

## Sucrose, Glucose, and Fructose Extraction in Aqueous Carrot Root Extracts Prepared at Different Temperatures by Means of Direct NMR Measurements

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Solutions obtained by heating carrot roots in water (stocks) are widely used in the food industry, but little information is available regarding the metabolites (intermediates and products of metabolism) found in the stock. The effect of treatment temperature and duration on the sugar composition of stocks was investigated directly by quantitative <sup>1</sup>H NMR spectroscopy, to understand the extraction mechanism when processing at 100 °C. Stocks prepared at three different temperatures (50, 75, and 100 °C) were investigated for up to 36 h. Three sugars (sucrose, glucose, and fructose) were detected and quantified. The concentrations of these three sugars reached a maximum after 9 h when the temperature of treatment was 50 or 75 °C. At 100 °C, the sucrose concentration reached a maximum after 3 h, whereas the concentration of glucose and fructose was still increasing at that time. Comparison of the kinetic composition of these carrot stocks with that of model sugar solutions leads to the proposal that the changes in stock composition result from sugar diffusion, sucrose hydrolysis, and hydroxymethylfurfural (HMF) formation.

**KEYWORDS:** Carrots (*Daucus carota* L.), stocks, sugars, <sup>1</sup>H NMR

### INTRODUCTION

Carrot stocks are traditionally made at home by heating roots of carrots (*Daucus carota* L.) in water. These stocks are also used in the food industry, but little is known about the metabolites found in the stock and the mechanisms of the extraction process. Such information on sugars is important because of (a) mechanisms for the extraction of sugar, which is an aim of this paper, could be the same as for the extraction of other compounds (such as environmental contaminants, other metabolites, etc.), (b) the nutritional interest of sugars in the diet (1), and (c) their impact on the possible development of flavor (which will be the aim of a future publication) through chemical processes between glucose and fructose and amino acids (such as Maillard reactions, HMF formation, etc.) (2).

The extraction of sugars from vegetable samples heated in water was previously studied for various reasons: stock analysis (3, 4), determination of vitamin losses (5, 6), and investigation into the release of nitrate from food products (7). Sucrose, glucose, and fructose are the major sugars present in carrot

roots: total sugar content ranges from 3.5 to 10.7% in fresh carrots (8–10). In raw carrots purchased at a local market, sucrose was the major sugar (representing 56.9% of total sugars), followed by glucose (24.6%) and fructose (18.5%) (11). However, a number of investigations has indicated that concentrations in free sugars vary considerably among carrot varieties and are influenced by environmental, agricultural, and storage conditions (12).

<sup>1</sup>H NMR spectroscopy allows rapid quantification in a single analysis of the major sugars, organic and amino acids, and phenolic compounds and thus obtains a metabolic fingerprint of a complex mixture (13–15), metabolic profiling being defined as the identification and quantification of a number of predefined metabolites in a plant sample. Many chromatographic methods such as GC/MS, LC/UV, and LC/MS have been used recently for this purpose (16–19). High-resolution NMR methods have been said to be less sensitive than GC/MS and LC/MS (20–22), but they avoid some preparation steps and provide the possibility of obtaining broad information in one measurement, which is useful for metabolic fingerprinting. <sup>1</sup>H NMR allows the detection of most proton-containing metabolites above a minimum threshold level (depending on the compound) (23).

The objective of this study was, thanks to direct quantitative

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determination of the sugars by  $^1\text{H}$  NMR analysis, to characterize the kinetics of sugar extraction at 100 °C and look for the mechanisms of stock preparation. The 50 °C temperature was chosen because pectinmethylesterase enzymes (EC 3.1.1.11, PME) will still be active during extraction (24), whereas PMEs are inactivated at 75 °C, and so the cell wall is differently degraded, and sugar extraction from the plant tissue may be modified between the two temperatures. Those two temperatures were used to understand what happened at 100 °C, which is the traditional way of cooking (25). The behavior of carrot stock at 100 °C was compared to that of sugar model solutions to provide hypotheses explaining the compositional changes in stock.

## MATERIALS AND METHODS

**Chemicals.**  $\text{D}_2\text{O}$  (99.9%) was from Euristop (Gif sur Yvette, France). TSP (98%) was from Aldrich (Saint Quentin Fallavier, France). NaOD and DCl were from Sds (Peypin, France). All other chemicals used were of reagent grade.

