Quantitative Analysis of Fructose Fate in a Plant Fermentation System

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There is a need for studies on the fermented plant hairy root cultures to learn more about the consumption and production of carbohydrates, sucrose, glucose, and fructose trends during plant metabolism and growth in fermented and biological systems. The multivariant quantitative analysis of sugars was carried out using a Fourier transform infrared spectrophotometer combined with a cylindrical internal reflection cell and a dedicated computer. Another analysis of the same samples was performed according to an enzymatic method. The obtained results were compared with the data of the spectrophotometry with very good agreement. Results achieved provide a new insight into the metabolism of cell cultures. The results demonstrate the possibility of analysis of fructose concentration in mixtures of glucose, sucrose, and various constituents of the media. Sucrose concentrations of 88 or 176 mM were diminished after 12 and 24 days, respectively, whereas glucose increased initially to reach maxima and fructose was building up steadily throughout the duration of study (28 days). The hairy roots were unable to consume fructose as assumed previously.

Keywords: *Fructose; FTIR; P-matrix; enzymatic method; hairy roots*

INTRODUCTION

Recent modern vibration spectroscopic techniques such as FT-Raman or FT-infrared spectroscopy provide specific molecular information of nearly any molecule in a short time.

Despite the wealth of information contained in vibrational spectra, these techniques have found to date very limited applications in biological cultures, especially considering measurements in aqueous solutions. The reason for this can be attributed to different facts, for example, the strong infrared absorption of water, which restricts the optical path length of cells to ~25 μm when using FTIR spectrometers. However, the use of FTIR spectroscopy has already proved to be of advantage concerning the simultaneous determination of glucose as well as the shortening of conventional detection schemes as shown in the case of sucrose determination in aqueous solutions.

Infrared (IR) spectroscopy is potentially a good method to investigate the fate of fructose since all sugars have characteristic IR spectra, but a serious hindrance to the analysis of biological fluids is that the most common solvent is water, which has very strong absorption bands in the mid-IR region between 4400 and 400 cm^{-1} which is almost totally absorbing at usable path lengths (Cook et al., 1972). Also, many biological systems scatter infrared considerably. However, attenuated total reflection (ATR) combined with FTIR can be used to overcome these problems (During, 1980). A version of ATR known as cylindrical internal reflectance (CIR) is most suitable for aqueous solution. As a quantitative tool, CIR has several advantages over the other methods for typical samples including biological fluids, fermentation broth, and organic solvents (During, 1980). The CIR cell has a fixed path length that, unlike a transmission cell, is almost insensitive to window wear and temperature and pressure changes.

The case to be evaluated in this study is hairy root cultures, usually produced by infection of *Beta vulgaris* plant with *Agrobacterium rizogenes* followed by their placement in growth medium (Hamill et al., 1987; Murashige et al., 1962; Gamborg et al., 1968) with sucrose as an energy source. During the metabolism of the plant, the disaccharide sucrose converted into glucose and fructose; thus, sucrose concentration decreases with time. Although it is known that glucose concentration increases initially and then falls, no information about fructose has been reported so far for this system. Only a few studies have been performed on the effects of nondigestible oligosaccharide in elderly Japanese subjects, suggesting that dietary supplementation with fructo- or galacto-oligosaccharide is beneficial to the large intestine (Hidaka et al., 1986; Masai et al., 1987; Mitsuoka et al., 1987; Hayakawa et al., 1990; Ito et al., 1990). These studies showed a higher activity of the colonic microflora in response to the consumption of oligosaccharide, as demonstrated by increased fermentation. Alles et al. (1996) reported on the "fate of fructo-oligosaccharide in human intestine", which concluded that the level of fructo-oligosaccharide controls the level of fermentation.

The most useful region of infrared for quantitative analysis of a wide range of typical biological solutes after water subtraction is the region 1500−800 cm^{-1}. However, there can be considerable overlap of bands in this region in complex mixtures. The high information

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content of the infrared spectra of biological compounds and the good reproducibility that can be realized in obtaining these spectra suggest the feasibility of performing multicomponent analyses in mixtures of biological compounds.

