a mixture of soluble oligosaccharides. Adding Termamyl gave higher starch concentrations with improved precision for standard and fruit tissue samples than did samples gelatinized without Termamyl. For example, starch concentrations measured from a typical ripe fruit sample were almost doubled when 50 μ l of Termamyl was used compared to gelatinization without the enzyme (Table 1), but were not increased further when enzyme volumes larger than 50 μ l were added.

A variant of the commonly used glucose oxidase procedure (Kilburn and Taylor, 1969) was substituted for the hexokinase method employed by Boehringer Mannheim (1979) for measuring hydrolyzed glucose, because each gave similar theoretical glucose recoveries (99.6% and 96.6%, respectively) from several standard starch hydrolysates ranging from 15 to 70 mg starch. The glucose oxidase method

was thus adopted routinely because of speed, convenience, and economy.

For common or comparable fruit samples, the TSS and starch methods described here have given results similar to those obtained with alternative procedures. For soluble sugars, this involved quantitative HPLC on common extracts as described above. For starch, the comparisons were made with fruit of similar maturity using the method reported here (Seager et al., 1992) and the Boehringer Mannheim method (MacRae et al., 1989b). The proposed starch method is similar to procedures developed specifically for cereal grains (Aman and Hesselman, 1984) and for nonfruit horticultural material (Rasmussen and Henry, 1990) but both the sugar and starch methods described in this paper should be applicable to a wide range of fruit tissue. Specifically, the use of a thermostable α -amylase during gelatinization of starch in kiwifruit resulted in much improved starch recoveries from these fruit samples. Further, the use of Termamyl may have a similar benefit in the analysis and quantitative recovery of starch from other fleshy fruit types; thus, this approach may warrant further study.

Kiwifruit, like most other fruit tissue, is inherently variable. Repeated analyses within the outer pericarp tissue of individual fruit gave a cv of $\approx 2\%$ to 10% and $\approx 5\%$ to 15% for sugar and starch analyses, respectively, depending on the stage of fruit maturity. Typically, variation between fruit for both analyses was 215%.

These methods were developed to meet the need to analyze the gross carbohydrate fractions of numerous fruit samples from kiwifruit vines. The procedures are practical and do not require expensive equipment, and there may be opportunities to improve or refine them further. Currently, their key advantages over existing methods, particularly the Boehringer Mannheim starch procedure, are simplicity, convenience, and speed, as one analyst can process 40 samples daily. These procedures may have applications in all aspects of kiwifruit research concerning the relationship between fruit carbohydrate composition and fruit development.

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Table 1. Effect of using a thermostable α -amylase (Termamyl) during starch gelatinization in standard and fruit samples.

Sample type	Vol of Termamyl (μ l)		
	0	25	50
<i>Theoretical recovery of released glucose (%)^a</i>			
Starch standard (mg)			
10	92.8 $\pm$ 12.7	101.2 $\pm$ 2.4	106.6 $\pm$ 1.5
35	86.7 $\pm$ 1.5	97.9 $\pm$ 2.3	101.2 $\pm$ 0.9
65	69.9 $\pm$ 2.0	81.2 $\pm$ 0.9	80.7 $\pm$ 0.6
<i>Starch (mg/g fresh wt)^a</i>			
Kiwifruit	1.9 $\pm$ 0.7	3.0 $\pm$ 0.3	3.5 $\pm$ 0.2

^aData are the means and SD of three replicate analyses from the same fruit using the methods described and the outer pericarp tissue from ripe fruit.

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