High-Performace Liquid Chromatographic Determination of Mono- and Oligosaccharides in Vegetables with Evaporative Light-Scattering Detection and Refractive Index Detection

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Abstract

Two high-performance liquid chromatographic methods are compared for the determination of mono- and oligosaccharides in vegetables using an NH_2 column and acetonitrile–water as the mobile phase. One method uses a gradient elution and evaporative light-scattering detection; method precision (relative standard deviation) ranges from 1.09 to 4.44%, and detection limits range from 0.014 to 0.061 mg/mL. The second method uses isocratic elution and refractive index detection; method precision (relative standard deviation) ranges from 1.16 to 4.34%, and detection limits range from 0.013 to 0.022 mg/mL. When samples are analyzed by both methods, the difference between the two mean values is not statistically significant.

Introduction

After water, carbohydrates form the second most abundant component of plants (1). Human dietary intake of these carbohydrates is important due to their diverse biological roles, which vary according to structure. Among carbohydrates of low molecular weight, digestible sugars are primarily energy sources but are also implicated in fat metabolism and, owing to their sweet taste, help to make food more palatable (2). Raffinose oligosaccharides have been demonstrated to induce flatus (3). Present in the seeds of legumes, they escape digestion and absorption in the upper digestive tract and instead are fermented by colonic bacteria to yield flatus gases, primarily H_2 and CO_2.

Determination of sugars can be carried out using volumetric (4,5) and enzymatic (6,7) methods, but they have the disadvantage of not being able to determine simultaneously and individually all the sugars present in a sample.

Chromatography has been shown to be a useful technique. Determination of carbohydrates by chromatography can be done in several ways, including thin-layer chromatography (8,9), ion chromatography (10), supercritical fluid chromatography (11), gas chromatography (12,13), and high-performance liquid chromatography (HPLC) (14,15), which probably gives the best results for the determination of sugars.

Most of the HPLC methods reported in the literature on the determination of mono- and oligosaccharides use an amino column and acetonitrile–water as the mobile phase with refractive index detection. Under these experimental conditions, Martin-Villa et al. (16) determined soluble sugars in raw and cooked vegetables using a ratio of 75:25 (v/v) acetonitrile–water. Knudsen (17) studied the composition of oligosaccharides in leguminous seeds using acetonitrile–water 65:35 (v/v). Van Den et al. (18) analyzed mono- and oligosaccharides in raw and cooked sweet potatoes using acetonitrile–water 72:28 (v/v) for mono-, di-, and trisaccharides and 60:40 (v/v) for the analysis of tetra- and pentasaccharides.

Recently evaporative light-scattering detection (ELSD) has become important in sugar analysis by HPLC, mainly because it allows the use of gradient elution. Herbreteau et al. (14) developed a method to analyze oligosaccharides using amino-bonded silica gel and a ternary eluent with ELSD.

The objective of this study was the comparison of two detection systems: ELSD and refractive index detection (RID) under the same experimental conditions for the determination of mono- and oligosaccharides in food vegetables by HPLC.

Table I. Method Precision Calculated for Each Sugar with Both Detectors using Pepper and Pea Samples for Monosaccharides and Oligosaccharides, Respectively

Sugar	ELSD RSD* (%)	RID RSD* (%)
Fructose	1.59	1.16
Glucose	2.02	1.48
Sucrose	1.09	2.82
Maltose	4.44	3.56
Raffinose	1.88	1.19
Stachyose	3.93	4.34

* Six replicates.

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Experimental

Samples

Six types of vegetables were used for this research: green beans (*Phaseolus vulgaris* L.), peppers (*Capsicum annuum* L.), peas (*Pisum sativum* L.), lentils (*Lens esculenta* L.), chickpeas (*Cicer arietinum* L.), and kidney beans (*Vicia faba* L.). They were purchased from a local market in Santiago de Compostela (Spain) in different states: fresh (green beans and peppers), frozen (peas), and dried (lentils, chickpeas, and kidney beans).

