PHYTOCHEMICAL ANALYSIS
Phytochem. Anal. **14**, 319–324 (2003)
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI**: 10.1002.pca.723

Carbohydrate Analysis of Floral Nectar Using Medium Infrared

Cesar Mateo Flores Ortiz,*[1] Ignacio Peñalosa Castro,[1] Luis Barbo Hernández Portilla,[1] Patricia Dolores Dávila Aranda[2] and Maria del Coro Arizmendi[3]

[1]Laboratorio de Fisiología Vegetal, UBIPRO, Facultad de Estudios Superiores Iztacala, UNAM, México
[2]Laboratorio de Recursos Naturales, UBIPRO, Facultad de Estudios Superiores Iztacala, UNAM, México
[3]Laboratorio de Ecología, UBIPRO, Facultad de Estudios Superiores Iztacala, UNAM, México

An instrumental method based on a chemometric model of the medium region of the infrared (MIR) was developed to analyse total sugar content and the proportions of glucose, fructose and sucrose. In order to construct the model, a set of 127 standard aqueous solutions of different sugars in the concentration range 0–20% (w/v) were prepared and analysed in the interval 4900–700 cm^{-1}. The MIR was transformed by normalisation, correction of baseline using the second derivative, and suppression of the signals of water and carbon dioxide. The region between 1150 and 950 cm^{-1} showed the highest correlation between signal and concentration. The correlation coefficient for total sugar content was 0.956, whilst those for glucose, fructose and sucrose were 0.982, 0.972 and 0.992, respectively. The method was validated using a set of 28 samples of nectar which had been assayed by chromatographic and refractometric methods. The method shows potential utility for the prediction of nectar sugar components.

Keywords: Medium range IR; FT-IR; carbohydrates; analysis of sugars; nectar; *Pachycereus pecten-aborigimum*; *Ceiba parvifolia*.

INTRODUCTION

Floral nectar is a rich nutrient solution that is offered by plants to pollinators (Simpson and Neff, 1983). The chemical content of nectar includes sugars, amino acids, lipids, phenols and anti-oxidants (Baker and Baker, 1983a). Sugars are the main constituent of nectars, representing at least 10% of solutes. Glucose, fructose and sucrose are the most common sugars and are by far the most important energy sources for the pollinators.

The composition of floral nectar depends on several intrinsic factors such as sexual form of the flower (Devlin and Stephenson, 1985; Wilson and Agren, 1989; Gillespie and Henwood, 1994), the floral stage (Pleasants, 1983), the structure of the floral nectary (Belmonte *et al.*, 1994; Davis *et al.*, 1998), and the flowering season and time of the day (Torres and Galetto, 1998). Intra-plant variations have also been reported (Freeman and Wilken, 1987). Additionally, the composition of nectar has been found to be associated with extrinsic environmental factors such as temperature, humidity and salinity (Bertsch, 1983; Hiebert and Calder, 1983).

Studies of nectar composition contribute to the relevant ecological information concerning plant–pollinator interactions. In general, it has been proposed that there is a close relationship between short tongue pollinators and nectars rich in hexose monosaccharides, whilst long tongue pollinators are related to sucrose-rich nectars (Baker and Baker, 1983b). Additionally, the sugar concentration and the amount of the nectar are strongly related to the morphology of the flower (concentrated or dilute nectars being found in cup or tube flower forms, respectively), and the number of visitors positively correlates with the amount of nectar per flower (Silva and Dean, 2000). All of the information that can be obtained from nectar composition requires high-quality analysis and this represents a formidable amount of work using conventional chromatographic techniques.

Total non-structural carbohydrates in plant samples may be readily determined by various techniques that do not discriminate between the sugar constituents (Buysse and Merckx, 1993). However, when identification and precise quantification of the sugars are required, the two most commonly employed protocols are HPLC and colorimetric enzymatic analysis (Velterop and Vos, 2001). Quantitative assays for total sugars have been performed using a refractometer (Kearns and Inouye, 1993), and by spectrophotometric analysis using the reaction with phenol in acid solution (Jolls *et al.*, 1994). For the quantitative and qualitative analysis of the ratio of hexose monosaccharides to sucrose in nectar, HPLC has been used with amperometric (Davis *et al.*, 1998) or refractometric detection, and enzymatic analysis (Davis, 1997). Additionally, GC analysis of derivatised carbohydrates has been used to study nectar composition (Forcone *et al.*, 1997). However, these qualitative/quantitative methods are, in general, expensive and time-consuming in terms of sample preparation.

