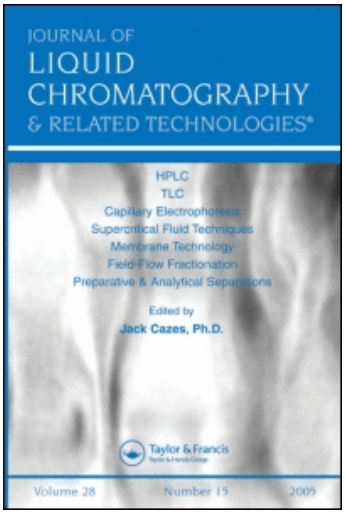

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HPLC Determination of Trehalose in *Selaginella lepidophylla* Plants

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ABSTRACT

A sensitive and selective assay of trehalose is presented for its determination in anhydrobiotic organisms that are able to tolerate lack of water because of their ability to synthesize large quantities of this disaccharide. The disaccharide was extracted with water and ethanol 80%, followed by normal bonded phase-high performance liquid chromatography and refraction index measurement. The assay demonstrated to be fast with good linear and high precision response. The column and detector performance show good retention, selectivity, and efficiency. Based on replicated analysis of standards over a range of 0.2–10 mg/mL, the method is accurate (90–100%) and precise (CV of 5% daily). The concentration of trehalose in *Selaginella lepidophylla* plants,

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subjected to hydration and dehydration treatments at seven different incubation times, ranged between 0.61 and 17.99 mg/mL. At the same time, the presence of fructose, glucose, and sucrose were analyzed. The standard deviation of plant samples were about 0.003–1.51%, where the detection limit was 0.01 mg/mL.

Key Words: HPLC; Trehalose; *Selaginella lepidophylla*; Anhydrobiotic organisms.

INTRODUCTION

Trehalose (α-D-glucopyranosyl-[I-I]-α-D-glucopyranoside) is an uncommon sugar in the plant kingdom.[1,2] This disaccharide, however, is often detected in symbiotic plant organs, where it is synthesized by the microbial partner.[3,4] Many seeds, some plants, yeast cells, fungal spores, and simple animals are capable of surviving almost complete dehydration, as little as 0.1% H_2O.[5] Many organisms that withstand desiccation express the disaccharide trehalose. Anhydrobiotic organisms are able to tolerate the lack of water because of their ability to synthesize large quantities of the disaccharide trehalose. No vertebrate has been shown to synthesize trehalose or to exhibit the degree of desiccation tolerance found in organisms that synthesize it.[6]

The mechanism by which trehalose mediates desiccation tolerance has not been completely determined, but seems to involve effects on both protein and lipid membranes. In general, trehalose is thought to replace the shell of water around macromolecules, preventing damaging effects during drying.[7]

Trehalose accumulation in plants has been reported only in *Selaginella lepidophylla* and *Myrothamnus flabellifolia*.[8–10] *S. lepidophylla* (Hook and Grev) is a desiccation-tolerant plant native to the Chihuahua desert of Texas and several states of Mexico, recognized for its ability to desiccate, curl up, and survive long dry periods. Adams et al.[8] found trehalose concentrations of 71–106 mg/g dry matter in desiccated leaves of *S. lepidophylla*. These authors proposed that one possible factor for the ability of *S. lepidophylla* to withstand desiccation may be the high accumulation of trehalose in leaves. During extreme drought, trehalose may have a protective effect on cell membranes and enzymes from denaturation.[8,11,12]

To determine whether trehalose could be expressed and detected, the samples are extracted and analyzed for high-pressure liquid chromatography analysis. Measurement of trehalose concentration in samples usually involves a purification of the sample on a Micro Bio-Spin Chromatography Column (Bio-Rad, Richmond, CA) with mixed-bed, analytical grade ion exchange

resin to remove charged molecules (trehalose is a neutral sugar) and concentrate the sample. For HPLC, an ionic interchange system was used at flow rate of 0.4 mL/min at 8°C and ED40 electrochemical detector. Water and 1 M NaOH were used as the eluent. The elution time for trehalose is approximately 30 min.[6,13,14]

The purpose of this article was the determination of trehalose in organisms that tolerate the desiccation (*S. lepidophylla*). The methodology can be utilzed in determining whether trehalose expression confers desiccation resistance in anhydrobiotic organisms.