Table II. Detection Limits Calculated for Each Sugar with Both Detectors

Sugar	ELSD (mg/mL)	RID (mg/mL)
Fructose	0.015	0.022
Glucose	0.014	0.013
Sucrose	0.018	0.016
Maltose	0.016	0.019
Raffinose	0.047	0.015
Stachyose	0.061	0.015

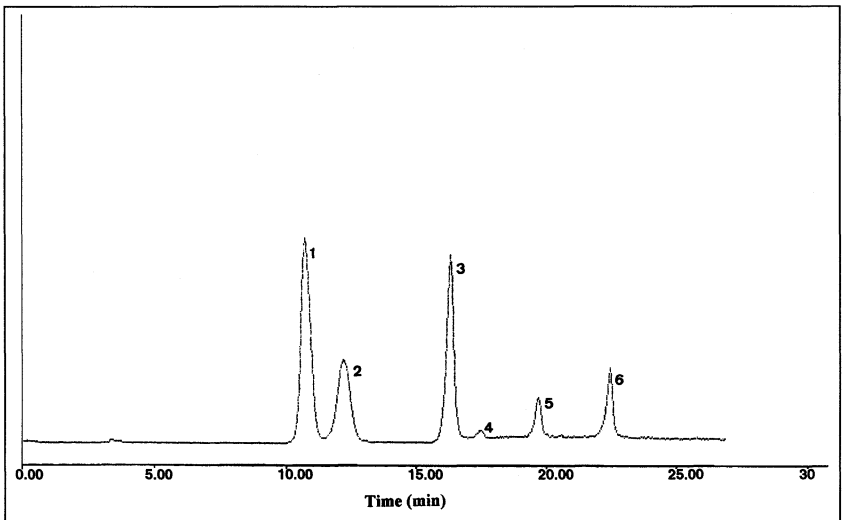

Figure 1. Chromatogram of a sugar standard using ELSD. Peaks: 1, fructose; 2, glucose; 3, sucrose; 4, maltose; 5, raffinose; 6, stachyose.

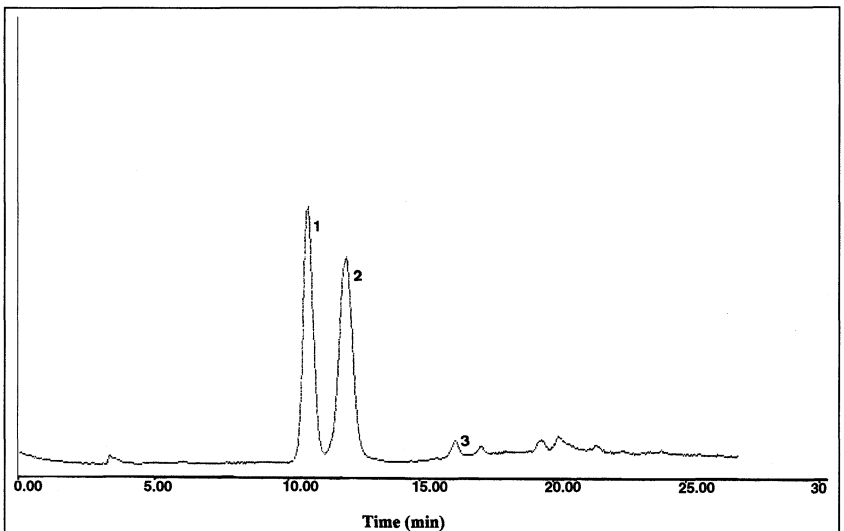

Figure 2. Chromatogram of a pepper sample using ELSD. Peaks: 1, fructose; 2, glucose; 3, sucrose.

Reagents

Fructose, glucose, and sucrose standards were from Merck (La Coruña, Spain), and maltose, raffinose, and stachyose were from Sigma (Madrid, Spain). Analytical-grade ethanol and HPLC-grade acetonitrile were from Scharlau (Barcelona, Spain).

HPLC apparatus

The HPLC equipment consisted of a Spectra Physics (San Jose, CA) HPLC apparatus comprising an 8700 XR ternary pump, a 20-µL Rheodyne (Cotati, CA) injection loop, an SP8792 column heater, and a 4290 integrator linked via Labnet to a computer running Winner 8086 software (Spectra Physics, operating system, MS.DOS 3.2). For separation, a 250 × 4.6-mm column packed with 5-µm Spherisorb NH_2 (Sugelabor, Madrid, Spain) was used. The RID was a Shodex (Showa Denko, KK) RI-71 model, and the ELSD was a ELSD II A Varex (Maryland).

Sample preparation

Samples (5–30 g, depending on the sugar content) were extracted by refluxing for 30 min with 80 mL of 70% ethanol. The extract was vacuum-filtered (Whatman [Maidstone, England] #541), and the filtrate filled to 100 mL with ethanol. A 5-mL aliquot of this solution was passed through a Waters Sep-Pak C_{18} column, filtered (0.45-µm pore-size membrane), and then injected into the chromatograph.

