# Determination of Mono-, Di-, and Oligosaccharides in Legumes by High-Performance Liquid Chromatography Using an Amino-Bonded Silica Column

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A high-performance liquid chromatography (HPLC) method for soluble sugars analysis, using an [[(aminopropyl)methyl]silyl]-bonded amorphous silica column, was applied to several dry legumes: chickpeas, lentils, white beans, pinto beans, and peas. Monosaccharide (ribose, fructose, glucose, and galactose), disaccharide (sucrose, maltose, and melibiose), and oligosaccharide (raffinose, ciceritol, and stachyose) composition of these samples was analyzed, with special interest on α-galactosides, because of their physiological role in inducing flatulent phenomena in humans after ingestion of legumes. Ciceritol (an inositol digalactoside) was the main sugar in chickpea samples and it was present also in lentils. In all the samples stachyose was found at higher levels than raffinose, and the content of these two flatulence-inducing sugars is higher in beans and lentils and lower in peas and chickpeas. However, great variability has been found in the sugar content of the different samples analyzed, probably due to genetic and environmental factors. The proposed method showed good results for the analysis of 10 simple and complex sugars in legumes, in a final time of about 40 min and in the same chromatographic run.

**Keywords:** *Soluble sugars; oligosaccharides; α-galactosides; legumes; HPLC*

## INTRODUCTION

Lentils, peas, beans, and chickpeas are widely cultivated all over the world and are considered the main legumes in human nutrition. Dry leguminous seeds are one of the main plant sources of proteins and carbohydrates. Carbohydrate fraction of legumes include monosaccharides (ribose, glucose, galactose, and fructose), disaccharides (sucrose and maltose) and oligosaccharides of the raffinose family (raffinose, stachyose, and verbascose) in which galactose is present in α-D-1,6-linkage.

α-Galactosides have important functions in the seeds of many plants. According to García and Mendoza (1992), some of these functions are being a storage and transport mechanism for carbohydrates; protecting vegetables against toxic effects of lectins and other toxins (as well as against desiccation), and acting as germination inhibitors when there is not enough water available; they also can play a role in cold acclimation of many plants.

The animal digestive system cannot degrade α-galactosides because of the lack of α-galactosidase. These oligosaccharides accumulate in the lower intestine and undergo anaerobic fermentation by bacteria with gas expulsion ($H_2$, $CO_2$, and traces of $CH_4$), causing the flatus effect and sometimes diarrhea and abdominal pain (Reddy et al., 1980; Fleming, 1981).

Quemener and Brillouet (1983) identified an α-D-digalactoside of pinitol first in chickpeas and after that in lentil and white lupin, and it was named ciceritol as originating from chickpea, *Cicer arietinum* L. This sugar was previously wrongly reported as manninotriose, and according to these authors, it seems to have no effect on inducing flatulence, perhaps due to a lower sensitivity of pinitol galactosides to hydrolysis by α-galactosidase.

Several works have been carried out on sucrose and oligosaccharides in legumes (Rao and Belavady, 1978; Fleming, 1981; Sosulski et al., 1982; Jood et al., 1985; Kuo et al., 1988; Carlsson et al., 1992; Frías et al., 1994; 1996) but only a few of them include the mono- and disaccharide fraction by its presence at a very different concentration. In these works, there are wide variations on the soluble sugar composition of different legumes, even from the same botanical species and variety. These differences can be due to genetic and environmental factors.

The analysis of legume sugars begins with extraction using hot aqueous−alcohol mixtures (methanol or ethanol). The extract is then evaporated and the residue is dissolved in water (Labaneiah and Luh, 1981; Sosulski et al., 1982; Kuo et al., 1988).