EXPERIMENTAL

Sample Preparation

Samples were subjected to hydration and dehydration treatments. *S. lepidophylla* plants (2.5 g) were homogenized in 15 mL H_2O or ethanol (80% v/v), and incubated at 50°C. The samples were extracted in water and ethanol from 0, 1, 2, 4, 8, 12, and 24 hr of hydration and 4, 8, 12, and 24 hr of dehydration. The suspension was centrifuged at 10,000 *g* for 30 min, and the supernatant removed and used to determine trehalose concentration by HPLC.

Analytical Method

HPLC analyses were carried out using a Varian Solvent Delivery System Pump Model 9012 and a Rheodyne Model 7125 injector (Rheodyne Inc., Cotati, CA) fitted with a 10 μL loop. For detection, a Varian Model 350 differential refractometer (RI) detector was used. A Star Chromatography Workstation Version 5.51 was used (Varian Associates Inc., USA). A Supelcosil LC-NH_2, 5 μm (4.6 mm × 25 cm) column was used with a 3 cm guard column (Supelco, Mex.) with 80/20 acetonitrile–water as the mobile phase at a flow rate of 1.0 mL/min. The mobile phase was filtered with 0.2 μm prior to use.

Quantitative determinations were done by comparison of three successive analyses on each of the water or alcoholic extracts, and the standard mixtures of sugars and trehalose. Descriptive statistic and regression analysis was applied to the data. The trehalose concentration was determined with reference to calibration standards of trehalose (20.6 mg/mL), fructose, glucose, and sucrose, at concentrations from 40 mg/mL (Sigma-Aldrich de México).

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RESULTS AND DISCUSSION

Detection Limit and Linearity of the Detector Response

Analytical working curves were determined for trehalose (Fig. 1). Linearity of the method was tested by analyzing the working standard solutions at different concentrations. The calibration curves of trehalose were constructed over the range of 0.2–10 mg/mL and showed good linear response. The regression coefficient was 0.99 ($n = 27$) for the trehalose standards. This allows the use of only one standard solution for routine analysis. The detection limits for trehalose was approximately 0.01 mg/mL. However, the minimum used level for routine analysis of trehalose is approximately 0.1 mg.

The column showed good resolution = 1.4, retention = 2.03, selectivity = 1.45, and column efficiency = 730 theoretical plates. Theoretically, absolute resolution indicates that the peaks are 99.7% separated to baseline, however, the descriptive resolution shows that the amount of separation is greater (Fig. 2). The theoretical plates indicate that the good resolution is due to column selectivity rather than the column efficiency.

Precision of the Method

The precision of the method was assayed by the analysis of 40 replicate standard samples in 20 days, which contained trehalose at 20.6 mg/mL. The calculated coefficient of variation was 10% for the trehalose areas. Daily

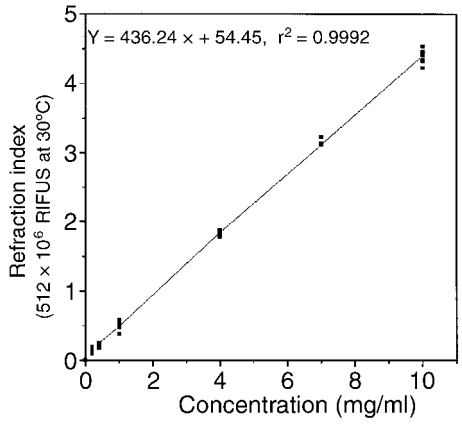

Figure 1. Trehalose standard curve.

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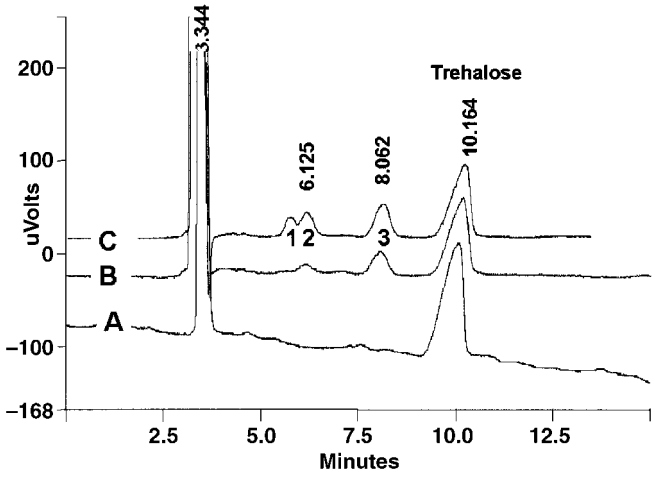