Rapid analytical methods have been developed using near infrared reflectance spectroscopy as alternatives to the traditional methods for studying carbohydrates (Osborne, 1981). However, few studies have focused on the use of medium infrared (MIR) reflectance since spectra in this region are complex and the water contained in biological material is a strong IR absorber. However, by using Fourier transform infrared (FT-IR) and attenuated

* Correspondence to: C. M. F. Ortiz, Laboratorio de Fisiología Vegetal, UBIPRO, Facultad de Estudios Superiores Iztacala, UNAM, México.
Email: cmflores@servidor.unam.mx
Contract/grant Sponsor: DGAPA, UNAM; Contract/grant number: IN203598.

Received 14 December 2001
Revised 11 July 2002
Accepted 2 August 2002

total reflectance (ATR) spectroscopic techniques, MIR spectroscopy has developed considerably (Cadet *et al.*, 1991). There are various mathematical methods that can be employed for processing IR data and one of them, partial least squares (PLS), has been successfully used on transmission MIR spectra for the analysis of carbohydrates (Kacurakova and Wilson, 2001).

The aim of the present work was to design a protocol for the qualitative and quantitative analysis of floral nectar carbohydrates based on MIR spectroscopic using PLS as the mathematical method for the construction of a chemometric model, and to validate this model against refractometric and chromatographic techniques.

EXPERIMENTAL

Floral nectar samples. Samples of floral nectar were obtained from the species *Pachycereus pecten-arboriginum* (Cactaceae) growing in the locality of Bahia de Kino, Sonora, Mexico, and *Ceiba parviflora* (Bombacaceae) growing in the locality of Tehuacán Puebla, Mexico. Aliquots (1 mL) of nectar were collected at night time using graduated pipettes and samples were placed in plastic tubes and maintained in the cold until analysis in the laboratory.

Measurement of MIR spectra. The method used was similar to that previously reported (Cadet and Offmann, 1997), but with minor modifications. MIR-FT spectra were collected on a Spectrum 2000 (Perkin Elmer, Boston, MA, USA) FT spectrophotometer. For ATR spectra, a crystal of zinc selenide was loaded with 1 mL of solution of either pure standard or the nectar sample. Data were recorded from 4900 to 700 cm^{-1} at a resolution of 4 cm^{-1} in the percentage transmittance mode. A combination of 32 scans resulted in an average spectrum.

Mathematical treatment. Multidimensional statistical analysis was performed with Quant software (Nicolet, Madison, WI, USA) using the PLS algorithm. The spectra modifications employed were normalisation, correction of baseline using the second derivative, and suppression of the signals of water and carbon dioxide. In order to build the model, a set of 127 standard solutions of varying concentrations (from 0 to 20%, w/v) of different sugars were prepared as depicted in Fig. 1.

Chromatographic analysis. Pure standards of sugars and the nectar samples were analysed by TLC using plates coated with silica gel G 60 (1 mm thick layers). Aliquots (5 µL) of solutions (containing 10 µg/mL) of pure standards of glucose, fructose and sucrose and an aliquot (1 µL) of nectar sample were applied separately to each plate. For elution, 16 mobile phases were assessed, but butanol:methanol:water (6:4:1) gave the best separation. Separated components were visualised with a mixture of water (4 mL), ethanol (51 mL), concentrated sulphuric acid (6.5 mL) and α-naphthol (0.5 ***g***). The amount of carbohydrate present in each spot on the plate was determined by densitometry using a Multimage Light Cabinet (Alpha Innotech San Leandro, CA, USA) image analyser running Alfaimager software.