In this study, a variety of algorithms including least-squares, factor analysis, and P-matrix approaches have been tested in preliminary investigation. These procedures require standard spectra of either pure components of the mixture or defined mixtures and can involve the use of the entire digitized spectrum of a "reduced" set of spectral descriptions, wavenumbers found by experiment that enhance the identity of each compound. It has been suggested that vibrational modes and energies of bands in biological molecules can be influenced by microenvironmental factors, for example, local pH and solute−solute or solute−solvent interactions. Also, temperature will modify infrared absorption intensities and/or frequencies. These effects may cause problems in quantitative analysis (Fink and Chittur, 1986). Methods such as P- and K- matrix can be less influenced by such effects since calibration is carried out using typical mixtures (Haaland et al., 1985), not pure material as would be employed for the Lambert−Beers law. The purpose of this study was to examine the fate of fructose that solubilized from sucrose in hairy root cultures by studying several samples of new system with particular emphasis on the problems of quantitative analysis in such complex systems.

MATERIALS AND METHODS

Materials. Plant tissue culture media of 88 and 176 mM (3 and 6% sucrose, respectively), Gamborg medium (Gamborg et al., 1968), and half-strength of ingredients of the following concentrations (mg/L) were used: $CaCl_2 \cdot 2H_2O$, 150.00; $CoCl_2 \cdot 6H_2O$, 0.025; $CuSO_4 \cdot 5H_2O$, 0.025; FeNa EDTA, 40.00; H_3BO_3, 3.00; KI, 0.75; KNO_3, 3000.00; $MgSO_4 \cdot 7H_2O$, 250.00; $MnSO_4 \cdot 4H_2O$, 13.20; $NaH_2PO_4 \cdot 2H_2O$, 169.60; $NaMoO_4 \cdot 2H_2O$, 0.25; $(NH_4)_2SO_4$, 134.00; $ZnSO_4 \cdot 7H_2O$, 2.00; inositol, 100.00; nicotinic acid, 1.00; thiamin hydrochloride, 10.00; and pyridoxin hydrochloride, 1.00.

The culture was initially created by infection of *B. vulgaris* plant leaf with *A. rhizogenes*. Two or three weeks after infection, hairy roots were produced from the site of infection. These were then transferred to liquid medium containing penicillin to kill any possible bacteria. After two to three generations, the culture was transferred to a penicillin-free medium and maintained at 25 °C with fortnightly subculture into fresh medium (Wilson et al., 1987).

For this experiment, the roots were grown in a 2 L fermentor, and samples were collected first on the days 4, 6, 8, 12, and 28.

The spectra of the samples and calibration standards were collected under the same conditions. Pure growth medium was used as a reference spectrum (background), which was subtracted from samples and standards. Concentrations of the three sugars in the fermentation samples were estimated using the P-matrix approach (Quant 32, Quantitative Software Application Manual, MO 91-0346, Digilab Inc.; Edward, 1984). Concentrations of the three components were plotted against time (days).

A second batch of samples was based on the same medium enriched with 176 mM sucrose, and 25 samples were collected daily from day 1 to day 24 and on day 28. All of these samples were analyzed according to the FTIR method and the enzymatic method using the same conditions. The sample volume was 4.5 mL, and to fill the cell, 1 mL of distilled water was added to each sample (the dilution factor was 1.222).

A series of calibration standards [sucrose (Analar grade), Fisons (scientific equipment), D-fructose (Analar grade, Aldrich chemicals), glucose (Analar, BDH)] were used without any further purifications. Twenty or 24 calibration standards were prepared. Seventeen of 20 calibration standards were used in the calibration step; the other 3 standards were used in the analysis step to validate the accuracy of the calibration. The most sensitive wavenumbers to change in concentration used in the analysis of the spectral region (reduced set) were 1094, 1088, 1058, 1040, 995, and 970 cm^{-1}. The P-matrix approach was used, and the concentrations of unknown fermented samples were plotted versus time in days. An FTIR60 (Bio-Rad Digilab Division) spectrometer was equipped with a deuterated triglycine sulfate (DTGS) photoelectric detector and CIR with a ZnSe crystal centered in the middle of the cell. Experimental work was done at 8 wavenumbers (cm^{-1}) resolution and 256 scans.

