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ROUTINE CITRUS JUICE ANALYSES USING HPLC WITH AMPEROMETRIC DETECTION

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Abstract. **Analytical methods useful for routine applications are ideally low-cost, simple to perform, and yield rapid results which are easy to interpret. Chromatographic methods, particularly of complex samples such as fruit juice, are often difficult, can be expensive and time-consuming, and may yield results devoid of interpretation. With the proper choice of detector, the chromatographic analysis of even the most difficult samples can often be simplified. During the past decade, there has been a rapid increase in the use of electrochemical (EC) detection methods in high-performance liquid chromatography (HPLC). The sudden popularity is due to the detector's extreme sensitivity, selectivity, and compatibility with reversed-phase and ion-exchange separations. Three HPLC/EC methods were developed and/or adapted for routine analysis of vitamin C, folic acid, sugars and sugar alcohols in citrus juice. Analysis time for each method was under 10 minutes. Analytical results for some fresh citrus juices are presented, along with statistical considerations.**

Introduction

Citrus juices are an important dietary component for the maintenance of good health, and can be useful in the treatment and management of a number of human diseases (9). Fresh orange, grapefruit and their juices, as well as commercially processed products, typically render a palatable blend of several key nutrients. The simple sugars glucose, fructose, and sucrose comprise the major carbohydate fraction and make up between 75 to 90% of the total soluble solids in juice; the relative amounts depend somewhat on season and variety. Although its nutritional value is not completely understood, the sugar alcohol myo-inositol is present at significant levels. Probably the most important water-soluble vitamins are vitamin C (L-ascorbic acid) and folic acid. Other nutrients include thiamine (vitamin B_1), vitamin B_6, niacin, riboflavin, pantothenic acid, bioflavonoids, potassium, other minerals and trace elements (18).

With the current emphasis on nutritional labeling and an increased consumer interest in nutrition, new demands are being placed on food and beverage industries to specify the quantity of nutritionally important components in foods. It is therefore important to have analytical methods that are accurate, rugged, and of low-cost. Methods that are rapid, simple to use or easily automated are preferable to more costly, time-consuming techniques.

Citrus juice is a complex matrix and analysis often requires significant sample cleanup, some type of separation, followed by an accurate measurement of the analyte, which is often present at very low levels. The usefulness of high performance liquid chromatography (HPLC) depends on the ability of the column to separate, and the detector to sense the component of interest. For complex mixtures, inadequate resolution due to limitations in column performance often requires that the detector be capable of discriminating the analyte from other coeluting substances.

During the past decade, there has been a significant increase in the popularity of electrochemical (EC) detection methods. Primarily used for the analysis of trace organic compounds in complex biological media, the usefulness of the EC detector stems from its selectivity, sensitivity, and relatively low cost. The thin-layer amperometric cell (Figure 1) is widely used because of the simplicity of design, small cell volume, and compatibility with several electrode materials. HPLC with amperometric detection permits routine analysis of picomole amounts of electroactive substances (8). Several of the nutritional factors in citrus juice are electroactive and amenable to amperometric detection.

The purpose of this work is to illustrate the utility of HPLC/EC for three routine applications in citrus juice analysis: Vitamin C (*L-ascorbic acid*), folic acid, sugars and sugar alcohols. Vitamin C exists in citrus juice in the reduced form and is easily oxidized on the surface of a glassy carbon electrode (1). Folic acid exists in reduced form as 5-methyltetrahydrofolic acid (5-MeTHF) or its polyglutamate derivatives (3). Sugars and sugar alcohols are electroactive in alkaline solution and were analyzed using high

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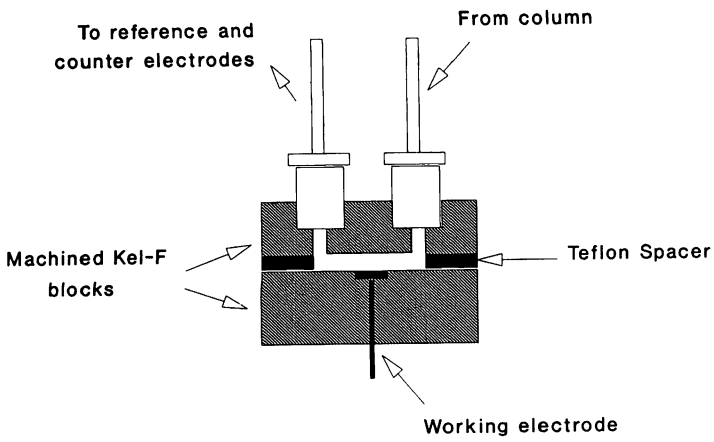

Fig. 1. A thin-layer amperometric detector cell design. (Adapted from Kissinger, P. T., ref. 8).

performance anion-exchange chromatography (HPAE) with pulsed amperometric detection (PAD) (7, 14).