**Sample Preparation.** Carrot (*Daucus carota* L.) roots were obtained from a local supermarket on the day of each experiment. After removing 0.5 cm sections from both ends (to eliminate leaves and secondary roots), the carrot roots were peeled by hand (the thickness is less than 1 mm). Each carrot was cut lengthwise into four parts to ensure homogeneous material was being compared. These four parts were cut lengthwise into disk sectors of a 5.5 mm thickness (to minimize edge effects). Thirty grams of disks from each part (sample) was weighed and washed in distilled water (to eliminate enzymes and metabolites, in particular, phenolics that could be modified by polyphenoloxidase (EC 3.1.), which were released by cutting).

A 250 mL three-necked glass round-bottomed flask heated by a temperature controlled water bath ( $\pm 2$  °C, Ikamag Ret, Staufen, Germany) was equipped with a thermometer ( $\pm 2$  °C), a magnetic stirrer, and a condenser that was used to prevent loss of water and other compounds. A total of 100 mL of distilled water at 50, 75, or 100 °C was added to each sample in three separate systems such as the one described previously. For each temperature, 1 mL of stock was taken at six time points between 0 and 36 h. These stock samples were lyophilized to eliminate water, which may cause NMR signal saturation; the resulting solids were dissolved in  $\text{D}_2\text{O}$  and lyophilized again (Lyolab A, LSL Secfroid SA, Aclens, Switzerland,  $-48.8$  °C, 1.1 mbar) to eliminate residual water. The resulting solids were redissolved in 1 mL of  $\text{D}_2\text{O}$ , and the pH was adjusted to 6.0 using NaOD (0.1 mol/L) or DCl (0.1 mol/L) to avoid chemical shift drift.

**Preparation of Sugar Model Solutions.** A 100 mL sucrose solution (100 mmol/L) was heated at the three temperatures (50, 75, and 100 °C). For each temperature, 1 mL of solution was taken at six different time points between 0 and 33 h. These samples of thermally treated sucrose solution were lyophilized, redissolved in  $\text{D}_2\text{O}$ , and lyophilized again. The resulting solids were dissolved in 1 mL of  $\text{D}_2\text{O}$ , and the pH was adjusted to pH 6.0 using NaOD or DCl (0.1 mol/L).

A total of 100 mL of a glucose and fructose solution (10 mmol/L for each sugar) was heated at 100 °C. A total of 1 mL of solution was taken at six different time points between 0 and 24 h. These samples of thermally treated glucose + fructose solution were treated as described previously for sucrose solutions.

No modification of the model solution pH was made because the stock pH varied only from 6.5 (distilled water pH) to 5 in 24 h of processing.

**Stock Model to Characterize the Extraction.** Three different carrot stocks were made according to the protocol described previously at 100 °C. Every 60 min, all water was removed and replaced by the same volume of water at 100 °C. The water was changed nine times over the time course of the experiment.

Each time the water was changed, 1 mL of stock was taken for sampling. These samples of stock were lyophilized; the resulting solids were dissolved in  $\text{D}_2\text{O}$  and lyophilized again. These solids were redissolved in 1 mL of  $\text{D}_2\text{O}$ , and the pH was adjusted to pH 6.0 using NaOD or DCl (0.1 mol/L).

**$^1\text{H}$  NMR Spectroscopy.** Two high-field NMR spectrometers were used: a 500 MHz Avance Bruker or a 300 MHz Avance Bruker (Wissembourg, France). The analysis of each solid extract was performed using 0.5 mL of lyophilized stock in  $\text{D}_2\text{O}$  (internal lock).

$^1\text{H}$  NMR spectra were recorded at 27 °C with 500 or 300 MHz Avance Bruker spectrometers using a 5 mm broadband inverse probe fitted with an autosampler. Each spectrum consisted of 64 scans of 32 K (500 MHz) or 65 K (300 MHz) data points with a spectral width of 6 kHz, an acquisition time of 5.3 s, and a recycle delay of 25 s per scan to allow complete relaxation and absolute quantification. The pulse angle was 90°. Spectra were acquired under an automation procedure (automatic shimming and automatic sample loading) requiring about 15 min per sample. Spectra were Fourier transformed with 0.3 Hz line broadening, phased, and baseline corrected using XWINNMR software (Bruker Biospin, Karlsruhe, Germany).

