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ORIGINAL PAPER

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Identification and quantification of soluble sugars in green beans by HPLC

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Abstract An HPLC method has been performed and validated for soluble sugar analysis in green bean samples (*Phaseolus vulgaris* cv. Perona), with good linearity, accuracy and precision.

Monosaccharides (fructose and glucose), disaccharide (sucrose) and the polyalcohol (myo-inositol) were identified in green beans samples, as well as two unknown compounds, whose content was less than 16% of total soluble sugars. Enzymatic assays using α-glucosidases were performed in order to clarify the identity of one of the components of this fraction (identified as myo-inositol). In all the batches of green beans analysed, fructose was the major sugar (0.88–1.44%), while sucrose (0.10–0.39%) and myo-inositol (0.08–0.66%) presented higher variability on the analysed samples.

Keywords Soluble sugars · Myo-inositol · Maltose · Green beans

Introduction

Soluble sugars have an important role in the sensorial properties of vegetable products, contributing also to their nutritive value. In green beans, this fraction is constituted basically by monosaccharides (glucose, fructose) and disaccharides (sucrose).However, not many authors have studied the occurrence of myo-inositol in this vegetable, whose presence has been suggested by [1, 2], acting as a precursor for uronic acids and phytic acid (which appears in the mature seeds). In the human body the importance of this compound is basically the formation of complex lipids, and some vitamin features have also been proposed for myo-inositol [3]. Oligosaccharides from the α-galactosydes family do not appear in beans until the maturity of the seeds, although [1] reported the presence of low levels of verbascose in green beans (0.2%).

Soluble sugar analysis of vegetal foods can be performed using different analytical techniques (polarometric, colorimetric, titration, enzymatic, microbiological, electrophoretic and chromatographic methods).

In complex mixtures of sugars, chromatographic methods allow its separation, identification and quantification. Liquid chromatography (HPLC) and gas chromatography have become the most useful techniques for soluble sugars analysis in vegetables. Gas chromatography requires sugar volatilisation using trimetilsilil (TMS) derivates or less frequently alditol esters, oxime-TMS ethers, acetil-TMS or alditol-TMS. This method has the disadvantages of being time-consuming and complicated, but it provides good resolution and accuracy in sugar quantification in complex mixtures [4].

HPLC is a faster and easier technique for sugar analysis on foods, since it does not requires derivatisation and provides great accuracy. The most applied detection system is differential refractive index. Spectrophotometric detection of coloured derivatives [5], pulsed amperometric detector for anionic exchange chromatography in carbohydrates separation [6], and high performance capillary electrophoresis methods with indirect UV detection [7], have also been used for soluble sugars separation and analysis.

The main stationary phases for HPLC sugar analysis are amino silica-bonded columns and ionic exchange resins [5, 6, 8]. HPLC soluble sugar analysis, using a silica-bonded column with amino groups and refractive index detection, is a fast, simple and accurate method [9], which has been applied by several authors [10, 11, 12, 13], with good resolution and absence of interference. Oruña-Concha et al. [14] applied a similar method for frozen green beans, quantifying fructose (as a major sugar), glucose and sucrose. This methodology is also suitable for complex sugar identification and quantification, as in the case of α-galactosydes, which appear in important levels in legume seeds [15].

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This work focuses on the optimisation, evaluation and application of an HPLC method for soluble sugar fraction analysis of green beans, including enzymatic assays to identify myo-inositol in this vegetable. The analytical method was validated for linearity, accuracy and precision and applied to different batches of green bean samples to establish the soluble sugar composition of this vegetable.

Material and methods

Samples. Five batches of Spanish flat pod green beans (*Phaseolus vulgaris*, cv. Perona), harvested in January, February and March 1996, as well as January and May 1997, were selected for analysis.

Previous assays. As different extraction conditions have been used by several authors [4, 12, 16] for soluble sugars extraction on different vegetal products, previous assays for extraction solvent (ethanol 80%, methanol 80%), time and number of extractions (30, 45, 60 min) and extract deproteinisation (with 10% lead acetate) were performed in order to optimise the analytical conditions.