Chromatographic methods allow the quantitation of individual sugars in complex mixtures. Cerning et al. (1975), Tanaka et al. (1975), and Bianchi et al. (1984) analyzed the sugar fraction of legumes by column and thin-layer chromatography. Amuti and Pollard (1977) and Rao and Belavady (1978) used paper chromatography; but nowadays HPLC and GC are the most suitable methods for sugar analysis in food samples. HPLC does not require derivatization and has been reported as a better method for oligosaccharides, whose high molecular weight is a difficulty for GC determination (Åman, 1979; Sosulski et al., 1982; Carlsson et al.,

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**Table 1. Calibration Parameters of Sugars Analyzed**

| sugar | equation[a] | coefficients: correlation ($r$) | coefficients: determination ($r^2$) (%) | concentration range (mg/mL) |
|---|---|---|---|---|
| ribose | $y = 247\,639x - 1\,114\,790$ | 0.9987 | 99.76 | 0.128−1.280 |
| fructose | $y = 148\,051x + 550\,180$ | 0.9996 | 99.93 | 0.206−1.290 |
| glucose | $y = 133\,380x - 268\,647$ | 0.9987 | 99.74 | 0.111−1.391 |
| galactose | $y = 142\,274x - 190\,646$ | 0.9995 | 99.90 | 0.133−0.663 |
| sucrose | $y = 162\,951x - 47\,295$ | 0.9997 | 99.94 | 0.264−1.650 |
| maltose | $y = 173\,306x - 54\,221$ | 0.9967 | 99.35 | 0.098−0.612 |
| melibiose | $y = 142\,333x - 369\,046$ | 0.9952 | 99.04 | 0.300−1.000 |
| raffinose | $y = 100\,557x - 168\,331$ | 0.9985 | 99.72 | 0.084−0.421 |
| stachyose | $y = 216\,892x - 845\,563$ | 0.9990 | 99.82 | 0.139−0.871 |

[a] $y$ = peak area; $x$ = amount of sugar (micrograms).

1992). Iverson and Bueno (1981), García and Mendoza (1992), Kosson (1992), and Sims (1995) also reported HPLC as an established and preferred method for the determination of individual sugars in carbohydrate mixtures, for its accuracy and simplicity.

Reverse-phase partition chromatography is one of the major types of chromatography in carbohydrate separation, and aminoalkylated silica gels are the most frequently used as stationary phase in combination with aqueous methanol or aqueous acetonitrile as mobile phase (Honda, 1984). Authors such as Kuo et al. (1988) and Basha (1992) used a cationic exchange resin column with an aqueous eluant, and temperatures of 90 °C for sugar analysis in legumes; however, an amino bonded column with acetonitrile/water (in proportions between 70/30 and 85/15) as mobile phase is more frequently used and does not require high temperatures for the column (Quemener and Brillouet, 1983; Henderson et al., 1986; Bernabé et al., 1993; López-Hernández et al., 1994).

This work aims to establish and apply simple extraction and analytical conditions to identify and quantify by HPLC, on the same analysis, most of the ethanol−water-soluble sugars in lentils, peas, beans, and chickpeas, with special regard to α-galactosides, including ciceritol, as a recently characterized sugar, using an amino-bonded silica column.

## MATERIALS AND METHODS

**Samples.** Dry seeds of five legumes have been considered for this study: lentils (*Lens sculenta* L.) from the United States, harvested in 1994 and 1995; dry peas (*Pisum sativum* L.) from the United States, harvested in 1994; white kidney beans (*Phaseolus vulgaris* L.) from Spain, harvested in 1994 and 1995 (cv. Cannellini); pinto beans (*Phaseolus vulgaris* L.) from Spain (cv. Pinta de León) and the United States, harvested in 1995; and chickpeas (*Cicer arietinum* L.) from Spain, harvested in 1994 (cv. Castellano and Pedrosillano).