**Identification.** Each major sugar was identified by peak assignment using  $^1\text{H}$  NMR spectra from pure compounds added to stock and by comparison of published data (23, 26). In cases where further confirmation of the assignment was required for other compounds, the stock samples were spiked with appropriate standards to confirm that the chemical shifts were identical.

**Quantification.** The electronic reference to access in vivo concentrations (ERETIC) method was used for quantification of absolute concentrations of metabolites (27) with calibration curves of  $\text{C}_1\text{H}-\alpha$  glucose with the 500 MHz spectrometer. The  $\text{C}_1\text{H}-\beta$  glucose peak was not easy to quantify because it was frequently inside the water peak even though the sample had been lyophilized twice before quantification. The ERETIC synthesized signal, transmitted by an additional antenna and received with the sample signal, allowed the estimation of absolute concentrations by comparing the reference peak area to the metabolite peak area with good accuracy and stability (26). Sodium salt of (trimethyl)propionic-2,2,3,3- $d_4$  acid (TSP) dissolved in  $\text{D}_2\text{O}$  was added to all samples to a final concentration of 0.01% for chemical shift calibration.

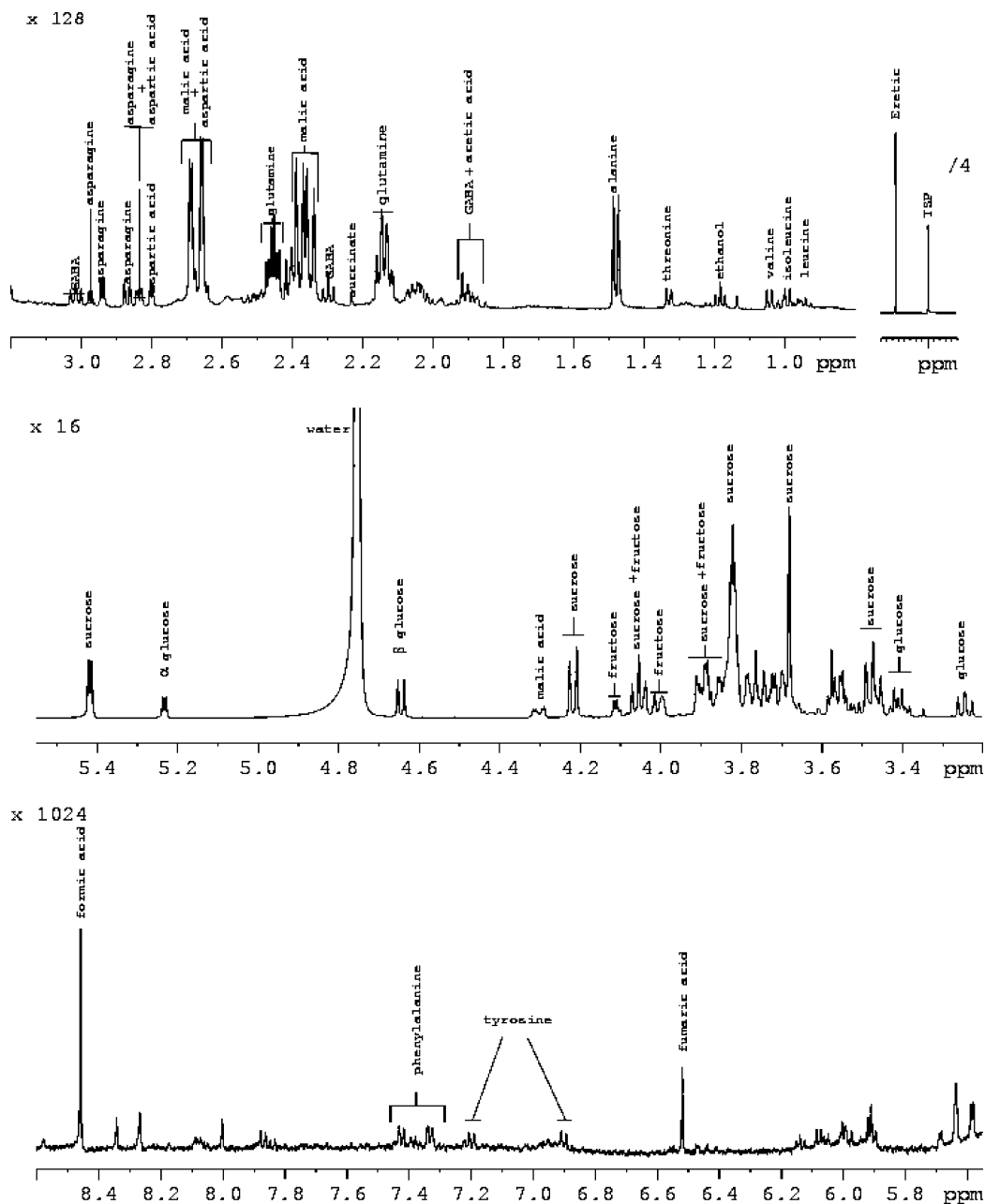
With the 300 MHz spectrometer: TSP was used for chemical shift calibration and for quantification of absolute concentrations of metabolites with calibration curves of  $\text{C}_1\text{H}-\alpha$  glucose and ( $\text{C}_3\text{H}$ ,  $\text{C}_4\text{H}$ )- $\beta$  +  $\text{C}_3\text{H}-\alpha$  fructofuranose (to take care of the different sugar forms). The  $\text{C}_1\text{H}-\beta$  glucose resonance could not be used for quantification because it was often overlapped by the water peak. A 0.02% TSP solution in  $\text{D}_2\text{O}$  was placed in a capillary tube that was added into each NMR tubes.

