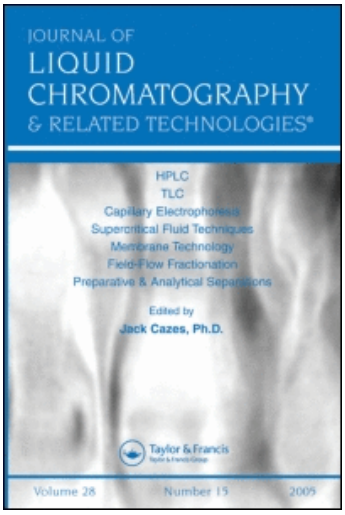

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IMPROVED METHODS OF OLIGOSACCHARIDE ANALYSIS FOR GENETIC STUDIES OF LEGUME SEEDS

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ABSTRACT

The analysis of low molecular weight carbohydrates in single seed of lentils has been carried out using two different high performance liquid chromatography (HPLC) methods. One used a reversed-phase column coupled to a refractive index detector (HPLC-RI), while the other utilised an anion-exchange phase column coupled to a triple-pulsed amperometric detector (HPAC-PAD). The latter was found to be more sensitive and could be used for the analysis of very small samples, hence allowing parts of a seed to be analysed and then grown and used for genetic studies.

INTRODUCTION

Legume seeds are an important source of protein for human and animal nutrition. Their value as a food, however, is limited by the presence of considerable amounts of α-

galactosides of the raffinose family, which have been implicated as the main cause of flatus (Calloway, 1971; Rackis, 1975; Reddy *et al.* 1984, Price *et al.* 1988). In consequence, the determination of the oligosaccharide content in pulses has been the subject of many investigations, many of which are linked to improving the quality of pulses for human nutrition (Price *et al.* 1988; Adsule, 1989).

Different methods have been proposed to remove raffinose oligosaccharides, including soaking, cooking, germinating or fermenting the seeds, or the meal, prior to incorporation into human diets (Rao and Belavady, 1978; Iyer *et al.* 1980; Reddy and Salunkhe, 1980; Jood *et al.* 1986, Kataria *et al.* 1990; Vidal-Valverde and Frias, 1992; Vidal-Valverde *et al.* 1992, 1993a,b). An alternative approach is to seek genetic variation for the α-galactosides and to use this in the development of improved varieties which have acceptable low levels of this compounds. If variants are found then it will become necessary to follow the segregation of the oligosaccharides in a breeding programme, and for this to accur it is necessary to develop semi-micro analytical methods which allow single seeds to be analysed non-destructively. In addition, it may be necessary to analyse the seed coat (testa) and the embryo of each seed separately, since the two can be genetically differents.

A number of methods have been used for the analysis of soluble carbohydrates in foods. Although they can be quantified by gas chromatography, their lack of volatility requires a time-consuming derivatization procedure (Knapp, 1971). High performance liquid chromatography (HPLC) methods, incorporating micro-particle silica-amino based columns and refractive index (RI) detection, are used widely and have given excellent results (Knudsen, 1986; Muzquiz *et al.* 1992; Arentoft and Sørensen, 1992; Vidal-Valverde *et al.* 1992; 1993a,b). Neither of these methods, however, has been carried out previously on the small amounts of sample required for single seed analysis.

It has been reported that high performance anion-exchange chromatography with pulsed amperometric detection (HPAC-PAD) will separate and quantify small amounts of sugars in a mixture of standard solutions (Rocklin and Pohl, 1983; Townsend *et al.* 1988; Wang and Zopf, 1989). Low-molecular weight sugars having pK values ranging from 12 to 14 can be separated by anion exchange, which allows their separation on a strongly basic hydroxide-form anion exchange column with highly alkaline eluents (Rendleman, 1973). HPAC-PAD equipped with a gold electrode is sensitive only to compounds containing

oxidizable functional groups including hydroxyl, amine and sulfide moieties; carboxylic acids and inorganic species do not interfere (Rocklin and Pohl, 1983).

The object of the present investigation was to assess the effectiveness of HPLC-RI and HPAC-PAD for determining the content of low-molecular weight oligosaccharides in individual lentil seeds.

MATERIALS AND METHODS

Reagents.