Figure 2. Analysis of trehalose: (A) standard of trehalose; (B) sample extracted 2 hr with ethanol; (C) sample extracted 2 hr with ethanol plus standard addition.

variability for nine replicate standards was 5% (calibration factor $= 5 \times 10^{-3}$, Standard deviation $= < 5.0 \times 10^{-4}$).

HPLC Analysis of Trehalose

High pressure liquid chromatography analysis showed a large peak present in the extract at 10.16 min [Fig. 2(B)] that corresponded to purified trehalose standard [Fig. 2(A)]. To confirm that this peak was trehalose, the extract was spiked with trehalose standard, resulting in the peak identification [Fig. 2(C)]. The quantity of trehalose in the ethanol extract was estimated to be 4.33–17.99 mg/mL. Peaks 1, 2, and 3 were identified as fructose, glucose, and sucrose, respectively.

A chromatogram, performing the separation of trehalose in *S. lepidophylla*, after 2 hr of hydration with water and alcohol under the chromatographic conditions used for all analyses in this report, is shown in Fig. 3.

The results show that trehalose is better extracted in alcohol. Extraction in 80% ethanol appeared to be the best extraction method.[8] Probably, trehalose is hydrolyzed to glucose when extracted with water, or by the action of the enzyme trehalase naturally present in the plant. The presence of glucose in the samples extracted with water (peak at 6.26 min) supports this. Chromatogram B also shows the presence of sucrose at 8.06 min in the samples

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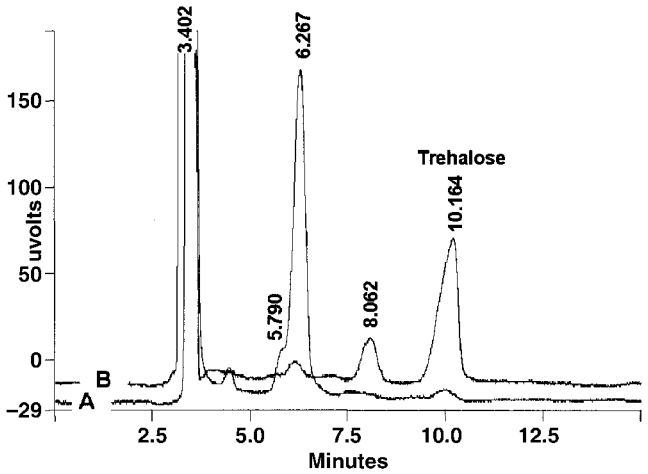

Figure 3. Analysis of trehalose. Sample extracted 2 hr with (A) water and (B) 80% ethanol.

extracted with ethanol. Chromatogram A shows traces of fructose (peak at 5.79 min) as well.

Direct analysis of the crude extract saves considerable sample manipulations that have been used in other studies to "clean-up," or clean the injector liner in gas chromatography.[8]

Recent studies have demonstrated that trehalose can be successfully resolved by HPLC, using gradient elution with water and 1 M NaOH and 5 μm particle size ion exchange columns. In these studies, the columns were either 250 or 300 mm in length and each analysis required 30–60 min.[6,13,14] However, if a further decrease of the analysis time could be obtained, a significant reduction in the total time required for multiple analyses would be achieved. This is especially important when numerous samples must be analyzed. In our study, we utilized no pretreatment to the sample. Normal bonded-phase HPLC was used and the total analysis time was 15 min.

Trehalose Concentration in *S. lepidophylla* Plants

A comparison was done between the disaccharide extracted with water and alcohol subjected to hydration and dehydration treatments at seven different incubation times. Although the total sugars do not differ (7.71–24.36 mg/mL extracted with water and 7.07–22.88 mg/mL extracted with ethanol 80%),

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Table 1. Trehalose concentration in *Selaginella lepidophylla* (in mg/mL).