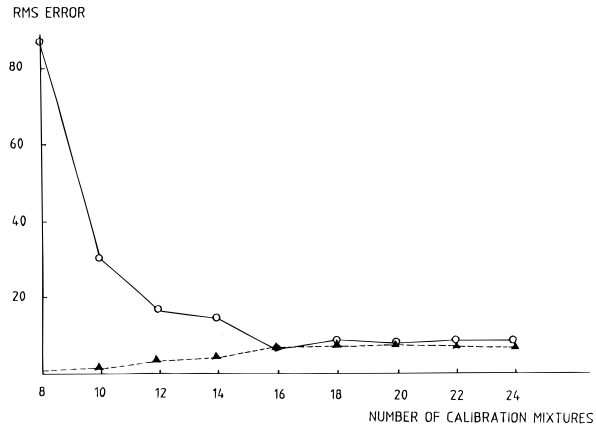

Figure 1. Fructose apparent (▲) and actual (○) RMS errors versus number of calibration mixtures and point at which the apparent and actual RMS errors converge to produce a minimum error in actual analysis.

Enzymatic Method. All fermentation samples were also analyzed for glucose and sucrose levels using an enzymatic method (Hudson et al., 1976). The enzymatic determination of glucose is based on a color-linked reaction of glucose with glucose oxidase and peroxidase. Determination of sucrose is carried out by stoichiometric inversion of sucrose to glucose and fructose by the use of invertase enzyme, followed by determination of glucose. The reaction was performed using a proprietary glucose test kit (Boehringer Mannheim, GOD-Perid, 124-036); a fructose kit was not available.

RESULTS AND DISCUSSION

Analytical Calibration and Analysis. By considering the IR spectra, detection limits for the solutions of three sugars were ~2.5 mM (3 times signal-to-noise), which is the lowest concentration after water subtraction to give a peak that is clearly distinguishable from the noise under the same acquisition conditions. The detection limits obtained for the three sugars should enable the quantification of the three sugars in the fermentation system because the expected sucrose concentrations in fermented media were about 88 and 176 mM.

Three-Component Analysis. Twenty-four calibration standard mixtures of different known concentrations of sucrose, glucose, and fructose were sampled. The calibration was generated in turn using 8, 9, 10, . . ., and 24. The apparent errors between the given and the measured concentrations of fructose were calculated at each set of standards. Three mixtures were analyzed as "unknowns", and the actual errors of fructose concentrations were calculated. Figure 1 shows a profile of apparent root-mean-square (rms) errors of the 24 standards and the actual root-mean-square errors of the 3 unknowns versus number of calibrations. This profile

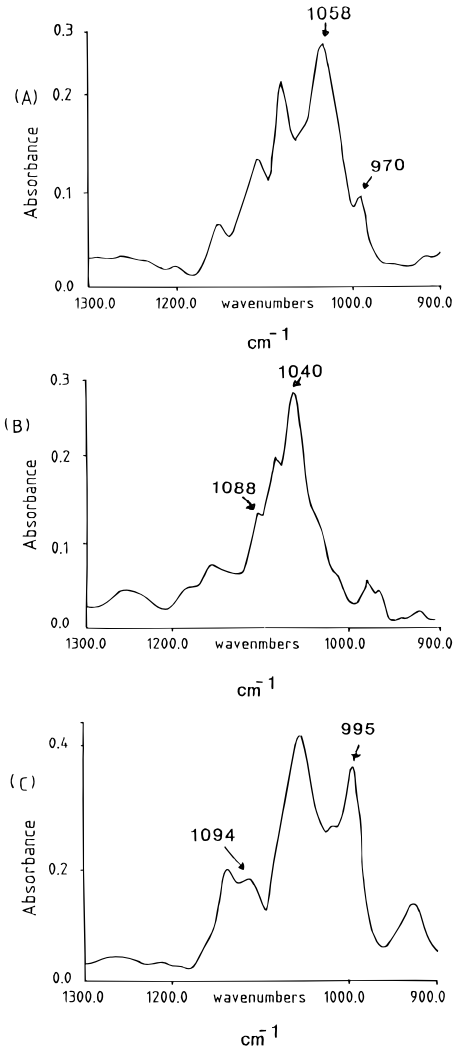

Figure 2. Typical spectra of (A) glucose, (B) fructose, and (C) sucrose. Wavenumbers (cm^{-1}) most sensitive to concentration, which include a reduced set of spectral descriptions, are indicated.

illustrates the minimum number of calibration standards to ensure the minimum number of calibration standards which provide minimum errors and the highest possible accuracy. The point at which the actual and the apparent errors meet corresponds to the minimum suitable number of calibration standards, which was found to be 16 mixtures. The number of calibration standards used was 17 and above for all analyses.