Enzymatic assays were performed in order to identify the unknown peak at 14.98 min. For this reason α-glucosidase activity enzymes were added to the extracts. Enzymes selected were:

Maltase (Sigma)
: Specific for maltose hydrolysis; 0.6 mg of powder (equivalent to 1 unit of maltase activity or 2.12 units of α-glucosidase activity) were added to 5 ml of each of those solutions: maltose standard (1 mg/ml), myo-inositol standard (1 mg/ml), maltose+myo-inositol standard (0.5 mg/ml each) and green bean extracts (about 0.5–1.5 mg/ml of maltose or myo-inositol), and they were incubated at 37 °C for 1 h.

Diastase (Fluka)
: Non specific for maltose, but with amylase, amyloglucosidase and pectinase activities; 10 mg of enzyme (13 amylase units) were added to 2 ml of each one of the following solutions: maltose standard (2.5 mg/l), myo-inositol standard (2.5 mg/l), a mixture of maltose+myo-inositol (1.25 mg/ml each) and green bean extract (about 0.5–1.5 mg/ml of maltose or myo-inositol); and they were incubate at 37–40°C for four days.

Procedure. The final analytical method applied was based on [12, 14] with some variations. Green bean samples were freeze-dried prior to the analysis in order to avoid their deterioration. González-Castro et al. [17] reported the stability of soluble sugars in green beans during freeze-drying process and in freeze-dried samples during several months. This effect has been shown by a comparative sugar analysis of fresh and freeze-dried green beans, with similar values in both cases. Freeze-dried samples were reduced to fine particles (diameter between 0.4 and 1.4 mm). Here 0.5 g dried and powdered green beans were extracted with 40 ml of 80% ethanol-water in a water bath with magnetic stirring at 55–60 °C3 for 45 min. The obtained extracts were vacuum filtered through glass fibre filters and extracts were evaporated by using a rotary vacuum evaporator at 40 °C. The concentrate was made up to 10 ml with distilled water and samples were passed through a previously washed (5 ml methanol followed by 5 ml water) Sep-pack C18 cartridge (Waters); 2 ml of filtrate was mixed with 8 ml acetonitrile to give a total volume of 10 ml. Samples were filtered through a 0.45-µm Millipore membrane (Millipore), and 100 µl of filtered samples were injected into the chromatographic system.

Instrumental equipment. A Waters Associates liquid chromatograph (Milford, MA, USA), equipped with isocratic 6000 A pump, U6 K injector, [[(amino propyl) methyl] silyl]-bonded amorphous silica column (µBondapack/carbohydrate analysis, 39 mm× 300 mm, particle size 10 µm), differential refractometer R401, and Waters Data Module 745 integrator was used. The mobile phase was acetonitrile:water (80:20). Operating conditions were flow rate of 0.9 ml/min and ambient temperature.

Results and discussion

Previous assays

From the solvent assays a very similar total soluble sugar content (21.11 and 21.55%) was obtained, respectively, for ethanol and methanol 80% in freeze-dried green beans, and for this reason, 80% ethanol has been chosen as extraction solvent due to its easier availability and low toxicity.

One extraction of 30 min and 45 min, as well as two extractions of 30 min each one with 80% ethanol, at 55–60 °C and magnetic stirring, gave results of 21.55%, 21.73% and 21.68% respectively for total soluble sugar content. From this assay, a simple extraction of 45 min was selected in order to minimize possible handling errors or losses during extraction, with a more complete extraction of sugars.

Several authors [5, 8] recommended extract deproteinisation when samples with a high protein content are analysed using this technique, due to the interaction between proteins and amino groups in the stationary phase. For this reason they recommended clarifying agents (1 ml of 10% lead acetate, barium hydroxide/zinc sulfate, dialysed ferroxychloride, alkaline copper sulfate, mercurous nitrate, trichloroacetic or phospho tungstic acid) to precipitate proteins in the samples, or purification through precolumns [18, 19, 20].