Chromatographic procedure

The method employing ELSD was carried out at ambient temperature using gradient elution of acetonitrile–water at a flow rate of 0.8 mL/min. Isocratic elution was employed for 7 min with a mixture of 78:22 (v/v) acetonitrile–water, which was followed until 12 min after loading with a gradient leading to a ratio of 60:40 (v/v) acetonitrile–water. Elution was continued isocratically with this mixture until 30 min after loading; at 35 min after loading, a new gradient led to the initial composition (78:22 [v/v] acetonitrile–water). Nitrogen (48 mm Hg) was used to nebulize the effluent coming from the column, and the evaporation temperature of the chromatographic eluent was 130°C.

The method employing RID was carried out at a constant temperature of 28°C using isocratic elution of acetonitrile–water (82:18 at a flow rate of 1.2 mL/min for monosaccharides and 68:32 at a flow rate of 0.8 mL/min for oligosaccharides).

Results and Discussion

Sample extraction

In developing the extraction process, two graduated series of ethanol (70 and 80%) and two extraction times (30 and 45 min) were tried. To determine the efficiency of the extraction of sugars, three preparations of the same homogenized sample of lentil were extracted by refluxing with 70 and 80% ethanol for 30 and 45 min. Both solutions extracted the same amounts of mono- and disaccharides, but the amounts of raffinose and stachyose extracted were higher using 70% ethanol. When the extraction time was varied, no major differences were observed in the amounts of the components extracted.

Optimization of chromatographic conditions for ELSD

In developing the method, several mobile phases as well as isocratic and gradient elution were tried. The initial isocratic mobile phase, acetonitrile–water (68:32, v/v), did not adequately resolve the peaks due to fructose and glucose. As the amount of acetonitrile in the mobile phase was increased, resolution of both sugars steadily improved. Good resolution was obtained with the acetonitrile–water (78:22, v/v) mobile phase, but it was at the expense of a longer elution time (more than 1 h). It was therefore necessary to assay several gradients of elution to allow adequate resolution of all the sugars in a reasonably short time. The gradient of elution employed is described in the chromatographic procedure in the Experimental section.

A chromatogram of a sugar standard mixture obtained using this gradient of elution and ELSD is shown in Figure 1. Figures 2 and 3 show the chromatograms for a typical pepper and pea sample, respectively, obtained under the same conditions.

Optimization of chromatographic conditions for RID

The isocratic acetonitrile–water (78:22, v/v) mobile phase employed with the ELSD to determine fructose and glucose did not adequately resolve both monosaccharides due to the deterioration of the NH_2 column, which is very susceptible to water (19). Several changes in the proportions of the mobile phase components were assayed (acetonitrile–water [80:20, 82:18, and 85:15, v/v]), and the flow rate was varied between 0.8–1.2 mL/min. The 82:18 mixture at a flow rate of 1.2 mL/min allowed good resolution of both monosaccharides in a shorter period of time.

The acetonitrile–water (60:40, v/v) eluent was employed for the analysis of the rest of the sugars to reduce analysis time (18), but it was necessary to increase the amount of acetonitrile to 68% because the peak due to the raffinose was in the tail of the extraction solvent. To improve the resolution, the ratios assayed were 68:32 and 70:30 (v/v), and the flow rates tried were 0.8, 1, and 1.2 mL/min. The best results were obtained with the mixture of acetonitrile–water (68:32) at 0.8 mL/min.

The typical chromatograms of a sugar standard mixture

Table III. Parameters of Calibration Lines* Obtained by ELSD

Sugar	Intercept (*a*)	Slope (*b*)	Correlation coefficient
Fructose	–1.36	4.7	0.9997
Glucose	–0.65	4.07	0.9997
Sucrose	–2.02	4.86	0.9996
Maltose	0.12	2.68	0.9995
Rafinose	–0.19	2.63	0.9998
Stachyose	–0.55	2.78	0.9995

* ($y = a + bx$)