Standard sugars (fructose, sucrose, raffinose and stachyose) were obtained from Sigma Chemical Co. Poole, Dorset, UK.

Sample preparation.

The Syrian Local Large (SLL) genotype of lentil (*Lens culinaris*) used in this study was obtained from the John Innes Institute germplasm collection. Single seeds were separated into the testa and the embryo components. Single embryos were ground in a Glen Creston grinder (type 31-700) and the resultant flour was used to extract the sugars.

Extraction procedure.

For the extraction of the α-galactosides, mono- and disaccharides, a range of weights of flour from single embryos were suspended in 5 ml of 80% ethanol, the sample boiled under reflux for 15 min, cooled and then centrifuged at 5000 rpm. The residue was extracted twice more, and washed with distilled water until no carbohydrate was detected (Molisch's test, Pearson, 1975). The supernatants were combined, concentrated in a Buchner Vortex evaporator and the residue used for chromatographic analysis.

HPLC determination.

The HPLC-RI analysis was performed on an HPLC chromatograph (Philips Analytical) equipped with a model PU-4100 pump, a Rheodyne Model 7000 sample injector (Berkeley, Ca) fitted with a 100μl stainless steel injection loop, and a model 132 optical reflection type differential refractometer detector (Gilson Associates). The chromatographic system was controlled by an IBM PC with 715 HPLC system controller software (Gilson Associates). A pre-column (3.2 mm i.d. x 4.0 cm) packed with C_{18} Porasil B and a μBondapack/Carbohydrate column (3.9 mm i.d. x 30 cm) (Waters Associates) at 35°C were employed, with a mobile phase of acetonitrile/water (75:25) which had previously been filtered through a millipore FH (0.45 μm) membrane (Waters Associates) and degassed with helium. The flow rate was 2 ml min^{-1}.

The HPAC-PAD analysis was performed on a Dionex (Sunnyvale, CA) LC gradient pump module and model PAD-II detector equipped with a solvent-compatible electrode. Sample injection was *via* a Dionex autosampler equipped with a 25 μl sample loop. Carbohydrates were separated on a CarboPac PA-100 pellicular anion-exchange resin column (4.0 x 250 mm) and a CarboPac PA-100 guard column (3 x 25 mm) (Dionex, Sunnyvale, CA) with a flow rate of 1 ml min^{-1} at ambient temperature. The mobile phase consisted of 145 mM sodium hydroxide solution, prepared with degassed water and 50% NaOH (BDH) solution. The mobile phase was degassed to prevent absorption of carbon dioxide and subsequent production of carbonate, which would act as a displacing ion and shorten retention times. Detection was by triple-pulsed amperometry with a gold electrode. The following working pulse potentials and durations were used for detection of low molecular weight sugars: E_1 = 0.05 V (t_1 = 300 msec); E_2 = 0.60 V (t_1 = 120 msec) and E_3 = -0.60 V (t_3 = 300 msec). The CHOH secondary hydroxyl groups are oxidized at E_1, E_2 which removes the reaction products, while E_3 cleans the electrode at a negative potential (Rocklin and Pohl, 1983). The response time of the PAD was set to 1 sec and the output range was set at 10000nA. Chromatographic data were collected and plotted using Dionex AutoIon 450 software.

For both HPLC-RI and HPAC-PAD systems, quantification of each sugar was accomplished by plotting the peak areas of the sample against those of standard solutions. Commercial ciceritol and verbascose standards were not available. Verbascose was isolated

from peas (Price, 1981) and ciceritol from lentils (Bernabe et al., 1993) and their identification confirmed on TLC silica gel plates as indicated previously (Vidal-Valverde and Rojas-Hidalgo, 1976). Insufficient amounts of these oligosaccharides were prepared for use as standards for HPLC, therefore, ciceritol (trisaccharide) and verbascose (pentasaccharide) were identified by their retention time and quantified, using raffinose (trisaccharide) and stachyose (tetrasaccharide) as standards.