As the main point of this paper is the changes of sugars, we decided not to concentrate stocks before analyses. Moreover, any preparative process can modify the chemical composition of stock. Using NMR, we can analyze the stock almost directly (only one lyophilization step, contrary to derivatization of sugars before GC-MS, for example). The sugar concentrations in each sample were calculated from concentrations in the NMR tube and stock volume. All experiments were repeated three times, on three different carrots.

## RESULTS AND DISCUSSION

**Evaluation of the Quantitative Determination of Sugars from Stocks Using  $^1\text{H}$  NMR Spectroscopy.** The first set of experiments was designed to identify the major metabolites in carrot root stocks. **Figure 1** shows  $^1\text{H}$  NMR spectra obtained at 500 MHz for a representative carrot root stock. Three regions could be identified corresponding to the amino acids region (0–3 ppm), the sugar region (3–6 ppm), and the phenolics region (6–10 ppm). Each major metabolite of the different extracts was identified after peak assignment using  $^1\text{H}$  NMR spectra from pure compounds associated with a comparison of published data (15, 23).

More than 21 metabolites could be identified. **Table 1** shows the shifts used for identification of the metabolites in stocks. This list comprises 3 sugars, 11 amino acids, 6 organic acids, and 1 alcohol.



**Figure 1.** Portions of a representative  $^1\text{H}$  NMR spectrum of carrot stock (75 °C, processing time: 6.00 h). For identification and position of resonances, see Table 1.

Absolute quantification of fructose by  $^1\text{H}$  NMR has to be carried out carefully due to resonance overlapping and coexistence of several isomers (28). Up to date, a controversy remains about the open forms of fructose that are only observed by some authors (28, 29). Nevertheless,  $\alpha$ -fructofuranose,  $\beta$ -fructofuranose, and  $\beta$ -fructopyranose are always considered as the major forms, and  $\alpha$ -fructopyranose as the minor one. To take into account the coexistence of the fructose isomers, a set of NMR spectra of different fructose solutions, from 0 to 50 mmol/L, was recorded, and a response factor was calculated for the signal at 4.12 ppm (corresponding to the cleanest peak).

Amino acids and organic acids were identified, but they were not studied in the present work. Their concentrations were higher than the detection threshold but lower than the quantitative level (results not shown).

**Kinetic Composition of Carrot Stocks Processed at 50, 75, and 100 °C.** Figure 2 shows the extraction of the three

major sugars at different times up to 36 h. The maximum sucrose concentration was observed at 3 h for a processing temperature of 100 °C and after 9 h for processing temperatures of 50 and 75 °C (Figure 2A). The changes in glucose and fructose concentrations were similar (Figure 2B,C). When processed at 100 °C, glucose and fructose concentrations increased with time. When processed at 50 and 75 °C, concentrations increased until 9 h and then remained stable.