Freeze-dried green bean samples contain about 20% of proteins; for this reason purification through Sep-Pack (able to retain macromolecules) was performed on the extracts. However, prior to Sep-Pack purification, an additional assay was also performed, consisting of adding 0.5 ml of 10% lead acetate to the alcoholic extracts. After that, they were centrifuged at 5000 rpm (3020 g) for 10 min, filtered, and the obtained liquid was evaporated, dissolved in 10 ml of distilled water and prepared for HPLC analysis. The results for total soluble sugars with and without lead deproteinisation were 17.53% and 17.82% respectively, with similar contents for individual sugars in both cases. For this reason, a deproteinisation step has not been considered as necessary for soluble sugar analysis of freeze-dried green beans.

Sugars identification

Retention times of soluble sugar standards were compared to those of the samples. Monosaccharides fructose and glucose, and the disaccharide sucrose were identified (Fig. 1). The peak at 14.98 min could be considered as maltose or myo-inositol, since both compounds coelute in this analytical condition. As the variation of chromatographic conditions could not separate both compounds, standard additions and enzymatic assays (using

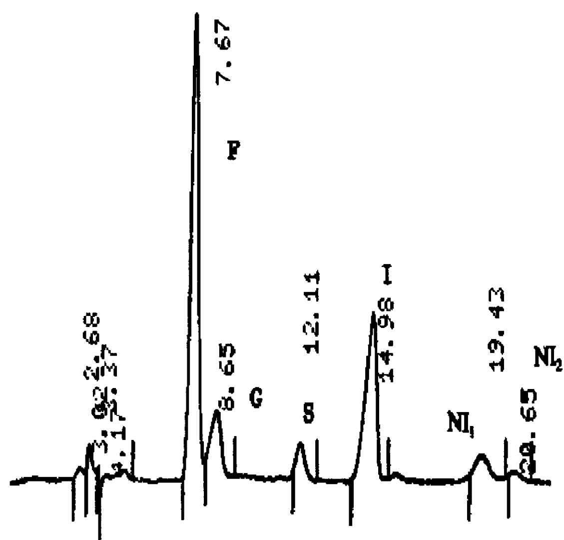

Fig. 1 Chromatographic profile of soluble sugars in green beans. F=fructose; G=glucose; S=sucrose; I=myo-inositol; NI_1, NI_2=unknown

maltase and diastase) were performed in order to identify the unknown peak. From the chemical structures of both compounds, maltose, but not myo-inositol, would be hydrolysed by these enzymes with a increase in the amount of glucose.

Maltase and diastase activities were evaluated on standards, samples and samples added with standards, in order to know if they also act in the presence of green beans extracts. The results obtained (Fig. 2) showed that both enzymes hydrolysed aqueous standards of maltase to glucose (100% hydrolysis for maltase, 95.3% hydrolysis for diastase) but not myo-inositol, and they also acted in mixtures of maltose+myo-inositol. However, diastase but not maltase showed activity hydrolysing maltose to glucose in the presence of green bean extracts (the activity of maltase could be inhibited by some compound present in them); diastase hydrolysed maltase to glucose (89.9% hydrolysis) in the extracts. None of these enzymes were active to hydrolyse the studied peak in green bean extracts. For this reason, it is concluded that, as this compound is not a substrate for maltose hydrolysis, it cannot be identified as maltose, and as it has been supported by previous studies and by standard additions, it can be identified as myo-inositol.

There were another two non-identified compounds at the end of chromatograms (19.43 and 20.65 min), named as NI_1 and NI_2, which could be di- or trisaccharides, with retention times not coincident with the standards of different soluble sugars assayed (melibiose, galactinol, maltotriose, raffinose).

Sugar quantification was performed by areas comparison, using mixtures of fructose, glucose, sucrose and myo-inositol as external standards. Non-identified peaks were quantified by using the precedent compound standard (myo-inositol), so results for NI_1 and NI_2 are expressed as myo-inositol.