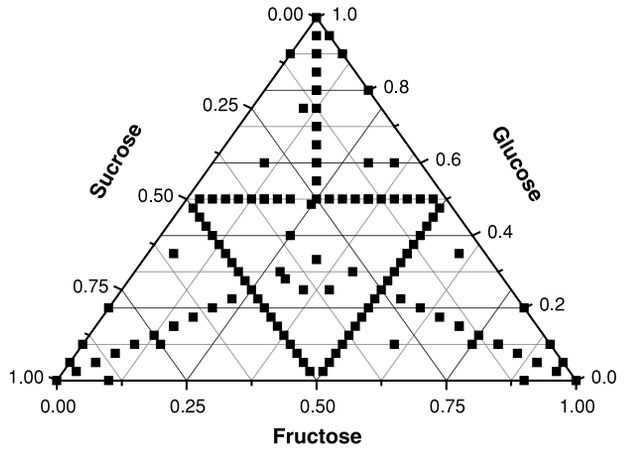

Figure 1. Array of concentrations of aqueous sugar standards employed to validate the chemometric model. Each square represents the composition of one of the 127 standard solutions employed. The concentration scales from 0 to 1 represent concentrations of sugar from 0 to 20% (w/v).

Refractometric analysis. In order to validate the quantification of total carbohydrate content obtained using the chemometric model, the total amount of carbohydrate was determined using an ABBE (MiltonRoy, Rochester, NY, USA) refractometer. Aqueous samples of pure glucose, fructose and sucrose in the concentration range 0–80% (w/v) were used as reference standards. For the analysis of nectar samples, collected solutions were diluted 1:10. All refractometric assays were performed at 25°C.

RESULTS AND DISCUSSION

The MIR of nectar (Fig. 2) shows three regions of absorption at 3700–2800, 1800–1470 and 1250–800 cm^{-1}, of which the first two arise almost entirely from the vibrations of water that is the natural solvent of the nectar. The region between 1150 and 950 cm^{-1}, however, shows the greatest difference between the signal of water and the sample of nectar and this area was selected for further consideration. This region is the same as that used by Cadet and Offmann (1997) in the analysis by MIR of sucrose in sugar cane juices. As can be see in Fig. 3(A), in this region the spectra obtained from aqueous sugar standards at concentrations of 12, 16 and 20% (w/v) showed maxima at 1063 cm^{-1}. Additionally, when these spectra were transformed to the second derivative (Fig. 3B), they gave rise to seven signals at 1116, 1076, 1064, 1033, 992, 980 and 966 cm^{-1}, which showed high correlation between signal and concentration of sugar. This transformation reduced the sensitivity of the signal but amplified the differences between spectra corresponding to each sugar. In addition, the use of the second derivative reduced problems related to the background absorption in real samples which was due to the presence of solid particles which could disperse the light (Rambla *et al.*, 1997).

The spectra, modified as typified in Fig. 3(B), of standard mixtures of glucose, fructose and sucrose in the proportions shown in Fig. 1, were used to build the chemometric model. The model was evaluated for its

