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Extraction, Separation, and Quantitation of Acid-Soluble Nucleotides in Citrus

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A method has been developed to extract the acid-soluble 5′-nucleotides from citrus juice. The nucleotide components of this extract are then effectively separated, identified, and quantitated by high-pressure liquid chromatography. Because of its simplicity, speed, and accuracy this method has the potential for monitoring changes in nucleotide concentrations in fruit during growth and maturation, disease or injury, and processing. Nucleotide patterns of several varieties of fresh citrus juice were determined.

Nucleotides function as essential intermediates in numerous biochemical and biosynthetic reactions in cells. They play an important role in energy transport and in the metabolism of sugars and organic acids. Such functions are of particular interest in the study of citrus fruit since the synthesis and accumulation of sugars and organic acids are important to juice flavor and quality. Buslig (1970) showed a correlation between the ratio of ATP/ADP and acidity in citrus fruit. There is also some evidence that in combination with other compounds, nucleotides may contribute to the characteristic flavors of certain fruits and vegetables (Luh and Chen, 1969; Kuninaka, 1960).

Nucleotides have been isolated from many fruits and vegetables (Luh and Chen, 1969) and from the juice vesicles of citrus fruit (Barmore and Biggs, 1972). However, traditional ion exchange chromatography is too slow and tedious to be practical for extensive study of nucleotide pools. Furthermore, the analysis times required, coupled with the susceptibility of nucleotides to hydrolysis, make the results of such investigations at best tentative. A rapid, sensitive method for the extraction, separation, and quantitation of nucleotides in citrus is necessary if their role in the development and quality of fruit is to be investigated.

MATERIALS AND METHODS

Apparatus. A Model ALC 202 high-pressure liquid chromatograph (HPLC), with two Model 6000 pumps, a U6K injector, Model 660 solvent programmer, and fixed wavelength (254 nm) UV detector (Waters Associates, Milford, Mass.), was used. A Model 3380A recording integrator (Hewlett-Packard, Avondale, Penn.) was used to record the chromatograms and quantitate the results. All solvents and samples were filtered with 0.22-μm membrane filters (Millipore, Bedford, Mass.). Extracts were lyophilized with a Model FD-ULT-6 freeze-dry unit (Thermovac Industries, Corp., Copiaque, N.Y.).

Columns. Glass columns, 0.7 cm i.d., filled to 6 cm with analytical grade anion resin, 50–100 mesh, chloride form (Bio-Rad, Richmond, Calif.) were used in the initial purification. The HPLC column was a prepacked Partisil 10-SAX anion exchange column (Whatman, Clifton, N.J.).

Reagents. Aqueous buffers were prepared from special purity potassium dihydrogen phosphate (Heico, Inc., Delaware Water Gap, Pa.). The 5′-mono-, di-, and triphosphate nucleotides of adenine (A), guanine (G), xanthine (X), cytosine (C), and uracil (U) were purchased from Aldrich Chemical Co., Milwaukee, Wis.

EXPERIMENTAL PROCEDURE

Preparation of Nucleotide Extracts. Samples (10 g) of fresh or processed single-strength orange juice were mixed with 15 ml of cold, 0.6 N perchloric acid and approximately 0.1 g of insoluble polyvinylpyrrolidone (Polyclar AT) and centrifuged at 16 000 rpm (3480 g) for 25 min at 0 °C. The supernate was removed, titrated to a pH between 5.7 and 6.0 with 15% KOH, and then filtered to remove the precipitated $KClO_4$ salts. This extract was then placed on an anion exchange column and washed with 10 bed-volumes of water. The nucleotides were eluted from the column in one group with 25 ml of 0.5 M HCl.

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Table I. Nucleotide Concentrations of Representative Orange Juice Samples (mg/100 ml of Fresh Juice)

Variety	CMP	AMP	UMP	GMP	UDP	CDP	ADP	GDP	UTP	CTP	ATP	GTP	Total
Pineapple	0.22	0.16	0.18	0.14	0.65	1.9	0.60	0.13	0.23	0.06	0.43	0.06	4.84
Pineapple	0.13	0.06	0.04	0.03	0.34	0.61	1.16	0.08	0.20	0.09	0.54	0.09	3.39
Valencia	0.28	0.05	0.03	0.02	0.76	0.28	1.48	0.43	0.13	0.08	0.53	0.05	4.11
Hamlin	0.08	0.18	0.13	0.08	(0.69)[a]	(0.71)	1.32	0.42	0.13	0.05	0.49	0.02	4.29
Murcott	0.21	0.06	0.04	(0.06)	0.23	(0.70)	2.6	0.12	0.03	0.04	0.16		4.19

[a] Values in parentheses are estimated. The fraction was determined to have more than one component.