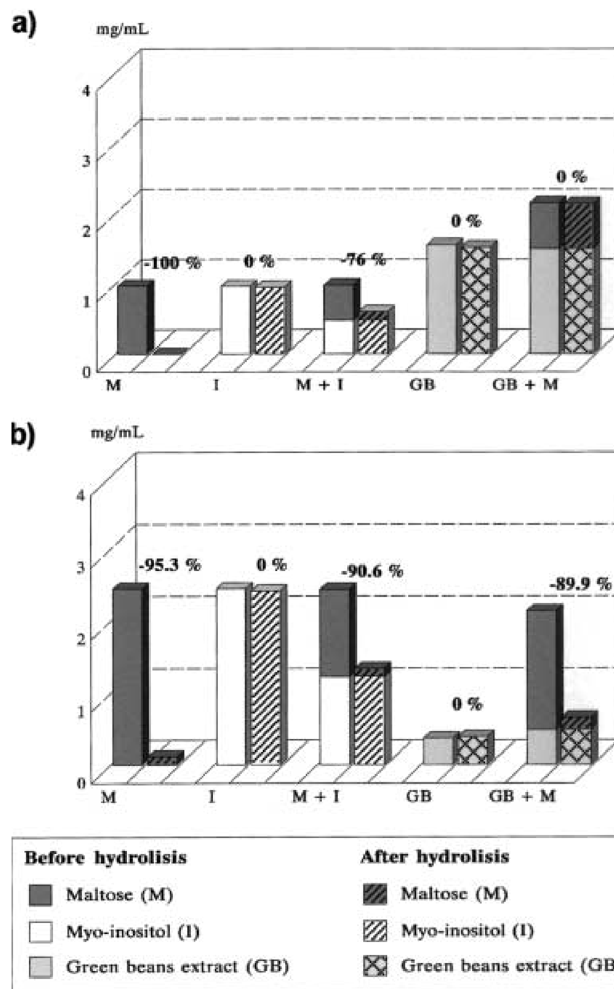

Fig. 2a, b Enzymatic assays on standards and green bean extracts (hydrolysis percents): **a** maltase; **b** diastase

Linearity

Different solutions of fructose (Merck), glucose (Merck), sucrose (Merck) and myo-inositol (Sigma) standards were prepared, in final concentrations between 0.1 and 2 mg/ml, and the relationship between peak area and concentration was evaluated after injection into the chromatographic system (Table 1). The determination coefficients (r^2) obtained were above 99.39%, with fructose and sucrose as those who showed the best linearity.

Accuracy

Accuracy of the analytical method and matrix effects in the samples was evaluated by calculating the recovery percentages obtained after the addition of different known concentrations of standards to a green bean extract. Good recovery percents (89.95–102.93%) were obtained, with the best ones for fructose and sucrose (Table 2).

Table 1 Linearity assays of the analytical method (n = 6)

Equation		Fructose	Glucose	Sucrose	Myo-inositol
		A=14,731,106 χ+61,496.3	A=13,651,251 χ–385,342	A=16,295.35 χ–47,295.6	A=4,950,200 χ+432,286.2
Regression coefficients	Correlation (r)	0.9996	0.9983	0.9997	0.9969
	Determination (% r^2)	99.918	99.660	99.940	99.390
Standard deviation	Slope	240,897.5	39,8763	199,390.7	223,855.9
	Intercept	168,895.7	349,188.7	246,489.9	324,361
Concentration range (mg/ml)		0.2–1.0	0.1–1.2	0.25–1.65	0.2–2

A=peak area
χ=concentration (mg/ml)

Table 2 Recovery assay on soluble sugars of green beans

Compound	Initial mount (mg) X±SD	Added amount (mg)	Total amount (mg) X±SD	% Recovery X±SD
Fructose	34.105±1.399	10.30	42.928±1.697	95.137±2.629
		20.60	54.936±3.387	96.255±5.934
Glucose	27.834±1.806	8.19	34.956±3.253	92.299±11.059
		16.38	44.650±1.509	93.509±3.161
Sucrose	28.755±1.703	9.99	38.455±2.281	97.095±5.759
		19.99	49.332±2.922	102.927±6.716
Myo-inositol	3.611±0.135	1.11	4.630±0.467	89.955±9.076
		3.33	6.633±0.521	92.299±9.076