The data for the known prepared mixtures of the three sugars using the P-matrix function using 17 and 20 calibration standards are shown in Table 1. The "reduction script" of the most sensitive to concentration wavenumbers (1094, 1088, 1058, 1040, 995, and 970 cm^{-1}) was used in the three-component analysis of a typical spectra of pure sugar concentration as shown in Figure 2.

The first and second batches of fermented hairy root samples were analyzed using calibration standards which followed the same trend and levels with respect to actual and apparent errors (Table 1 and Figure 1). Between 17 and 20 calibration standards of the three component sugars were used to cover concentrations between 20 and 375 mM.

The first batch of samples of the hairy roots (which were grown on culture medium enriched with 88 mM

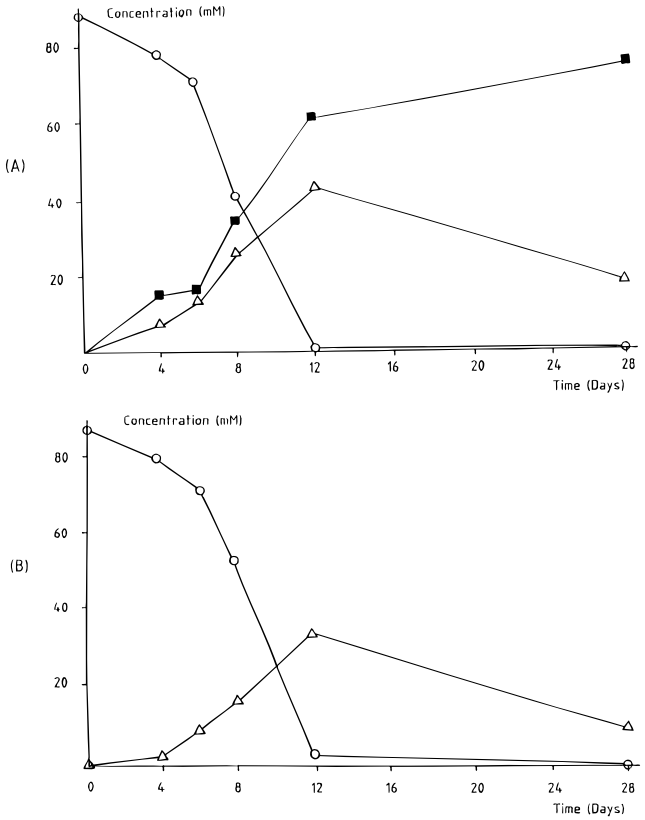

Figure 3. (A) Variation of sucrose (○), glucose (△), and fructose (■) concentrations (mM) during metabolism of hairy roots versus time (days) by FTIR techniques. (B) Variation of sucrose (○) and glucose (△) concentrations (mM) of the same samples by enzymatic method versus time (days).

Table 1. Three-Component Mixture Results Using 17 (A) and 20 (B) Calibrations

sugar	concentration (mM, known)	P-matrix estimation (mM)
	(A) 17 Calibrations	
glucose	20.00	20.22
	20.00	18.16
	40.00	38.86
fructose	20.00	18.31
	60.00	58.31
	20.00	20.47
sucrose	20.00	21.75
	60.00	60.45
	20.00	22.65
	(B) 20 Calibrations	
glucose	60.0	56.61
	50.0	54.57
	50.0	47.73
fructose	375.0	368.48
	250.0	244.82
	225.0	232.29
sucrose	25.0	29.26
	200.0	197.86
	220.0	216.19

sucrose), collected on days 4, 6, 8, 12, and 28, were analyzed by FTIR techniques and enzymatic method. Figure 3 shows profiles of sucrose, glucose, and fructose concentration (mM) versus days of collection. Sucrose concentration decreased to a minimum on day 12, while the concentrations of both glucose and fructose increased up to day 12, after which time the fructose concentration continued to increase and glucose concentration decreased. The two methods of analysis confirm these trends with respect to glucose and