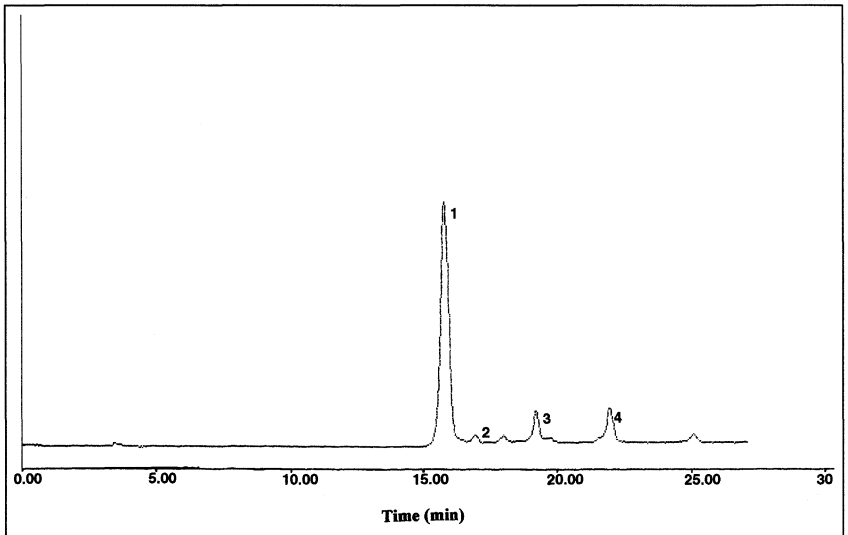

Figure 3. Chromatogram of a pea sample using ELSD showing the sugars detected. Peaks: 1, sucrose; 2, maltose; 3, raffinose; 4, stachyose.

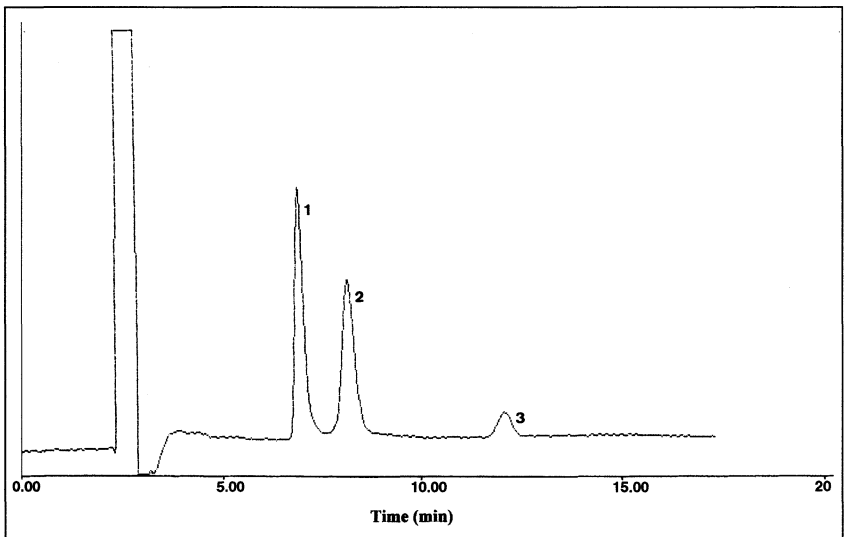

Figure 4. Chromatogram of a monosaccharide and sucrose standard using RID. Peaks: 1, fructose; 2, glucose; 3, sucrose.

obtained by the RID method are shown in Figure 4 (monosaccharides and sucrose) and Figure 5 (oligosaccharides). Figures 6 and 7 show chromatograms for a typical pepper and pea sample, respectively.

Table IV. Parameters of Calibration Lines* Obtained by RID

Sugar	Intercept (*a*)	Slope (*b*)	Correlation coefficient
Fructose	–1.70	6.77	0.9999
Glucose	–1.89	6.42	0.9999
Sucrose	–0.82	11.72	0.9999
Maltose	–0.14	10.12	0.9974
Raffinose	–0.35	10.43	0.9999
Stachyose	–0.047	10.12	0.9999

* ($y = a + bx$).