X=mean (n=3)
SD=standard deviation (n–1)

Table 3 Precision assay of the analytical method (g/100 g on dry basis)

		Fructose	Glucose	Sucrose	Myo-inositol
Repeatability	X	12.909	4.400	3.950	5.699
	SD	0.527	0.311	0.283	0.292
	CV	4.086%	7.078%	7.156%	5.120%
Reproducibility	X	11.534	3.490	4.131	5.666
	SD	0.623	0.271	0.294	0.287
	CV	5.400%	7.753%	7.118%	5.062%

X=mean (n=10)
SD=standard deviation (n–1)
CV=coefficient of variation

Table 4 Soluble sugars content of green beans (% on wet basis)

Batch	Fructose X±SD	Glucose X±SD	Sucrose X±SD	Myo-inositol X±SD	NI_1 X±SD	NI_2 X±SD	TOTAL X±SD
1	0.885±0.027	0.364±0.013	0.167±0.012	0.082±0.008	0.028±0.004	0.213±0.019	1.756±0.036
2	0.904±0.067	0.440±0.042	0.291±0.016	0.159±0.035	–	0.162±0.039	1.906±0.104
3	0.975±0.020	0.464±0.002	0.151±0.005	0.124±0.043	–	0.074±0.015	1.784±0.079
4	1.443±0.014	0.351±0.039	0.395±0.008	0.293±0.020	0.082±0.014	0.353±0.057	2.916±0.045
5	1.316±0.027	0.285±0.008	0.107±0.007	0.664±0.042	0.122±0.013	0.048±0.009	2.535±1.317
Mean values	1.051±0.245	0.386±0.074	0.227±0.102	0.269±0.229	0.085±0.052	0.252±0.107	2.198±0.498
% CV	28.358	19.111	45.131	85.219	61.118	42.362	22.683

X=mean value (n=3)
SD=standard deviation (n–1)
NI_1, NI_2=non identified compounds
CV=coefficient of variation

Precision

Repeatability of the analytical method has been evaluated by the analysis of the same sample, ten times in the same day. Reproducibility was studied from ten different extractions of the same sample of freeze-dried green beans, in different days. Results (Table 3) showed similar values of repeatability and reproducibility, below 7.8%, with the best precision for fructose and myo-inositol.

Application to samples

The analytical method previously described has been applied to five different batches of Spanish flat pod

green bean samples (*Phaseolus vulgaris* cv. Perona). The composition of the soluble sugar fraction in the analysed green beans (Table 4) agree with those reported in [1, 14]. Total soluble sugar content in green beans ranged from 1.756 g/100 g to 2.916 g/100 g, with a mean value of 2.198 g/100 g on fresh basis.

This fraction included fructose as the major sugar (47.8% from the total soluble sugars), glucose, sucrose and myo-inositol. Unknown compounds were less than 16% of total sugars. There were no evidence of α-galactosides oligosaccharides in green beans, as these compounds are synthesized in the seeds during the ripening process, which agrees with [16].

Myo-inositol, sucrose and unknown compounds showed great variability between the different batches of green beans (coefficient of variation above 42%), which may be attributed not only to varietal and environmental factors, but also to maturity stage of the product, as soluble sugars are involved in respiration, synthesis and degradation processes in the vegetable, and myo-inositol content is influenced by phytic acid transformations.

Conclusions

Myo-inositol has been found in green beans. The applied HLC method allows separation and quantification of four soluble sugars (fructose, glucose, myo-inositol, together with two minor compounds) in freeze-dried samples of this vegetable, with good linearity, accuracy and precision. These sugars are always present in green beans, with fructose as the major one and the others in variable amounts.

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