Materials and Methods

Reagents

L-ascorbic acid was obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). 5-methyltetrahydrofolic acid (5-MeTHF, barium salt) was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. An estimated 80% purity was calculated from the absorbance at 290 nm of a freshly prepared standard solution using a published molar absorptivity of 2.9×10^4 (11). Reagent grade anhydrous dextrose (D-glucose), D-fructose, sucrose (Fisher Scientific, West Haven, CT) and myo-inositol (Sigma) were dried under vacuum at 65°C for no less than 3 hours. Other reagents included tetrabutylammonium dihydrogen phosphate (TBAP, 1.0 M solution, Aldrich), glacial acetic acid, concentrated sodium hydroxide, and meta-phosphoric acid (Fisher), sodium acetate, concentrated phosphoric acid (Mallinckrodt, Paris, KY), concentrated perchloric acid (J. T. Baker, Inc., Phillipsburg, NJ), and ethylenediamine tetraacetic acid, disodium salt (Na_2EDTA, Sigma).

Samples

Juices from ten early season citrus cultivars were hand expressed from fruit picked at the Florida Citrus Arboretum (Winter Haven, FL). Ten mL portions of juice samples were clarified by centrifuging at 10,000 RPM and 2°C for 15 min.

Preparation of Standards and Samples

Ascorbic acid assay. A stock solution was prepared by weighing 50.0 mg of L-ascorbic acid into a 100 mL volumetric flask and diluting to mark with the HPLC mobile phase. A working standard was prepared by diluting the stock solution 100-fold with the mobile phase. Samples were prepared by pipetting 1 mL of clarified juice into a 100 mL flask and bringing to mark with 0.04 M perchloric acid. Samples were filtered through 0.45 μm nylon syringe-type filters prior to injection.

Folate assay. A primary stock solution of 5-MeTHF was prepared by dissolving approximately 12.5 mg of the salt in 100 mL of 0.05 M phosphate/acetate buffer (adjusted to pH 7.2 with concentrated NaOH) containing 4.0 g ascorbic acid and 5.0 g meta-phosphoric acid. This was kept frozen at –20°C util needed. Secondary stock solutions of approximately 1.25 μg mL^{-1} folate and 400 μg mL^{-1} ascorbate were prepared fresh daily by dilution of the primary stock with HPLC mobile phase A. One mL of each clarified juice was pipetted into separate 1.5 mL microcentrifuge tubes. The pH was adjusted to about 5.0 using 1.0 M NaOH. This was followed by the addition of 0.2 mL of a conjugase enzyme suspension, necessary to deconjugate higher-order polyglutamate forms to 5-MeTHF monoglutamate (3). The samples were incubated in a water bath at 37°C for 90 min, cooled rapidly on ice, then centrifuged at 10,000 RPM for 5 min. The samples were finally filtered through 0.45 μm nylon syringe-type filters and transferred to amber autosampler vials of 300 μL capacity for injection. Samples were kept cold and shielded from light prior to injection.

Sugar and sugar alcohol assay. A primary stock solution containing sucrose, glucose, and fructose in a ratio 2:1:1 (approximately 10% W/V total sugar) and myo-inositol (0.2% W/V) was prepared by weighing the dried solids into a 100 mL volumetric flask and diluting to volume with HPLC grade water. This was kept frozen until use. Juice samples were prepared by 100-fold dilution with HPLC grade water. The standard sugar mixture was treated in an identical manner. All samples and standards were filtered through 0.2 μm nylon syringe-type filters prior to injection.

HPLC Methods

General. The HPLC system consisted of Waters (Milford, MA) Model 510 and Model 6000 LC pumps, a Model 721 system controller, and Model 710B WISP Autosampler. The EC detector was an E.G.&G. Princeton Applied Research Model 400 (Princeton, NJ). Data were acquired with a 20-bit A/D converter (CSI Model 160S, Autochrom Inc., Milford, MA) at a rate of no less than 2 Hz. Peak height or area calculation was performed using APEX Chromatography Software (Autochrom, Inc.) with the aid of a 286 AT-style computer (CompuAdd Corp., Austin, TX).