A 10-ml aliquot of this eluate was then lyophilized and the residue taken up in 1 ml of water. All solutions used for titrating, washing, and eluting nucleotides were stored at 6 °C until immediately before use. Titrating and eluent collection flasks were kept in ice baths during these procedures and stored at −8 °C before and after HPLC analysis.

HPLC Determination of Nucleotides. The procedure was similar to that used by Scholar et al. (1973) to measure nucleotide concentrations in human blood. A comparison of the performance of various anion resins for nucleotide analysis by HPLC is reported by Hartwick and Brown (1975).

A 25-μl sample of the filtered extract was injected onto the HPLC anion-exchange column. A concave gradient was used in which the low concentration eluent was 0.0225 M KH_2PO_4, at pH 3.74, and the final concentration was 0.675 M KH_2PO_4, at pH 4.77. Column temperatures were ambient (21–23 °C) and flow rate was 0.9 ml/min with a column pressure of 900 psi. All 12 nucleotides were eluted in 45 min and detected at 254 nm. Retention times, identifications, and concentrations of individual components were computed and recorded immediately by the recording integrator. CMP (cytidine 5′-monophosphate) was used as an internal standard in each case.

The retention times of the mononucleotides were found to be extremely sensitive to column equilibration between sample runs. The chromatograms obtained were reproducible by reversing the solvent program and holding at initial conditions for 10 min before making the next injection. Once a week the column was washed overnight with a 1 M KH_2PO_4 solution.

Identification of Nucleotides. Standard nucleotide solutions (0.4 mM) were extracted and determined under conditions identical with those given above. Nucleotide fractions were then tentatively identified on the basis of these retention times. Identities were verified by enriching samples with standards. In addition, the three cytidine nucleotides and all mononucleotides were confirmed by determining UV absorbance ratios of eluted samples at 280/260 and 250/260 and comparing these with corresponding ratios for the eluted standards. Adenine and guanine nucleotides were confirmed by TLC analysis on DEAE-cellulose prepared sheets using isobutyric acid/NH_4OH/H_2O (57:4:39) as solvent (P-L Biochemicals, 1968). Purity of components was also verified by comparing width at half-height of peaks with those of the corresponding standard (Khym, 1975).

Nucleotide recovery was determined by processing standard solutions through the extraction procedure and also by adding known amounts of standard solutions to the initial juice samples. All samples were run in duplicate to provide data on reproducibility of the method.

RESULTS AND DISCUSSION

A chromatogram of standard solutions of the acid-soluble 5′-nucleotides found in citrus juice is shown in Figure 1. Individual components are labeled and their retention time indicated. Under the conditions used a retention time window of ±4% was sufficient to identify all nucleotide components in standard and juice mixtures.

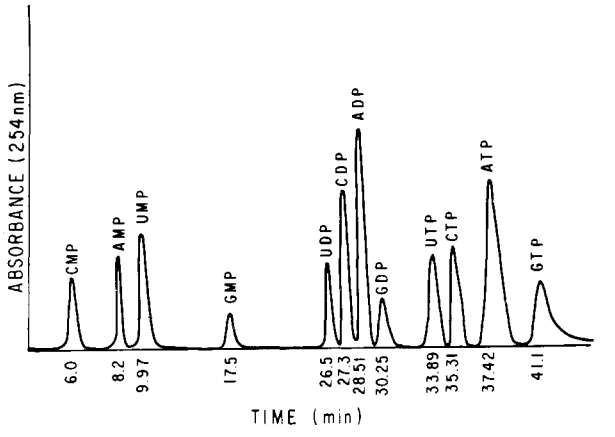

Figure 1. Chromatogram of mono-, di-, and triphosphate nucleotides of adenine, guanine, cytosine, and uracil. For experimental details, see text.

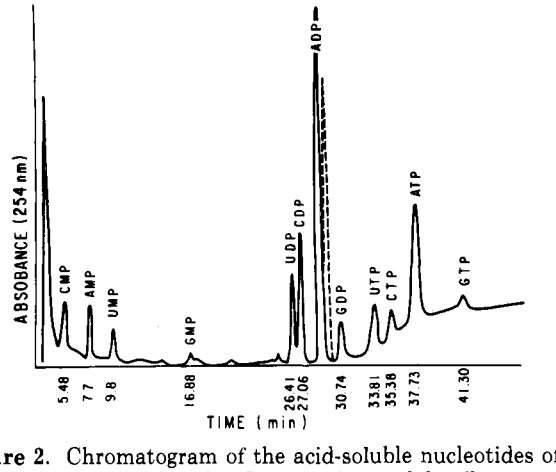

Figure 2. Chromatogram of the acid-soluble nucleotides of a typical orange juice sample. For experimental details, see text.