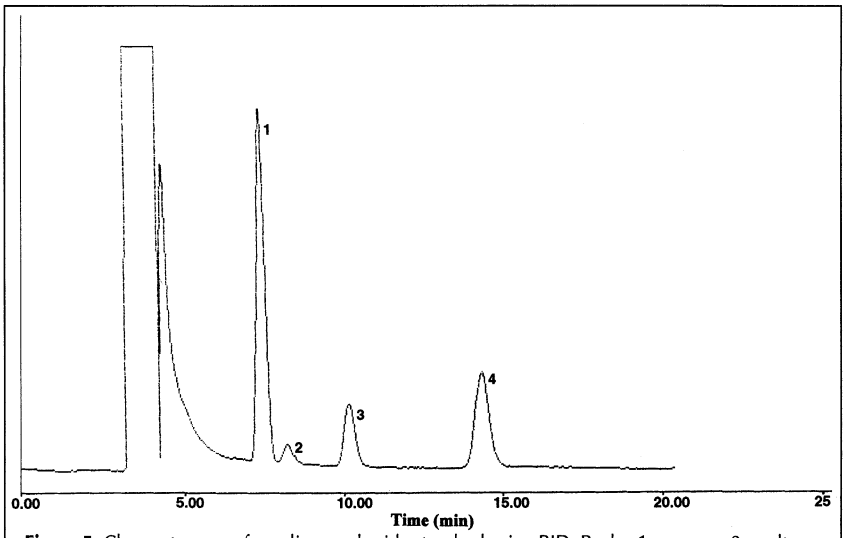

Figure 5. Chromatogram of an oligosaccharide standard using RID. Peaks: 1, sucrose; 2, maltose; 3, raffinose; 4, stachyose.

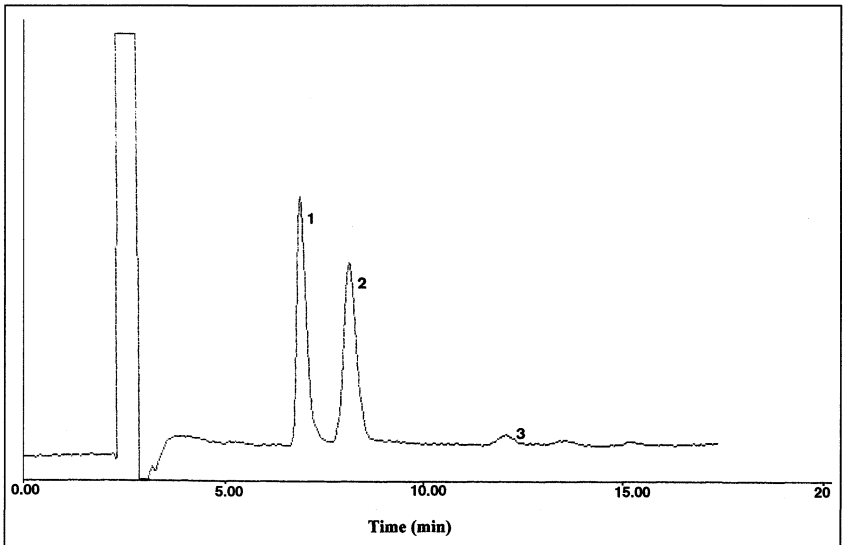

Figure 6. Chromatogram of a pepper sample using RID showing the sugars detected. 1, fructose; 2, glucose; 3, sucrose.

Comparison of both chromatographic methods

The major advantage of ELSD over RID is that it permits the use of gradient elution. Furthermore, RID is susceptible to ambient effects such as temperature and can produce negative peaks, which are difficult to quantitate.

Comparison of the chromatograms obtained for both methods indicates that the first peak appearing in the RID chromatogram (ethanol 70%) was not detected by the ELSD method. This was due to the fact that ethanol was more volatile than the mobile phase.

To determine the precision of the method for monosaccharides, six aliquots of the same homogenized pepper sample were each subjected to the complete procedure and injected in duplicate. For oligosaccharides, the same procedure was made in peas. The relative standard deviations (RSDs [%]) for both methods are given in Table I. RSDs for all sugars with both detectors were less than 5%.

Comparison of the mean values of these data using the two-sample analysis option of the Stat-Graphics package (version 2.6) indicated that there was no statistically significant difference between them ($p \leq 0.05$). The detection limit of each carbohydrate was calculated in accordance with American Chemical Society guidelines (20). Similar results were obtained with both detectors, as shown in Table II.

Both methods were calibrated using a series of sugar standards (four concentration levels in the range of analytical interest). Linear regression of the area of each sugar (y) on the concentration of the standard (x) yielded the equations given in Table III for ELSD and Table IV for RID. Correlation coefficients for these data excedeed 0.997, but RID for all the sugars except maltose gave a higher linear response than ELSD.

Recovery percentages were evaluated by spiking samples of pepper with a mixed standard, then subjecting them to the rest of the procedure, and detecting them by ELSD. Recovery percentages for all sugars were higher than 94.6%

Samples

Table V lists the mean and standard deviation of each sugar in several types of vegetables. The results were based on six preparations of identical samples for peppers and peas and three preparations for the rest of vegetables.