Ascorbic acid assay. The mobile phase was prepared by dissolving 8.2 g of sodium acetate and 150 mg of disodium EDTA with approximately 500 mL of HPLC grade water in a large beaker. The pH was adjusted to 5.0 with acetic acid. This solution was then transferred into a 1 L volumetric flask and diluted to mark with HPLC grade water. The solvent was filtered through a 0.45 μm filter and degassed by sparging with helium. The analytical column was a ZORBAX ODS (DuPont, Wilmington, DE), 4.6 mm x 25 cm, with 5 μm packing. The mobile phase flow rate was 0.8 mL min^{-1}. Injection volumes were 20 μL. External standard calibration was made within every four sample injections. Separation was at ambient temperature. The detector was equipped with a glassy carbon electrode operated at + 700 mV (vs. Ag;AgCl, 3M NaCl) with the sensitivity at 100 μA full scale.

Folate assay. Mobile phase A was 10% methanol in 0.05 M phosphate/acetate buffer (pH 5.0) containing 0.005 M TBAP. Mobile phase B was identical except that it contained 30% methanol. Both solvents were filtered through a 0.45 μm filter and degassed by sparging with helium. Separations were performed at ambient temperature. A 10-port switching valve (Valco, Houston, TX) was confi-

gured for sample clean-up and backflush; its use is described in detail elsewhere (20). A UV detector (Waters Model 441, 254 nm, sensitivity = 2.0 AUFS) was employed to detect unretained ascorbate as it eluted from the precolumn. The EC detector was equipped with a glassy carbon electrode operated at +200 mV (vs. Ag;AgCl) with the sensitivity at 10 nA full scale. The precolumn was a Waters Nova-Pak C18, 3.9 x 75 mm, with 4 μm packing. The flow rate was 1.0 mL min^{-1} and injection volumes were 20 μL. External standard calibration was made within every four sample injections. Data were corrected for the purity of the standard and dilution which accompanied addition of enzyme.

Sugar and sugar alcohol assay. Sodium hydroxide (50% W/W) was diluted to 0.14 M (pH 13) with filtered (0.45 μm) HPLC grade carbonate-free water and used as the mobile phase. Care was taken to exclude carbon dioxide. Anion-exchange separation was performed with a Dionex (Sunnyvale, CA) CarboPac PA1 column (4 x 250 mm) at ambient temperatures (23 ± 2°C). No guard column was used in order to minimize band spreading. Injection volumes were 20 μL. External standard calibration was made within every four sample injections. Tubing and fittings were stainless steel. The pumping rate was 1.0 mL min^{-1}. The detector was equipped with a single gold working electrode and operated in the pulse mode at potentials (vs. Ag;AgCl): E1 = +50 mV (167 ms), E2 = + 650 mV (167 ms), and E3 = –950 mV (500 ms). Sensitivity was set at 100 μA full-scale and current was sampled during E1.

Results and Discussion

Vitamin C is one of the principal nutritional components of citrus and many consumers rely on citrus juice as a primary source of this vitamin. Thus, it is important that an ascorbic aid assay of citrus products be carried out regularly. Figure 2 is an overlay of typical chromatograms obtained using the described method. The analysis is complete in under four minutes. Some tailing is evident, however no attempt was made here to improve the separation; quantitation was satisfactorily performed by by measuring peak height. Analysis of several fresh and processed juices in our laboratory (2) gave a range of ascorbic acid values from 43.1 mg to 61.2 mg per 100 mL of juice, agreeing well with literature values. Repeatability is estimated to be ±0.5 mg based on replicate injections, corresponding to a coefficient of variation of about 1%. Standard calibration curves constructed by successive dilution of the standard were linear (R^2 = 0.9999) over the range 12 to 100 mg per 100 mL juice.

Folates are nutritionally essential due to their role as coenzymes in the biosynthesis of nucleic acids, amino acids and proteins. Citrus juice, in particular orange juice, has been reported to be a good source of dietary folate (6, 16).

Microbiological assay using *L. casei* (5) has been employed almost exclusively for quantitating folate in citrus juices. Unfortunately, the disparate growth response of this microorganism to polyglutamate forms ($glu_{n>3}$) (17) raises doubts as to the reliability of the results.