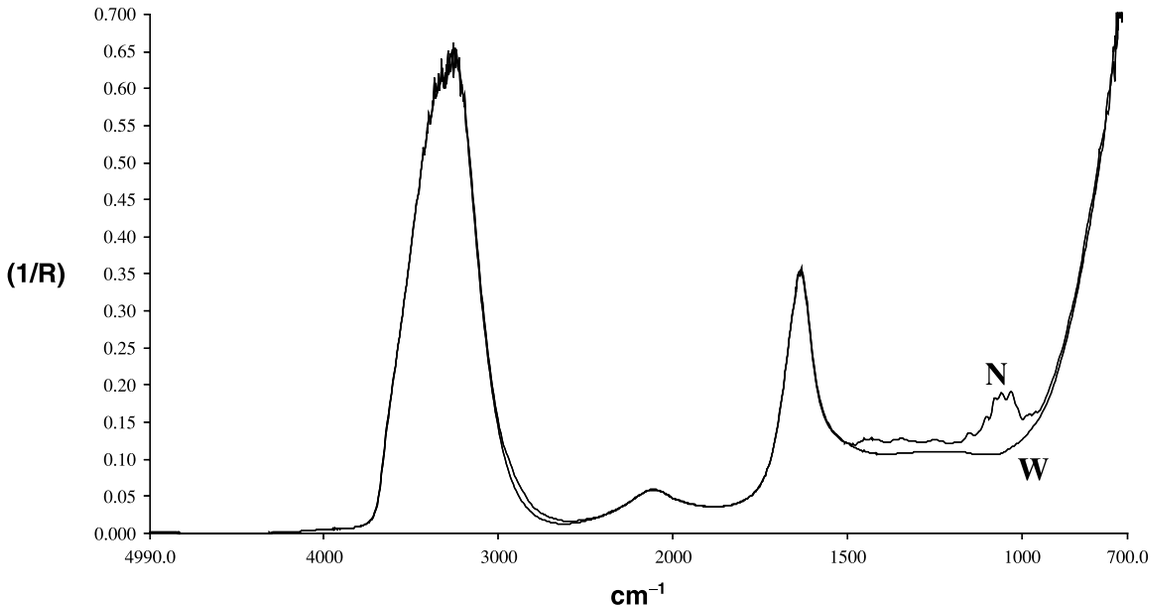

Figure 2. The MIR spectra of water (W) and of a typical nectar sample (N) in the MIR range 4990–700 cm^{-1}.