Hours of extraction	Water extraction				Ethanol extraction			
	Fructose	Glucose	Sucrose	Trehalose	Fructose	Glucose	Sucrose	Trehalose
Hydration								
0	2.09	21.66	ND	0.61	NT	0.91	3.83	15.47
	0.06 ± 2.85	0.57 ± 2.61		0.02 ± 3.72		0.05 ± 5.88	0.16 ± 4.06	0.37 ± 2.41
1	1.53	16.19	ND	1.38	NT	0.87	3.09	12.62
	0.08 ± 5.49	0.37 ± 2.27		0.04 ± 3.04		0.08 ± 9.09	0.20 ± 6.46	0.07 ± 0.54
2	0.66	15.41	ND	ND	NT	0.55	2.19	11.72
	0.31 ± 5.8	0.84 ± 5.48		ND		0.003 ± 0.57	0.01 ± 0.45	0.03 ± 0.25
4	NT	15.52	ND	3.98	NT	1.11	3.78	17.99
		0.57 ± 3.69		0.04 ± 0.94		0.02 ± 1.98	0.10 ± 2.77	0.06 ± 0.34
8	NT	18.06	ND	1.54	NT	1.17	2.11	11.87
		1.19 ± 6.58		0.04 ± 2.56		0.42 ± 5.83	0.01 ± 0.31	0.13 ± 1.07
12	1.04	7.2	ND	0.46	NT	0.81	1.93	4.33
	0.17 ± 5.85	0.13 ± 1.98		0.01 ± 1.0		0.32 ± 3.35	1.51 ± 0.30	0.02 ± 0.37
24	NT	7.06	ND	0.65	NT	0.85	0.8	6.38
		0.13 ± 1.89		0.08 ± 2.49		0.08 ± 9.18	0.07 ± 9.04	0.08 ± 1.33
Dehydration								
2	NT	11.72	ND	1.57	NT	1.49	2.29	9.67
		0.20 ± 1.77		0.07 ± 4.39		0.07 ± 4.78	0.05 ± 2.03	0.07 ± 0.24
4	NT	15.76	ND	ND	NT	1.22	2.2	10.5
		0.21 ± 1.35				0	0.15 ± 6.66	0.24 ± 2.32

(*continued*)

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Table 1. Continued.

Hours of extraction	Water extraction				Ethanol extraction			
	Fructose	Glucose	Sucrose	Trehalose	Fructose	Glucose	Sucrose	Trehalose
8	NT	10.88 0.46 ± 4.21	ND	ND	NT	0.87 0.02 ± 2.16	1.45 0.05 ± 3.60	12.87 0.38 ± 2.97
12	NT	12.04 0.71 ± 5.93	ND	4.65 0.003 ± 0.07	0.73 0.11 ± 5.52	ND	2.53 0.06 ± 2.20	14.01 0.88 ± 6.28

Note: NT = not tested (fructose was present only at the detection level in many cases, so quantitative data were not obtained); ND = not detected; data represent the mean, standard deviation ± variation coefficient of three independent replicates.

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the change in concentration of glucose and sucrose (mg/mL) was highly notable. The yield of fructose is lower for the extractions with water and was not detected in ethanol (Table 1). The average trehalose concentration was, however, highly variable between different solvents, ranging from around 0.46 mg/mL for extraction with water at 0 hr to 17.99 mg/mL with ethanol at 4 hr (Table 1). The trehalose yields in water extracts had a narrow range, from 0.46 to 4.65 mg/mL, in contrast to the yields from alcohol extracts, which varied from 4.33 to 17.99 mg/mL. The apparent stoichiometry between the lower values in glucose and sucrose, and increase in trehalose concentration, suggests that glucose and sucrose serves as the carbon source for trehalose synthesis.[8]

The shift in the free sugar pool is clearly seen in the compositional data and the water vs. ethanol 80% extraction. In particular, a relative increase in sucrose was observed in the ethanol extract. The concentration of trehalose increased upon ethanol extraction, while a decrease in glucose level was observed.[15]

The proposed HPLC method has been demonstrated to be suitable for analysis of trehalose in organisms that tolerate the desiccation. It represents a considerable alternative over the existing methodologies for determination of trehalose, both in analysis time and consumption of chemicals. The described method can be used to determine whether trehalose expression confers desiccation resistance in anhydrobiotic organisms.

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