**Preparation of Samples.** The analytical method for sugar determination on legumes was derived from those applied by Labaneiah and Luh (1981) and Kuo et al. (1988) and modified by Sánchez-Mata (1996). In this study, to establish the best conditions for extraction of complex sugars from legumes, different solvents were evaluated. Murphy et al. (1972) compared 40%, 60%, and 80% ethanol for sugar extraction of legumes, obtaining a more complete extraction with the last concentration. In the present work, methanol−water 80% [used by Fleming et al. (1981), Sosulski et al. (1982), and Basha (1992)] and ethanol−water 80% [used by Bernabé et al. (1993), López-Hernández et al. (1994), and Frías et al. (1996)] have been assayed. Different extraction systems were also evaluated—reflux (Labaneiah and Luh, 1981; Frías et al., 1996) and magnetic mixer (Carlsson et al., 1992)—as well as 1−3 extractions during 30 and 45 min. Final conditions selected for our analysis were as follows: all samples were reduced to fine particles and well mixed to obtain representative samples for analysis. Triplicate subsamples of 1.5 g of powdered legumes were extracted twice with 40 mL of 80% ethanol−water in a water bath with a magnetic stirrer at 55−60 °C for 45 min. After each extraction the samples were centrifuged for 30 min at 3000 rpm (1900*g*) and the supernatants were pooled and filtered. This extract was reduced in volume by using a rotary vacuum evaporator to evaporate the ethanol. The concentrate was made up to 10 mL with distilled water. Then the samples were passed through a previously washed (5 mL of methanol followed by 5 of mL water) Sep-Pak C18 cartridge (Waters, Milford, MA); 2 mL of filtrate was mixed with 8 mL of acetonitrile to give a total volume of 10 mL. Before injection the samples were filtered through a 0.45 μm Millipore membrane (Millipore, Bedford, MA). Aliquots (250 μL) of filtered samples were injected.

**Instrumentation.** A Waters Associates liquid chromatograph (Milford, MA), equipped with a 6000A pump, a U6K injector, and a differential refractometer R401, was used. The mobile phase was acetonitrile−water (80:20). Operating conditions were flow rate, 0.9 mL/min, and ambient temperature. All chromatograms were recordered on a Waters Data Module 745 integrator. The chromatographic column used was a Waters μBondapack/carbohydrate analysis, 3.9 mm × 30 cm, filled with [[(aminopropyl)methyl]silyl]-bonded amorphous silica, particle size 10 μm.

Standard sugar (Merck, Darmstadt, Germany) solutions were prepared; 100 μL aliquots of these solutions were injected into the chromatographic system and the resulting peak areas were plotted against concentration for the calibration curve, using the external standard method (Table 1).

A sample of chickpea cv. Castellano was selected as the most representative for recovery assays, adding known amounts of standards of ribose, fructose, glucose, galactose, sucrose, maltose, raffinose, and stachyose, corresponding to the sugars found in the sample. The recovery of ciceritol has not been possible to study, due to the absence of a commercial standard available. After the addition, the sample was analyzed under the same experimental conditions.

## RESULTS AND DISCUSSION

**Linear Range and Recovery Efficiency.** The equations obtained for the calibration curve of each sugar are shown in Table 1. In every case the relationship between the concentration and the peak area obtained was linear, with determination coefficients higher than 99%.

The results of recovery assays are shown in Table 2. Sucrose and maltose showed the lowest recovery percentages, which may be due to their low solubility in alcohol. The others sugars showed recovery percentages between 93.33% and 101.45%.

**Analysis of the Samples.** The sugar profiles of the legume samples analyzed under the conditions previously described are shown in Figures 1−5. The analyses were performed considering the eleven peaks that appears between 4.11 and 38.61 min as minimum and maximun retention time that corresponds to nine

**Table 2. Recovery Percents of Sugars Added to Chickpea Samples**

| sugar | initial amount[a] (mg) | added amount (mg) | found amount[a] (mg) | % recovery |
|---|---|---|---|---|
| ribose | 0.282 ± 0.000 | 2.00 | 2.175 ± 0.036 | 101.45 |
| fructose | 3.134 ± 0.203 | 4.00 | 6.897 ± 0.097 | 93.327 |
| glucose | | 2.00 | 1.941 ± 0.041 | 97.050 |
| galactose | | 2.00 | 1.998 ± 0.636 | 99.900 |
| sucrose | 57.219 ± 0.002 | 20.00 | 53.981 ± 2.660 | 83.811 |
| maltose | 9.222 ± 0.002 | 8.12 | 15.237 ± 0.080 | 74.115 |
| raffinose | 13.032 ± 0.609 | 10.00 | 22.869 ± 2.276 | 98.364 |
| stachyose | 18.541 ± 0.030 | 20.00 | 37.543 ± 5.897 | 95.010 |

[a] Values in the column are average of three determinations ± standard deviation ($n - 1$).