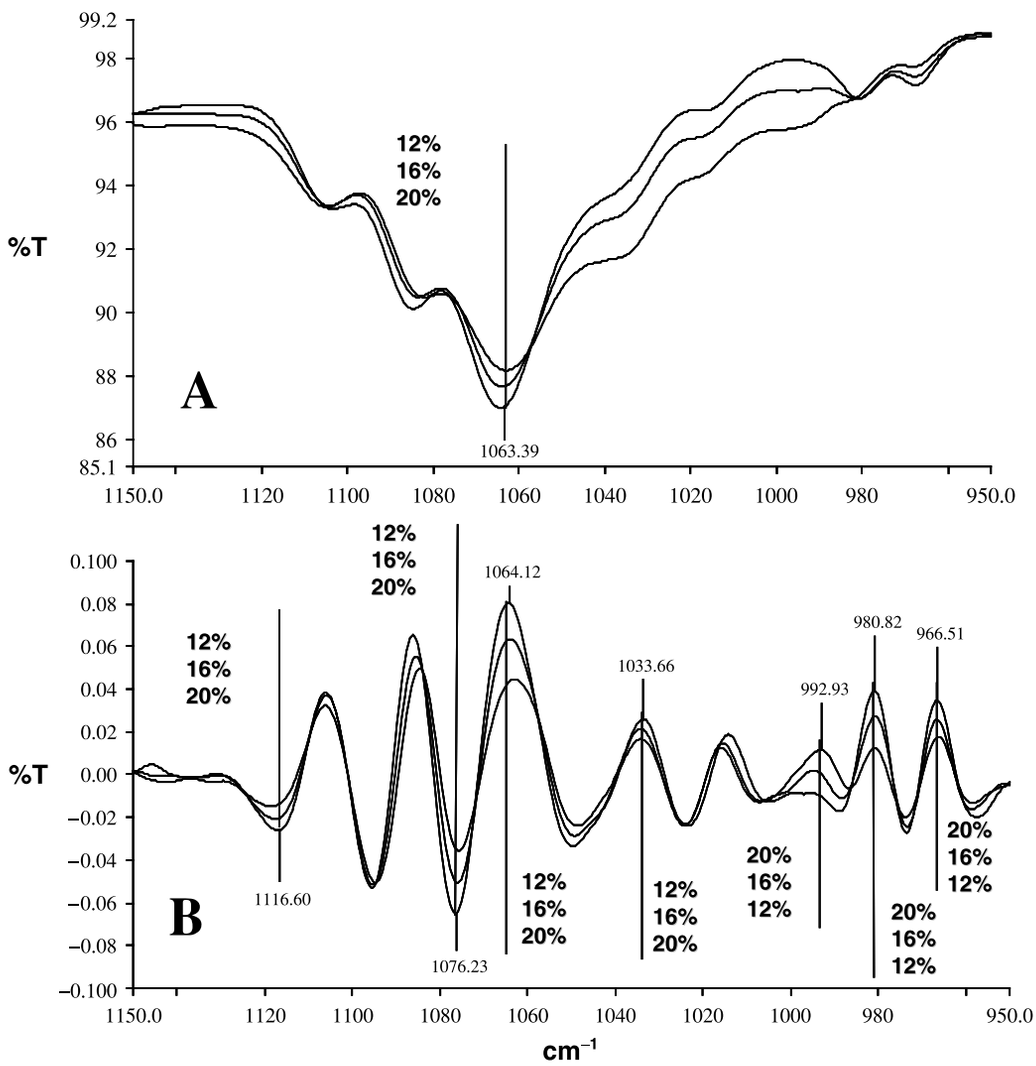

Figure 3. MIR/ATR spectra of aqueous sugar standards with concentrations of 12, 16 and 20% (w/v) in the range 1150–950 cm^{-1} (A) and the transformed second derivatives of these spectra (B).

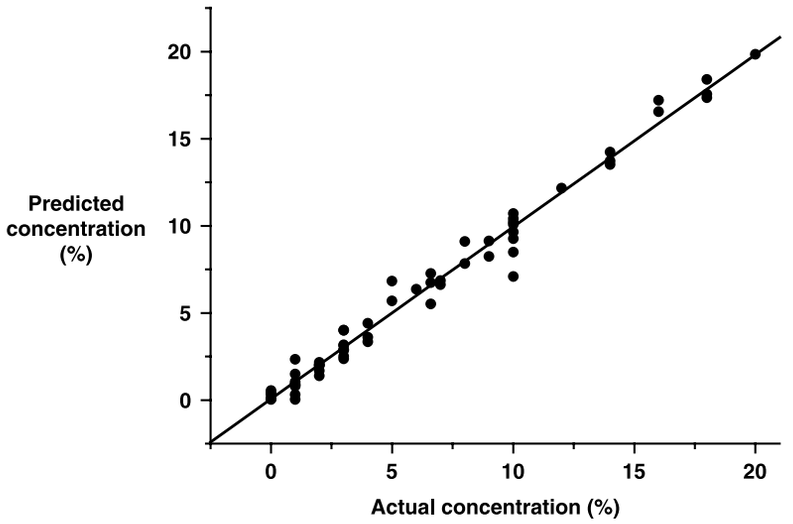

Figure 4. Correlation between the actual content of total sugar and that predicted by the chemometric model.

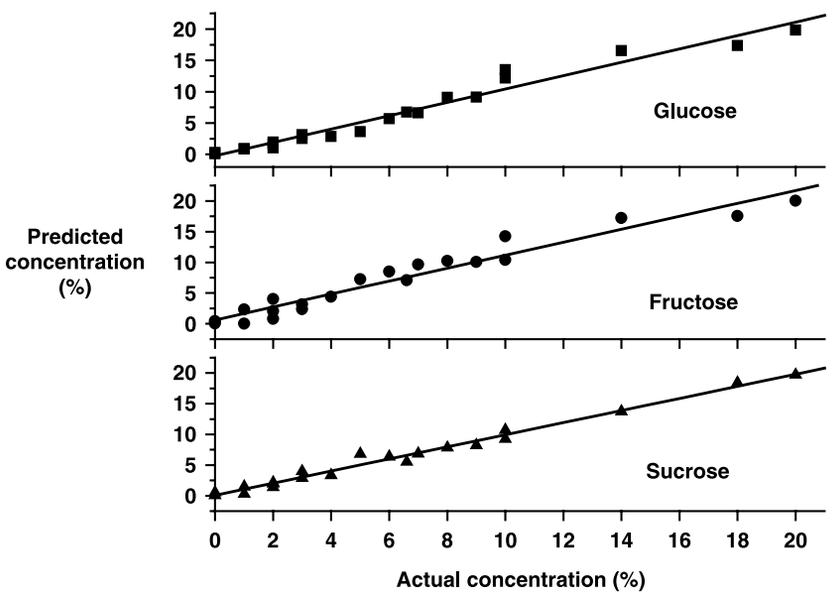

Figure 5. Correlation between the actual contents of glucose, fructose and sucrose and those predicted by the chemometric model.

ability to predict the total sugar content of 80 synthetic standards of varying concentration of sugar. A high correlation coefficient (0.965) between the actual and the predicted concentration was obtained (Fig. 4). The correlation of the model showed that the level of detection with high accuracy was near to 1 ***g*** of sugar/100 mL which is appropriate for the measurement of the typical sugar content in real nectar samples (Bernardello *et al.*, 1999). Additionally, standard sugar mixtures, assayed by chromatographic and refractometric methods, were used to validate the prediction of the model for each sugar separately. Figure 5 shows the correlation between actual and predicted concentrations for glucose, fructose and sucrose producing correlation values of 0.982, 0.972 and 0.992, respectively.