To explain the increase in glucose and fructose concentrations at a processing temperature of 100 °C, tests were made using the same analytical method on model solutions. In particular, sucrose hydrolysis and sugar dehydration were considered as possible mechanisms of sugar evolution, in addition to extraction from plant tissue, and were tested accordingly. Sucrose hydrolysis was proposed to contribute to the increase in glucose and fructose concentrations at 100 °C, associated with the decrease in sucrose at the same temperature. If only sucrose

**Table 1.** Resonance Assignments with Chemical Shifts and Spin–Spin Coupling Patterns of Metabolites Identified in 500 MHz  $^1\text{H}$  Spectrum of Carrot Stock

compound	assignment	$^1\text{H}$ multiplicity	$^1\text{H}$ amount	$\delta^1\text{H}$ (ppm)
Acetic acid	C2H <sub>3</sub>	s	2	1.92
Alanine	C3H <sub>3</sub>	d	3	1.48
Asparagine	C3H <sub>2</sub>	dd	2	2.92
Aspartate	C3H <sub>2</sub>	dd	2	2.82
Ethanol	C1H <sub>2</sub>	t	2	1.18
Formate	C1H	s	1	8.47
Fructose	$\beta(\text{C3H} + \text{C4H}) + \alpha\text{C3H}$	m	3	4.12
Fumarate	C2H + C3H	s	2	6.53
GABA	C2H <sub>2</sub>	t	2	2.31
Glucose	$\alpha\text{C1H}$	d	1	5.25
	$\beta\text{C1H}$	d	1	4.66
Glutamate	C3H <sub>2</sub>	m	2	2.07
Glutamine	C4H <sub>2</sub>	m	2	2.45
Isoleucine	C6H <sub>3</sub>	d	3	1.01
Leucine	C5H <sub>3</sub> + C6H <sub>3</sub>	dd	6	0.96
Malate	C2H	dd	1	4.30
Phenylalanine	C5H + C6H + C7H	m	5	7.40
Succinate	C2H <sub>2</sub>	s	4	2.43
Sucrose	glucopyranosyl–C1H	d	1	5.41
Threonine	C4H <sub>3</sub>	d	3	1.33
Tyrosine	C6H + C8H	d	2	6.88
Valine	C4H <sub>3</sub>	d	3	1.00
	C5H <sub>3</sub>	d	3	1.04

<sup>a</sup> Abbreviations: d, doublet; dd, doublet of doublets; m, complex multiplet; s, singlet; t, triplet. Chemical shifts were determined at pH 6 in D<sub>2</sub>O and expressed as relative values to that of TSP at 0 ppm (23).

hydrolysis was occurring, this assumption could be checked by correlating the sucrose concentration to the glucose plus fructose concentration. However, Maillard reactions or dehydration reactions of hexoses (30, 31) can occur when reducing sugars and amino acids are present, so the glucose and fructose are probably consumed by those reactions at the same time that they are extracted or created by sucrose hydrolysis.