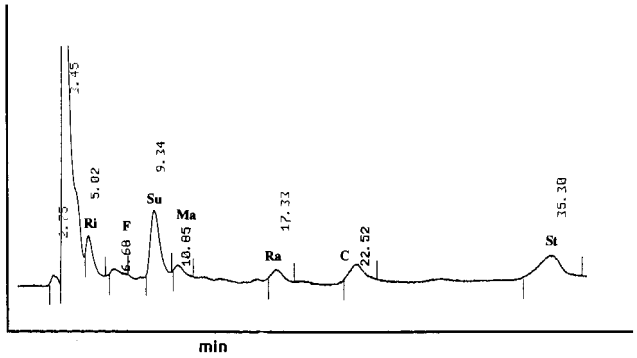


**Figure 1.** Chromatographic profile of sugars on lentil (*Lens sculenta* L.). Ri, ribose; F, fructose; Gl, glucose; Ga, galactose; Su, sucrose; Ma, maltose; Me, melibiose; Ra, raffinose; U, unknown; C, ciceritol; St, stachyose.

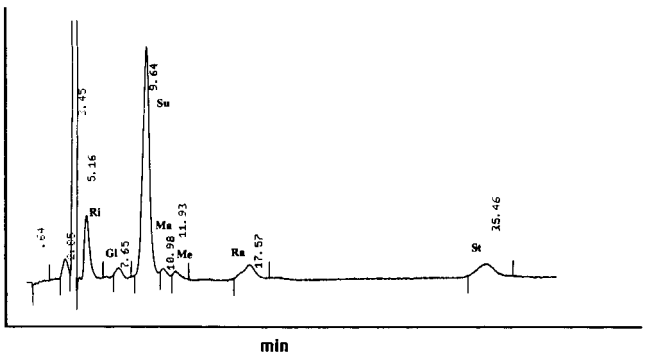


**Figure 2.** Chromatographic profile of sugars on dry pea (*Pisum sativum* L.). Abbreviations are given in the caption to Figure 1.

identified peaks (with external standards), a peak supposed to be ciceritol, and an unknown peak.

Table 3 shows the results of sugar analysis on the samples. By comparison of the HPLC sugar profiles of samples with the commercial standards available, the following sugars have been identified and quantified: monosaccharides (ribose, fructose, glucose, and galactose), disaccharides (sucrose, maltose, and melibiose), and oligosaccharides (raffinose and stachyose). The pentasaccharide verbascose reported by Phillips and Abbey (1989) in peas and lentils has not been found in any of the samples analyzed. These authors also reported its absence in beans, and Quemener and Brillouet (1983) and Díaz-Pollán (1994) did not find it in chickpea samples.

According to other authors (Quemener and Brillouet, 1983; Bernabé et al., 1993; Díaz-Pollán, 1994; Frías et al., 1994), we have considered the peak found between raffinose and stachyose as ciceritol. This sugar has been

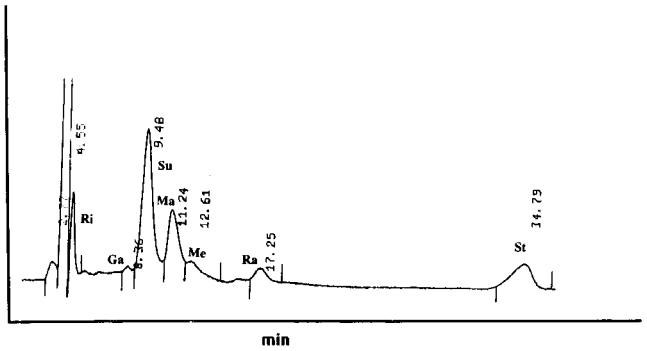


**Figure 3.** Chromatographic profile of sugars on white bean (*Phaseolus vulgaris* L. cv. Cannellini). Abbreviations are given in the caption to Figure 1.

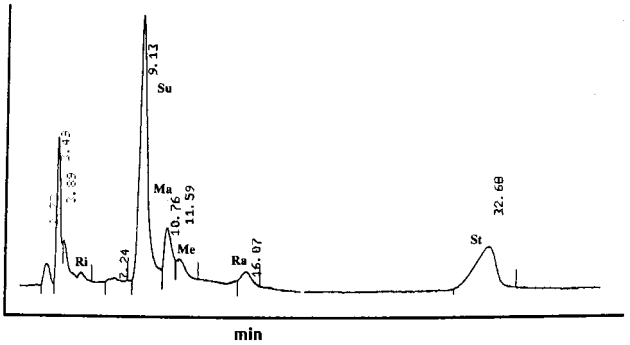


**Figure 4.** Chromatographic profile of sugars on pinto bean (*Phaseolus vulgaris* L. cv. Pinta de León). Abbreviations are given in the caption to Figure 1.