It was determined earlier (19) that ascorbate was the main obstacle to selective detection of 5-MeTHF in citrus juice when using HPLC with amperometric detection (E = +200 mV vs. Ag;AgCl). It was therefore necessary to reduce the level of ascorbate in the samples by using a cleanup procedure. A 10-port switching valve enabled this extraction to be carried out automatically. At pH 5, 5-MeTHF is essentially anionic and the use of an ion-pairing reagent (TBAP) in mobile phase A enhances its retention on a C18 column (13). A small percentage of methanol (10%) facilitated the washing of less tightly bound, and possibly interfering, compounds (such as ascorbate) through the precolumn while 5-MeTHF was retained. Increasing the methanol content to 30% in mobile phase B provided sufficient solvent strength to elute 5-MeTHF. Ion-pairing reagent (TBAP) was also added to mobile phase B in order to preserve the integrity of the "ion-pairs", otherwise peak shape deterioration was noted. Figure 3 is a typical chromatogram obtained by using the switching technique. The large band which elutes about 2.5 min after column switching is probably residual ascorbate, and perhaps other unretained compounds electroactive at +200 mV. Peaks were usually symmetrical in shape and peak height was used for the calculations. A series of

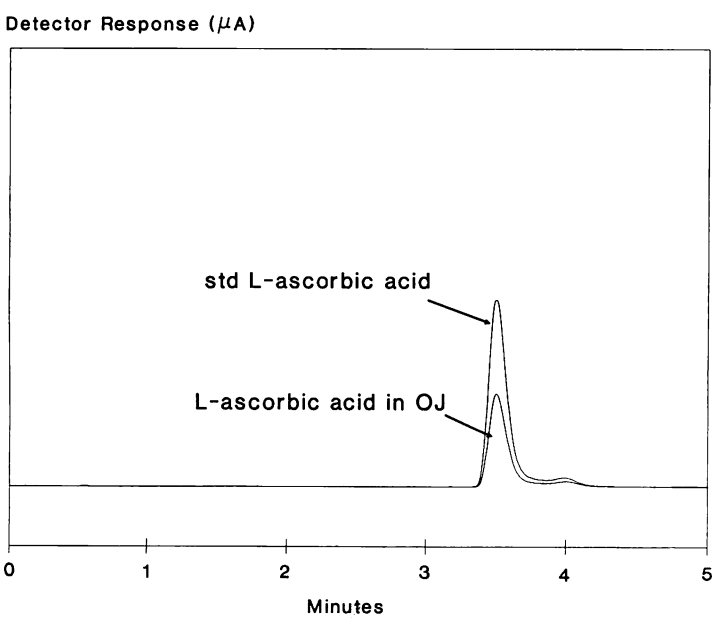

Fig. 2. Reversed-phase HPLC/EC chromatogram of standard L-ascorbic acid and L-ascorbic acid in orange juice.

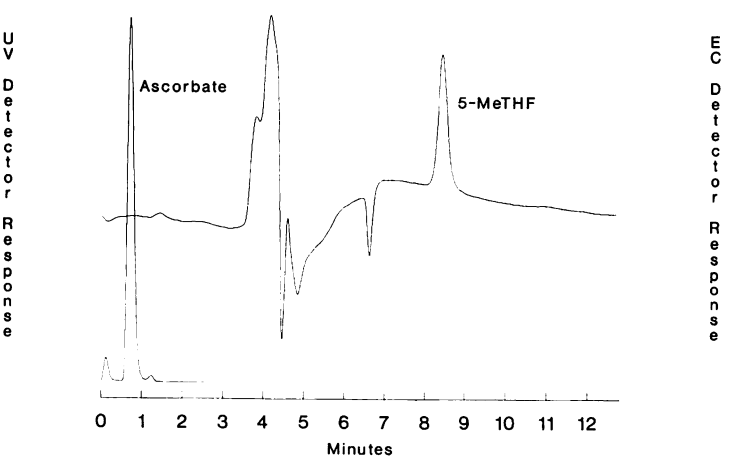

Fig. 3. Reversed-phase HPLC/EC chromatogram of standard 5-MeTHF in an ascorbate solution by direct-injection with column-switching. Chromatographic conditions described in text.

standard dilutions over the range 0.25 to 0.125 μg mL^{-1} resulted in a linear detector response, (R^2 = .09992). In order to estimate the reproducibility of the HPLC method, repetitive analyses (N = 14) of a single unpasteurized juice sample (12 °Brix) were carried out. Results ranged from 0.237 to 0.265 μg ml^{-1}, with a mean of 0.247 μg mL^{-1} and a standard deviation of 0.007 μg mL^{-1}. This corresponds to a coefficient of variation of about 2.8%.