Figure 2 is typical of the nucleotide profiles of fresh juice samples tested. The quantitative data on this sample are included in Table I (no. 2). In some juice samples a major component is eluted at or near the retention time of ADP (dotted lines at t_R = 28 min in Figure 2). The identity of this component is not as yet certain. It is, however, cleanly separated from ADP in those cases in which it occurs by rechromatographing the appropriate HPLC fraction.

The quantitative recovery of total and individual nucleotides was found to be quite sensitive to temperature, pH, and time spent on the initial purification column. The triphosphates in particular are easily hydrolyzed to the di- or monophosphate forms. Under the conditions specified, however, it is possible to keep the recovery of individual and total nucleotides in standard and fortified samples to within ±5% of their initial values. Analysis of a standard nucleotide mixture showed 95.2% recovery of triphosphates, 98.1% recovery of diphosphates, and 96% recovery of monophosphates. Lowest recovery was obtained for GTP, 94.6%, and GMP, 94.7%.

The HPLC procedure gave excellent resolution at ambient temperatures. Repeated injections of the same samples were consistent within ±3%. Duplicate samples agreed within ±10% on both individual and total nucleotide concentrations. Column performance was remarkably stable over a 6 to 9 month period and duplicate columns were practically identical. Columns used showed 3500 to 4500 theoretical plates for GMP at initial program conditions.

At the present time nucleotide concentrations in more than 150 samples of fresh and processed juice have been measured. Three orange, one mandarin, and two grapefruit varieties are included. All fruit tested had attained commercial maturity. Typical total nucleotide concentrations in the samples tested range from 3.4 to 7 mg/100 ml of fresh juice. These values are somewhat higher than the 2.61 to 5.77 mg/100 g pulp reported by Barmore and Biggs (1972) for juice vesicles of the same type of fruit.

Table I shows concentrations of individual nucleotides found in several of the juice samples investigated. These values differ from the estimated quantities reported by Barmore and Biggs (1972). We report higher diphosphate and triphosphate concentrations. This may be due to differences resulting from the use of juice as opposed to juice vesicles. More probably however it reflects the increased efficiency of the Partisil column and shorter analysis times made possible by the high-pressure liquid chromatograph. This has significantly reduced the possibility for hydrolysis which plagues conventional LC methods.

Using the luciferin–luciferase system of Buslig and Attaway (1969), Barmore and Biggs (1972) reported ATP concentrations of 0.52, 0.55, and 1.33 mg/100 g of pulp in Pineapple oranges. The average ATP concentration in ten Pineapple orange samples tested is 0.44 ± 0.09 mg/100 ml of fresh juice.

The major advantages of the method over that previously reported (Barmore and Biggs, 1972) are improved resolution, ease and simplicity of sample preparation, and a 90% reduction in the time a sample spends on the anion exchange column. No data on percent recovery from fortified samples are available for comparison with the values found in this study.

As the structural units of the nucleic acids, nucleotides are valuable indicators of the metabolic state and maturity of fruit. Furthermore, the nucleotides identified have been shown to be related to sugar content (Hassid, 1967), organic acid levels (Buslig, 1970), and possibly flavor (Schinneller, 1972) of citrus juice. Luh and Chen (1969) suggest that nucleotides act in combination with other compounds to influence the taste of some fruits and vegetables.

Preliminary work indicates that after harvesting the nucleotide concentrations in citrus fruit are influenced by storage, handling, and processing. Thus, flavor changes that occur during these procedures may be in part related to changes in nucleotide concentrations. The method developed to measure such nucleotide concentrations is sensitive, convenient, and accurate.

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Separation of Pigments, Flavonoids, and Flavor Fractions from Citrus Oils by Gel Permeation Chromatography

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Concentrated cold-pressed oils of citrus and a hexane extract of orange peel were separated into three fractions, color, flavonoid, and volatile flavor, by gel permeation chromatography. Yields of pigment from the cold-pressed oils and peel extract, respectively, were as high as 116 and 350 mg/kg peel. Components of the color fractions from cold-pressed oils and peel extract were separated by thin-layer chromatography (TLC) and compared. Constituents of the flavonoid fractions were identified by comparison of TLC data with those of authentic samples. Twenty-three components of the volatile flavor fraction from tangerine oil were identified by gas–liquid chromatography. Three esters, citronellyl acetate, decyl acetate, and 1,8-*p*-menthadien-9-yl acetate, not previously reported as tangerine oil constituents, were among the identified components.

Analysis of high-boiling components of natural product mixtures, such as citrus essential oils, has been difficult because adequate means for their rapid separation into manageable fractions free of interfering compounds have not been available. The most commonly used technique, distillation, is inadequate for separating high-boiling components of citrus oils because they decompose at high temperatures, even under high vacuum (Moshonas, 1971). Adsorption chromatography (Moshonas, 1971) is often

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