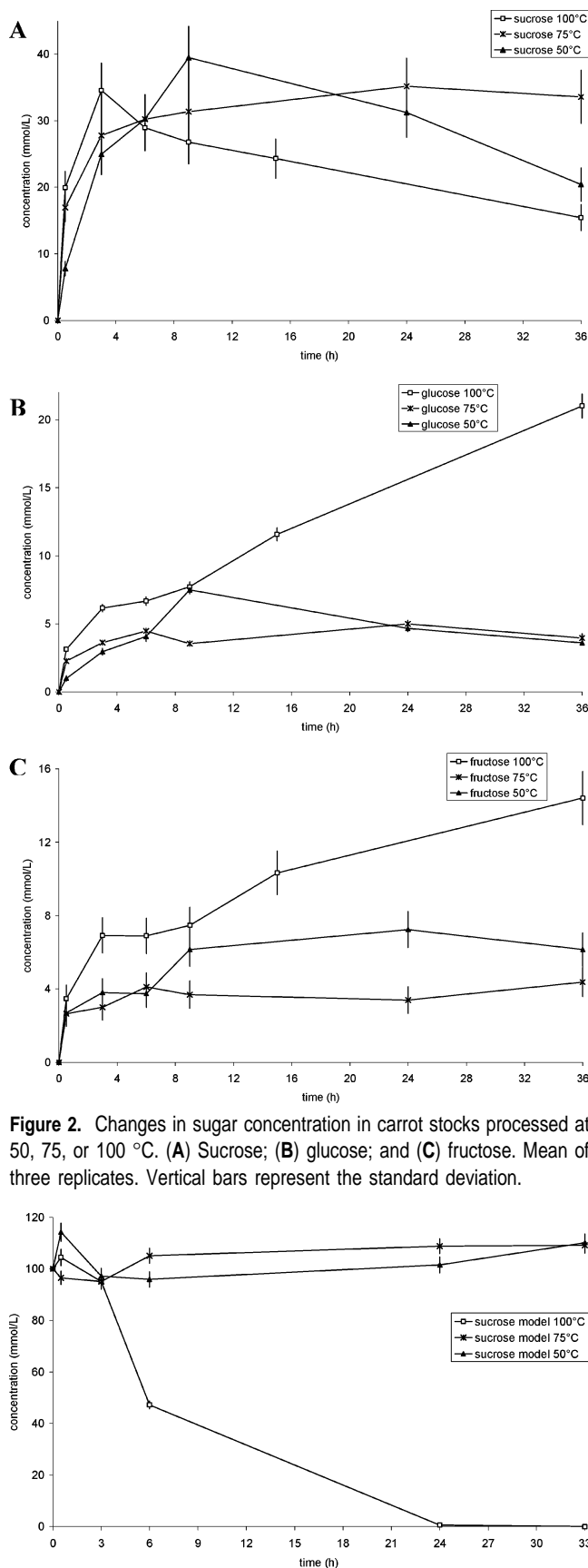
Solutions of amino acids and fructose or glucose (in similar concentrations as in the stock) were studied to understand the Maillard reaction contribution (results not shown). As no amino acid consumption could be observed, the hypothesis that Maillard reactions were occurring was dropped.

Solutions of sucrose alone or glucose + fructose were heated to follow the hydrolysis of sucrose and dehydration reactions of glucose and fructose. The kinetics of the concentration of the three sugars was followed by  $^1\text{H}$  NMR analysis.

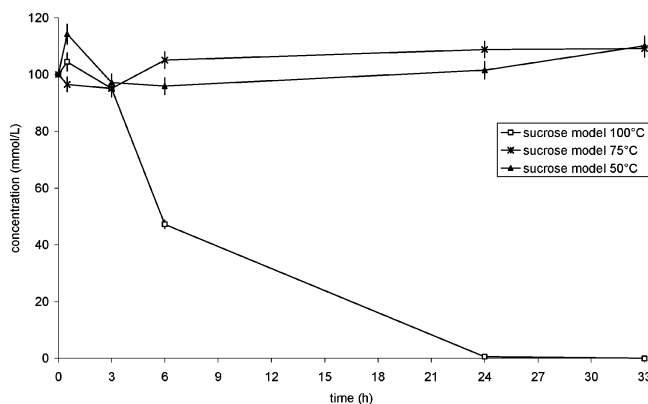
**Kinetic Hydrolysis of a Sucrose Model Solution Heated at 50, 75, and 100 °C.** Three sucrose solutions (100 mM) were heated at 50, 75, and 100 °C for up to 33 h. The decrease in sucrose concentration was followed by  $^1\text{H}$  NMR analysis (Figure 3).

Results show that chemical hydrolysis of sucrose occurred only at 100 °C, between 3 and 24 h of the process. Hence, sucrose chemical hydrolysis should be considered as an important factor to explain changes in sugar concentration in the processed carrot stocks. However, as this particular experiment did not explain the sucrose decrease in carrot stock at 50 °C after 9 h, other reactions must have contributed to this decrease.

In Figure 2, it has been calculated that 60 and 40% of fructose and glucose, respectively, were not recovered after sucrose hydrolysis at 100 °C. This is in accordance with literature about sugar dehydration: fructose dehydration is faster than glucose dehydration (30, 31). To visualize sugar dehydration, the following experiment was carried out.