The soluble sugars found in green beans were fructose and glucose. These occurred at similar levels in peppers, whereas fructose was more abundant than glucose in green beans. Peppers contain sucrose, too; however, its amount was only 8% of the total sugars.

Unlike green beans and peppers, in which fructose and glucose accounted for 90–100% of the total soluble sugars, the concentrations of

Table V. Sugar Content of Several Vegetables (mg/100 g sample)

	Fructose (mean ± SD)	Glucose (mean ± SD)	Sucrose (mean ± SD)	Maltose (mean ± SD)	Raffinose (mean ± SD)	Stachyose (mean ± SD)
Pepper*	1073 ± 17.1	1269 ± 25.6	197.9 ± 2.56	undetected	undetected	undetected
Green bean†	1360 ± 19.5	410 ± 13.8	undetected	undetected	undetected	undetected
Pea*	undetected	undetected	1277 ± 13.9	85.5 ± 3.8	217.4 ± 4.1	371.2 ± 14.6
Lentil†	161.8 ± 74	77.6 ± 5.22	1110 ± 26.19	305.6 ± 30	425 ± 5.60	2330 ± 64.8
Chickpea†	80.27 ± 3.32	76.8 ± 4.89	2440 ± 103.6	undetected	475.8 ± 15.3	1869 ± 63.1
Kidney bean†	undetected	180.6 ± 6.81	3652 ± 62.13	57.24 ± 1.11	205.5 ± 3.83	1997 ± 66.5

* Six preparations of identical samples.
† Three preparations of identical samples.

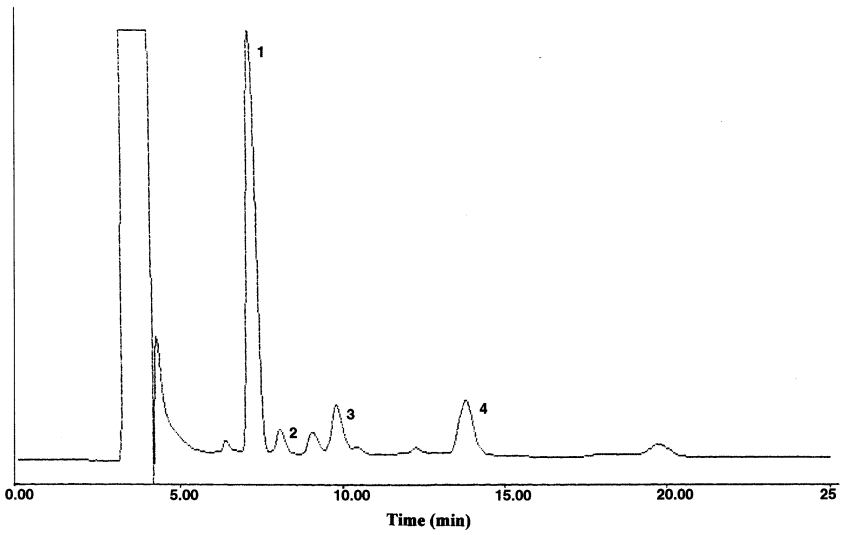

Figure 7. Chromatogram of a pea sample using RID showing the sugars detected. Peaks: 1, sucrose; 2, maltose; 3, raffinose; 4, stachyose.

both sugars in legume seeds was very low (those in peas were not detected) due to the duration of the maturity process in the conversion of sugar starch occuring in seeds. Sucrose was the most abundant sugar in the seeds, with the exception of lentils, in which stachyose was the major sugar. All seeds contained raffinose and stachyose; the amount of these oligosaccharides varied between 30 (peas) and 68% (kidney bean) of total soluble sugars.

Carbohydrates are primarily energy sources and are not customarily characterized and quantitated individually in the most widely utilized food composition tables. Generally these tables distinguish between mono- and polysaccharides and between digestible and indigestible for unspecified varieties of vegetables.

For green beans and peppers, these data are lower than those reported in the literature for digestible sugars (1,21,22). Raffinose and stachyose, as well as the carbohydrates not hydrolyzed by human digestive enzymes, belong to the group of indigestible carbohydrates. Our values of digestible carbohydrates compare well with those reported in the literature but not with those of the indigestible carbohydrates because there are many different carbohydrates in this group.

Conclusion

The reproducibility and linear regression show that both methods are suitable for the determination of mono- and oligosaccharides in vegetables. However, ELSD allowed the use of gradient elution through which all sugars were determined in a single injection. In this way, the analysis time was also reduced.

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