Over 100 commercial juices have been analyzed for folate using the method (20). HPLC results were generally higher than those obtained by microbiological assay. There are two reasons why higher results are to be expected when using the HPLC method. First, no conjugase enzyme treatment of juice samples was employed prior to the microbiological assay. Tamura (17) has shown that the response of *L. casei* to folates is dependent on the polyglutamyl chain length, with poorer response elicited for folate compounds with greater than three glutamic acid residues. The higher levels of 5-MeTHF monoglutamate following the enzyme treatment, confirmed by the HPLC analyses (19, 20), lends support to earlier evidence that polyglutamate forms are present in citrus juices (15). Secondly, calibration of the microassay method was performed using the oxidized folic acid monoglutamate (pteroylglutamic acid) rather than the reduced 5-methyl substituted derivative. There is evidence that *L. casei* responds better to the former, a factor which would undoubtedly lead to underestimation of the level of 5-MeTHF in the juice samples (4). The HPLC/EC method was used here to analyze the folate content of fresh-squeezed juice from ten early-season citrus cultivars. Results are given in Table 1.

Analysis of citrus fruits for carbohydrates is of special interest since varietal, geographic, seasonal, and maturity differences can dramatically affect the composition. Myo-inositol (i-inositol or hexahydroxycyclohexane) is a commonly occurring sugar alcohol widely distributed in both plants and animals. Its discovery in citrus juice (12) and subsequent quantitation (10, 22) showed that citrus was a rich source of this compound. In spite of its potential importance to the citrus industry both from nutritional and economic perspectives, the development of suitable methodology for its routine analysis has largely been ignored.

Since carbohydrates exhibit only weak absorbance in the UV-Vis region, refractive index (RI) has been the conventional means of detection after HPLC separation. However, as a measure of a bulk property of solution, RI does not offer selectivity for sugars over other compounds. The

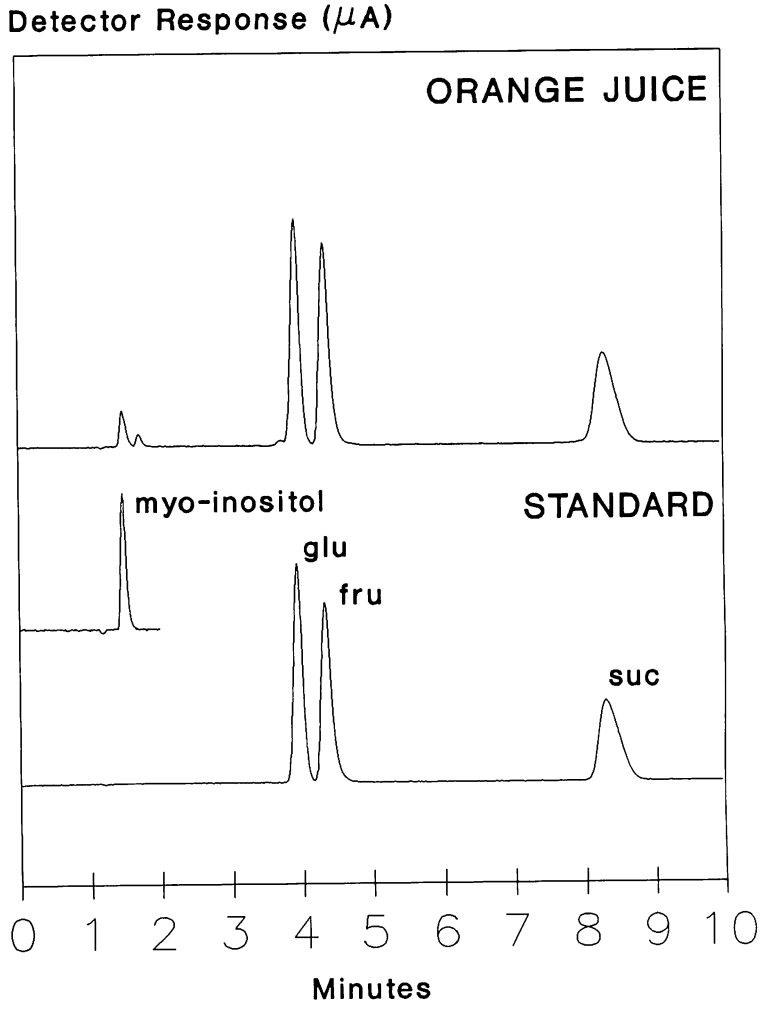

Fig. 4. HPAE/PAD chromatograms of sugars and sugar alcohols in orange juice and in standard solution.