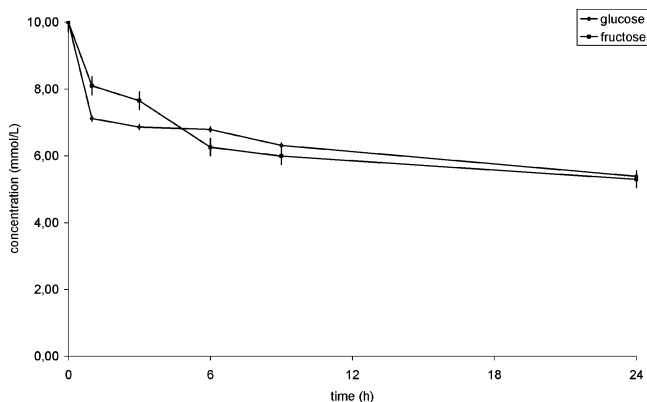
**Figure 2.** Changes in sugar concentration in carrot stocks processed at 50, 75, or 100 °C. (A) Sucrose; (B) glucose; and (C) fructose. Mean of three replicates. Vertical bars represent the standard deviation.



**Figure 3.** Changes in sucrose concentration in sucrose solution processed at three temperatures. Mean of three replicates. Vertical bars represent the standard deviation.

**Kinetics of a Glucose + Fructose Model Solution Heated at 100 °C.** Glucose and fructose solutions (10 mM each sugar)



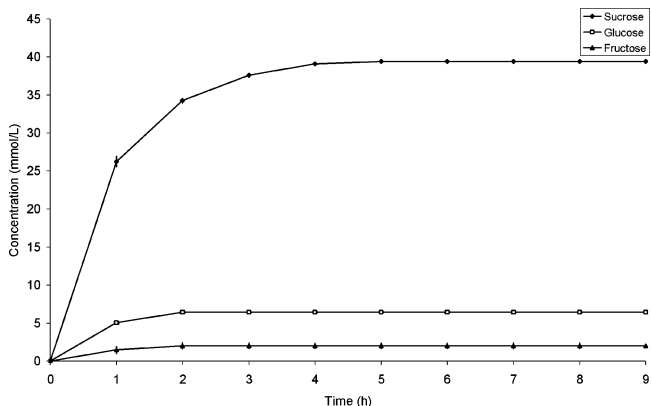


**Figure 4.** Changes in glucose and fructose concentrations in glucose plus fructose solution processed at 100 °C. Mean of three replicates. Vertical bars represent standard deviations.

were heated at 100 °C for up to 24 h (in triplicate). The decrease in sugar concentration was monitored by  $^1\text{H}$  NMR (**Figure 4**): 40% of sugars disappeared at 100 °C. The loss in glucose and fructose during stock preparation seems to show that glucose and fructose are consumed at the same time as they are extracted or formed by sucrose hydrolysis. It was assumed that these sugars could be dehydrated to form HMF (30, 32). To test this assumption, a fructose solution was heated at 100 °C during 17 h (**Figure 5**). Characteristic peaks (9.45, 7.55, and 6.75 ppm) of HMF (33) were found in the fructose solution and in the glucose solution (data not shown). This result is in accordance with the assumption that during the production of stock, the extraction of sugars, the hydrolysis of sucrose, and the resulting production of glucose and fructose through this hydrolytic process, as well as the dehydration of glucose and fructose, all occur simultaneously.

**Extraction from Plant Tissue without Hydrolysis of Sucrose at 100 °C.** To further understand the extraction of sugars from the carrots and to avoid the sucrose hydrolysis reaction, another experiment was carried out, where stock was replaced by pure water at hourly intervals. The 1 h time interval was chosen because previous analysis showed that sucrose hydrolysis occurs after more than 1 h. The solutions removed from the experimental system after each 1 h interval were

analyzed by  $^1\text{H}$  NMR, and cumulated concentrations were calculated to display pure sugar extraction (**Figure 6**). Glucose