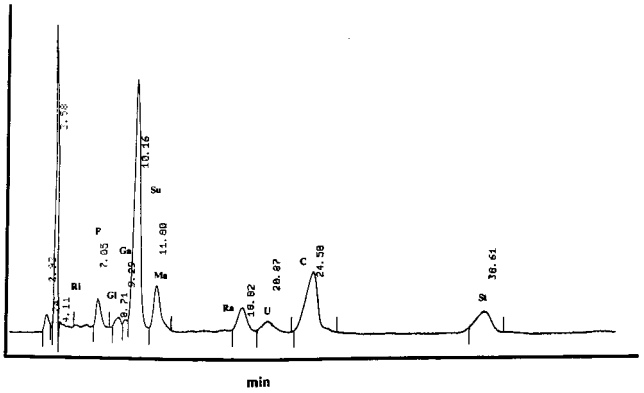


**Figure 5.** Chromatographic profile of sugars on chickpea (*Cicer arietinum* L. cv. Castellano). Abbreviations are given in the caption to Figure 1.

previously reported as manninotriose by other authors (Fleming, 1981; Sosulski et al., 1982). However, Quemener and Brillouet (1983) identified it as ciceritol by enzymatic assays and GC/MS, and Bernabé et al. (1993) confirmed this identification by NMR techniques. Vidal-Valverde et al. (1993), Frías et al. (1994), Díaz-Pollán (1994), and Frias et al. (1996) also identified this sugar in chickpeas and lentils. As these authors reported, in our samples, this sugar represents 22−29% of the total sugars of lentils and 36−43% of the total sugars of chickpeas. Because of the lack of a commercial standard for ciceritol, the quantification of this sugar has been done with the calibration curve of raffinose corrected by its molecular weight.

The total soluble sugar content of samples analyzed varied between 4.323 g/100 g for dry pea and 7.596 g/100 g for chickpea cv. Castellano. For lentils, the total

**Table 3. Mono-, Di-, and Oligosaccharides in Legumes, on Wet Basis**

| | | saccharide[a] (wet basis, g/100 g) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ribose | fructose | glucose | galactose | sucrose | maltose | melibiose | raffinose | ciceritol | stachyose | total |
| lentils | 1994 | 0.631 ± 0.176 | 0.098 ± 0.000 | tr | tr | 1.042 ± 0.403 | 0.129 ± 0.049 | tr | 0.796 ± 0.087 | 1.356 ± 0.099 | 1.639 ± 0.185 | 6.003 ± 0.575 |
| | 1995 | 0.648 ± 0.175 | 0.034 ± 0.004 | tr | tr | 1.425 ± 0.026 | 0.196 ± 0.065 | tr | 0.732 ± 0.149 | 1.819 ± 0.206 | 1.714 ± 0.080 | 6.486 ± 0.345 |
| dry peas | | 0.616 ± 0.059 | | 0.071 ± 0.021 | | 2.452 ± 0.059 | 0.114 ± 0.012 | 0.090 ± 0.000 | 0.483 ± 0.000 | | 0.640 ± 0.083 | 4.323 ± 0.252 |
| white beans | 1994 | 0.186 ± 0.092 | 0.158 ± 0.000 | | 0.072 ± 0.000 | 1.448 ± 0.121 | 0.404 ± 0.131 | 0.054 ± 0.000 | 0.270 ± 0.118 | | 1.650 ± 0.113 | 4.589 ± 0.375 |
| | 1995 | 0.048 ± 0.003 | 0.172 ± 0.005 | | 0.031 ± 0.000 | 2.009 ± 0.116 | 0.874 ± 0.121 | 0.177 ± 0.001 | 0.513 ± 0.040 | | 2.491 ± 0.204 | 6.009 ± 0.472 |
| pinto beans | USA | 0.156 ± 0.000 | | | | 2.651 ± 0.037 | 0.795 ± 0.030 | 0.093 ± 0.025 | 0.248 ± 0.043 | | 1.960 ± 0.086 | 5.736 ± 0.133 |
| | Spain | 0.301 ± 0.081 | | | | 3.487 ± 0.151 | 0.787 ± 0.063 | 0.181 ± 0.015 | 0.338 ± 0.065 | | 2.061 ± 0.095 | 7.048 ± 0.093 |
| chickpea | Castellano | 0.033 ± 0.008 | 0.231 ± 0.056 | 0.065 ± 0.020 | 0.050 ± 0.011 | 2.281 ± 0.242 | 0.568 ± 0.049 | | 0.629 ± 0.095 | 2.786 ± 0.204 | 1.171 ± 0.145 | 7.596 ± 0.485 |
| | Pedrosillano | 0.191 ± 0.041 | 0.288 ± 0.009 | | | 1.092 ± 0.077 | 0.613 ± 0.051 | | 0.569 ± 0.088 | 2.514 ± 0.277 | 0.743 ± 0.093 | 5.886 ± 0.328 |