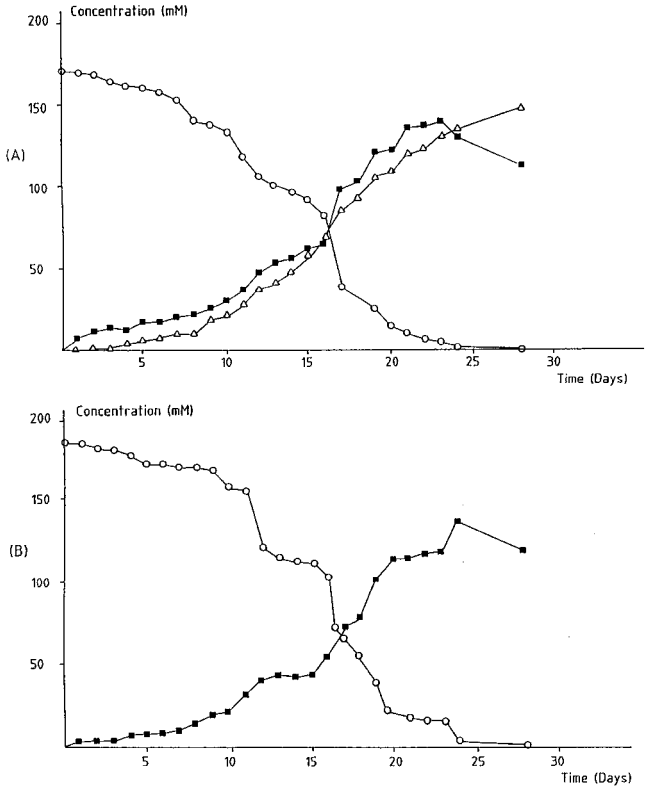

Figure 4. (A) Variation of sucrose (○), glucose (■), and fructose (△) concentrations (mM) during hairy roots metabolism by FTIR technique versus time (days). (B) Variation of sucrose (○) and glucose (■) concentrations (mM) during hairy roots metabolism by enzymatic method versus time (days).

sucrose. The second batch of samples were the hairy roots grown on medium enriched with twice the initial concentration of sucrose (176 mM), and samples were collected on days 1−24 and 28 and analyzed. Figure 4 shows profiles of sugar concentration trends during the 28 days. The sucrose content of the enriched medium was diminished during 24 days, whereas fructose increased to 150 mM on day 28. The glucose content of the medium increased to 145 mM at day 24 and then started to decrease and reached 108 mM at day 28. Generally, hairy roots seem to hydrolyze sucrose in 12 days, and at twice the concentration the sucrose was hydrolyzed in 24 days. The root seems to hydrolyze sucrose and selectively use glucose for growth and metabolism. The trends of fructose building up, which are clearly shown in Figures 3 and 4, demonstrate that the roots were unable to use fructose as an energy source. It is assumed that during the fermentation processes and hairy root metabolism, the fructose was consumed by the plant, similar to glucose (Berlin et al., 1986). Sugars can be hydrolyzed in the medium and preferentially consumed by plant cells and tissues (Pierik, 1987). It was reported previously that leaves from strawberry plants grown in vitro in the presence of sucrose showed ribulose biphosphate carboxylase activity and a negative carbon balance (Debergh and Zimmerman, 1991). These results clearly showed that this is not the case and have raised some questions about the current knowledge of metabolism in this system. Accumulation of sucrose in roots was reported previously and indicates a regular uptake of sucrose by the root system (Brokowaska and Kubik, 1990). Since in vitro cultures have a reduced function of chloroplast, it is necessary for plants to substitute for carbohydrates by others added to the medium that could be directly metabolized by the tissues (Hisajima et al., 1985; Maretzki et al., 1974). Confidence in fructose results was enhanced by the similarity of data and trends of sucrose and glucose in the two methods of analysis.

In these experiments we report for the first time that levels of glucose, fructose, and sucrose have been measured simultaneously by a spectroscopic method and showed that the previous supposition about the time course of fructose concentration may be mistaken.

In the fermented system the phenomena that can be noticed are that this plant may select an energy source from among those available and that the consumption of glucose seems to follow a regular manner irrespective of the initial concentration or the sugar content. Another observation is that the hairy root contributes positively in hydrolyzing sucrose into glucose and fructose in a certain mechanism, and this will be the objective of our future studies.

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