The developed method was applied to predict the total sugar content and the proportions of glucose, fructose and sucrose in 28 samples of nectar obtained from *Pachycereus pecten-aboriginum* and *Ceiba parvifolia.* Table 1 shows the predicted proportions for each sugar and the difference between the actual concentrations and the predicted values for total carbohydrates. From these results it is possible to observe that the minimum and maximum values of total sugar were registered in nectar samples from *C. parvifolia* [13.14 and 25.41 (w/v), respectively]. In addition, the results showed that *P. pecten-aboriginum* produces a nectar rich in hexose monosaccharides, whilst *Ceiba parvifolia* produces a sucrose-rich nectar.

Even though the results of the analysis of solutions of varying concentrations of sugars showed a satisfactory correlation between actual carbohydrate content and the predicted value, negative values obtained with real samples of nectar indicated that the model has some inconsistencies. These may be occasioned by the content of some additional metabolites present in the sample, or to fermentation of the nectar which might modify the matrix of the solution and produce artefacts. However, the possibility of using MIR as an easy, cheap and non-destructive technique for obtaining an

Table 1. Total sugar and proportions of glucose, fructose and sucrose in samples of nectar from *Pachycereus pecten-aborigimum* and *Ceiba parvifolia* as predicted using the chemometric model

	Predicted sugar composition			Total carbohydrates		
Nectar sample	Glucose (w/v)	Fructose (w/v)	Sucrose (w/v)	Actual[a] (w/v)	Predicted (w/v)	Difference (%)
Pachycereus pecten-aborigimum						
1	10.66	11.17	−1.71	19.51	20.20	3.52
2	7.34	6.36	6.37	19.63	20.20	2.89
3	7.81	7.33	2.96	18.12	18.30	0.98
4	3.41	6.62	8.01	17.93	18.30	2.09
5	8.21	6.81	2.99	17.90	18.30	2.21
6	7.60	4.47	7.77	20.16	20.20	0.20
7	6.31	5.95	2.84	14.95	15.40	3.00
8	8.89	8.23	2.61	19.50	19.84	1.76
9	7.94	4.95	4.87	17.79	17.90	0.63
10	9.27	5.32	5.13	19.78	19.84	0.32
11	7.53	7.48	2.77	17.37	17.90	3.03
12	9.49	8.78	1.24	19.56	19.70	0.71
13	7.17	6.47	1.26	14.92	14.99	0.49
14	9.67	7.73	−2.04	15.12	15.44	2.14
Ceiba parvifolia						
1	11.50	1.09	7.36	19.65	20.00	1.79
2	5.99	1.76	11.38	18.73	19.16	2.32
3	0.49	8.43	8.98	17.37	17.91	3.11
4	1.92	3.56	7.63	12.77	13.14	2.91
5	4.69	4.67	10.55	19.79	20.00	1.05
6	2.07	5.81	14.57	21.55	22.50	4.42
7	0.98	4.58	14.40	19.44	20.00	2.86
8	2.23	8.24	10.96	21.08	21.55	2.21
9	1.81	8.08	15.38	24.90	25.41	2.03
10	−0.16	4.20	10.87	14.72	15.00	1.88
11	1.61	6.61	15.76	23.46	24.16	2.99
12	−0.51	4.29	11.09	14.71	15.00	1.94
13	−1.41	4.99	14.16	17.30	17.91	3.55
14	−0.58	0.94	13.63	13.97	14.16	1.34

[a] Values obtained using a refractometric method.

accurate analysis of the total sugar content, and the proportions of glucose, fructose and sucrose separately, in a high number of floral nectar samples has been demonstrated.

Acknowledgements

The authors wish to thank Dr. Alfonso Valiente for the generous donation of the nectar samples. The research was supported by grant DGAPA, UNAM, IN203598.

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