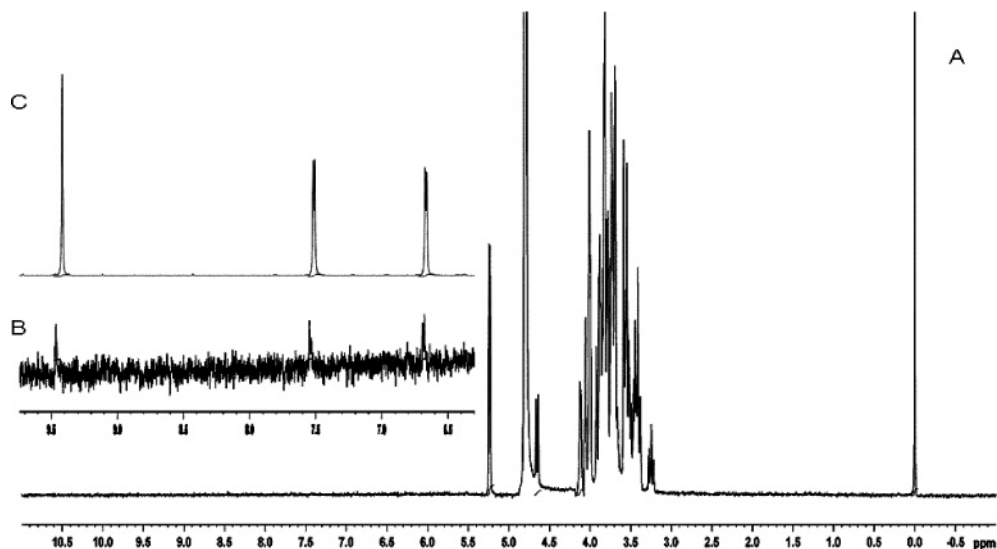


**Figure 6.** Kinetics of sugar extraction only in a model stock solution heated at 100 °C. Mean of three replicates. Standard deviations are hidden by the symbols.

and fructose were extracted in 2 h, even though the sucrose needed 5 h to be completely extracted. Glucose, fructose, and sucrose concentrations reached 6.4, 2.0, and 39.4 mmol/L, respectively. It has been noted that some glucose and fructose are lost each time we change the water by HMF formation. But, the question here was only to know for how long these sugars could be extracted, and for that we do not use the values of the concentrations but only look at the asymptotic regime.

All these results point to the following mechanism: at 100 °C, sucrose is extracted between 0 and 5 h and hydrolyzed after 1 h. Between 1 and 5 h, both extraction and hydrolysis occurs. At 75 °C, only sucrose is extracted, although at 50 °C, both extraction and other reactions occur. At 100 °C, glucose and fructose are extracted in the first 2 h. Between 1 and 2 h, both sugars are produced by sucrose hydrolysis; some of these sugars have been dehydrated into HMF since the beginning of the treatment. At 75 °C, fructose and glucose are merely extracted; however, at 50 °C, while fructose is extracted, glucose is consumed, probably by enzymatic reaction(s).

In conclusion,  $^1\text{H}$  NMR quantification of sugars in a complex solution, here carrot stock, is quick, straightforward, and allows the detection of a number of compounds simultaneously (e.g.,



**Figure 5.** Representative  $^1\text{H}$  NMR spectrum. (A) Glucose and fructose solution after 17 h of heat treatment at 100 °C. (B) Expanded spectrum portion showing HMF formation. (C) HMF solution.

amino acids, organic acids, etc). Migration of sugars from plant tissue into water depends on temperature and time. At 100 °C, extraction of sucrose occurs with hydrolysis, and glucose and fructose concentrations result from the balance between formation through sucrose hydrolysis and disappearance through HMF formation. At 75 °C, there seems to be only sugar extraction. At 50 °C, the mechanism needs further investigation.

#### ACKNOWLEDGMENT

We are grateful to Frédéric Lejard, Céline Berger, and Eric Vouland for support (Diana Ingrédients); Juan Valverde, Rachel Edwards Stuart, and Robert Méric for technical assistance (Collège de France); and Mickaël Maucourt (INRA Bordeaux), Patrick Varoquaux (INRA Avignon), Marie-Christine Ralet, Jean-François Thibault (INRA Nantes), and Douglas Rutledge (INRA INAPG) for helpful discussions.

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Received for review January 18, 2006. Revised manuscript received April 5, 2006. Accepted April 9, 2006.