HPAE/PAD method is particularly well-suited for the analysis of complex samples such as fruit juices which contain components that may interfere with RI detection. Figure 4 illustrates the chromatogram obtained under the described conditions. Analysis time was under 10 min.

Multi-level calibration using five successive dilutions of the standard sugar mixture exhibited linear responses for the three sugars and myo-inositol. Sucrose data fit slightly better to a quadratic equation. The standard deviations of the method were calculated from replicate injections (n ≥

Table 1. D-glucose, D-fructose, sucrose, myo-inositol, and folic acid in fresh-squeezed juice from 10 early season citrus cultivars.

Cultivar	glucose (%W/V)	fructose (%W/V)	sucrose (%W/V)	myo-inositol (%W/V)	5-MeTHF (μg/mL)
Bearss lemon	1.53 ± 0.02*	1.49 ± 0.04	0.3 ± 0.3	0.039 ± 0.006	0.073 ± 0.007
Persian lime	0.91	0.86	0.2	0.040	0.084
Page orange	2.50	2.64	5.8	0.062	0.098
Hamlin orange	1.94	2.22	5.6	0.041	0.244
Orlando tangelo	1.35	1.46	4.0	0.110	0.095
Minneola tangelo	1.75	1.97	4.4	0.090	0.095
Robinson tangerine	2.47	2.70	5.8	0.043	0.111
Star Ruby grapefruit	1.40	1.50	3.0	0.047	0.111
Duncan grapefruit	2.09	2.14	3.4	0.076	0.110
Marsh grapefruit	1.83	1.90	1.9	0.039	0.083

*error estimates are standard deviations based on replicate (n≥5) analyses

5) of a standard sugar mix, similar in concentration to the samples analyzed (21). These were found to be 0.02, 0.04, 0.3, and 0.006 %(W/V) for glucose, fructose, sucrose, and myo-inositol respectively. The HPAE/PAD method was used to analyze the sugar content of fresh-squeezed juice from ten early-season citrus cultivars. Results are given in Table 1.

Conclusion

HPLC with amperometric detection can be a useful tool with which to routinely analyze electroactive compounds in citrus juice. A simple, reversed-phase, isocratic HPLC method was illustrated in which vitamin C analysis is complete in less than four minutes. An automated column-switching technique allows the determination of citrus juice folate by direct injection of filtered juice into the HPLC system. Treatment of juices with a conjugase enzyme prior to analysis is recommended for quantitating total folate. Analysis is complete within eight minutes. A high performance anion-exchange method with pulsed amperometric detection can be used routinely to quantitate glucose, fructose, and sucrose, as well as the sugar alcohol, myo-inositol. Run times are less than ten minutes.

The amperometric detection system was found to be rugged and extremely selective, providing superior sensitivity where needed. The methods illustrated are simple and require no sample preparation other than dilution and filtration.

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MODELS FOR SEASONAL CHANGES IN °BRIX AND RATIO OF CITRUS FRUIT JUICE

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Additional index words. Oranges. Forecasting method.

Abstract. **Seasonal changes in °Brix and °Brix/% acid ratio of Florida early season oranges and California 'Washington Navel' oranges were fitted by linear models. The constant term varied with different climatic conditions. The rate of change was 0.0256 °Brix/day and 0.0735 ratio/day for Florida early season oranges and 0.0579 ratio/day for California "Washington Navel" oranges, respectively. An application of the models for early prediction of changes in °Brix and °Brix/% acid ratio throughout the harvesting period was developed.**

Producing high quality products is a common goal of both citrus growers and processors. High quality processed citrus juices must come from high quality fruit. Traditionally, producing high quality fruit is a responsibility of growers. Fruit quality varies with varieties and stages of maturity as well as other factors throughout the season. The processors receive whatever fruit is available as it matures throughout the season and process this into juice

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