[a] Values are averages of three determinations ± standard deviation ($n - 1$). tr = nonquantifiable levels.

soluble sugar content was more constant than the other legumes (Lineback and Ke, 1975; Díaz-Pollán, 1994).

In all of them, monosaccharides represented only 4.64−12.14% of the total sugar content of the samples, and the highest proportions were those of peas and lentils, with higher ribose content. In both of them, this pentose is the major monosaccharide, and it is the only one present in pinto bean samples. Its content as a free sugar in legumes has not been previously reported. For white beans and chickpeas, fructose showed the highest content of the monosaccharide group. Other monosaccharides, such as glucose and galactose, were minorities and have been detected in some legumes at trace levels; in other cases, they were not detected. This fact agrees with the studies of others authors such as Phillips and Abbey (1989).

Sucrose was the main sugar of peas and the two samples of pinto beans, representing, respectively, 56.72%, 46.21%, and 49.47% of their total soluble sugar content. These results agree with those of Rao and Belavady (1978), Jood et al. (1985), and Sosulski et al. (1982). According to Phillips and Abbey (1989), the presence of maltose in legumes was found. Melibiose was only quantified in peas and beans.

α-Galactosides are, from a physiological point of view, the most interesting group of the soluble sugar fraction of legumes. They have been found to be the main fraction of the sugars present in lentils, white beans, and chickpeas, representing respectively 64.45%, 45.91%, and 62.69% of their total sugar composition, and 25.97% and 36.26%, respectively, in peas and pinto beans, which content was surpassed by sucrose.

The main α-galactoside in chickpeas was ciceritol, followed by stachyose and raffinose; in lentils ciceritol appeared in a similar quantity to stachyose, and the third in importance is raffinose. In all the samples, stachyose content was higher than that of raffinose, as it has been described for leguminous seeds (Rossi et al., 1984; Kuo et al., 1988). While there is a positive correlation between flatulence phenomena in animals and stachyose and raffinose ingestion (Fleming, 1981), this property has not been showed with ciceritol, despite its α-galactoside chemical structure, and this has been atributed to its lower sensitivity to α-galactosidase (Quemener and Brillouet, 1983). This is the reason to consider these two sugars of the samples as those responsible for the flatulence phenomenon that follows the ingestion of legumes.

Similar sugar contents have been found on lentil samples from different years, and great differences between white beans harvested in consecutive years. For chickpeas, differences between cultivars were expected.

Values for free sugars in legumes analyzed agree with the range published; that confirms the viability of the method applied, which can be used to quantify all the simple and complex sugars presents in legumes on the same chromatographic run.

From this study, we can conclude that stachyose and raffinose represent a proportion of the total sugar content of the analyzed legumes that varies between 22.15% (for chickpeas) to 49.99% (for white beans). The absolute content of flatulent α-galactosides in white beans, lentils, pinto beans, chickpeas, and peas was, respectively, 2.462, 2.440, 2.303, 1.560, and 1.123 g/100 g of raw product, with clearly lower values of flatulent

carbohydrates in peas and chickpeas compared to those of beans and lentils.

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