RESULTS AND DISCUSSION

A prerequisite for studying the inheritance of low-molecular weight carbohydrates in legumes is to optimize the conditions for analysing the minimum amount of sample. The embryo and testa can be separated from single seeds and analysed separately; a single lentil seed weighs between 20 and 100 mg, with 5-15% of this weight corresponding to the testa. High performance liquid chromatography with a refractive index detector (HPLC-RI) and high performance anion-exchange chromatography with a triple-pulsed amperometric detector (HPAC-PAD) were used to quantify the monosaccharide, fructose; the disaccharide, sucrose and the oligosaccharides of the raffinose family (raffinose, ciceritol, stachyose and verbascose) in the embryos from seeds of the lentil genotype SLL. The results from the two methods were compared to establish the optimum conditions for analysing the composition in single embryos.

Table 1 shows the retention times (t_R, capacity factor (k') and number of theoretical plates (N) of the fructose, sucrose, raffinose and stachyose detected by HPLC-RI and HPAC-PAD. Figure 1 shows the chromatograms of these sugars in a standard mixture (a) and SLL lentil embryo sample (b) analysed by HPLC-RI, whilst Figure 2 shows the standard mixture (a) and SLL lentil embryo sample (b) analyzed by the HPAC-PAD system.

A standard curve was plotted out for each available standard. The equation constant, the correlation coefficient, the concentration range and the detection limit for each sugar are illustrated in Tables 2 and 3. The correlation coefficients of all standard sugars were always greater than 0.99. The concentration range analysed and the detection limit were between 10- and 100-fold higher using the HPAC-PAD system compared with HPLC-RI. This system also showed a good detection limit for fructose, sucrose, raffinose and stachyose, again with a higher sensitivity than for HPLC-RI.

TABLE 1

Chromatographic parameters in the detection of low molecular weight sugars by HPLC-RI and HPAC-PAD[a]

	Parameters					
	HPLC-RI			HPAC-PAD		
Sugars	t_R	k'[b]	N[c]	t_R	k'	N
Fructose	2.90	1.38	1098	5.40	1.97	5184
Sucrose	5.00	3.10	1600	7.90	3.34	3994
Raffinose	9.00	6.38	2304	15.10	7.30	8635
Stachyose	16.50	12.52	3951	17.00	8.34	6400

[a] HPLC-RI: μBondapack/Carbohydrate column (3.9 x 300 mm) (Waters Associates) at 35°C; eluent acetonitrile/water (75:25). HPLC-PAD: CarboPac PA100 (4.0 x 250 mm) (Dionex); eluent, 145 mM NaOH.

[b] $k' = (t_R - t_M)/t_M$, where t_R = retention time of solute and t_M = retention time of solvent front.

[c] N, number of theoretical plates = $16\ [t_R/W]^2$, where t_R represents retention time and W represents peak width.

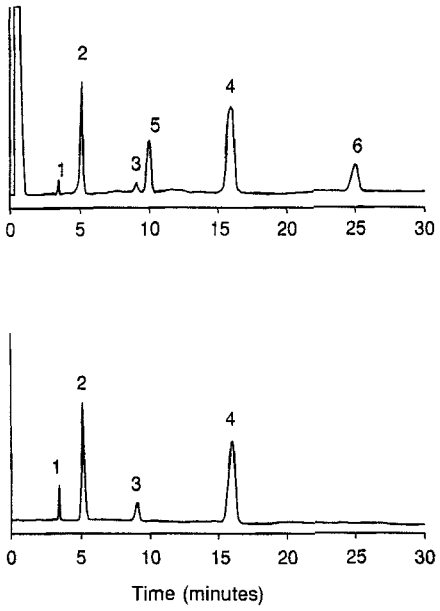

FIGURE 1.- Chromatograms obtained using HPLC-RI system: μBondapack/ Carbohydrate column (3.9 x 300 mm) (Waters Associates) at 35°C; eluent acetonitrile/water (75:25). (a) Standard solution; (b) SLL cotyledon lentil sample, where: 1.- Fructose; 2.- Sucrose; 3.- Raffinose; 4.- Stachyose; 5.- Ciceritol; 6.- Verbascose.

The amount of fructose, sucrose, raffinose, ciceritol, stachyose and verbascose in lentil embryo meal was analyzed by HPLC-RI system using different sample sizes (from 10 mg to 100 mg) (Figure 3). The results obtained showed good agreement for samples ranging between 40 mg and 100 mg. When the weight of the sample was in the range 20 mg to 40 mg the amount determined for each sugar was slightly lower, and with 10 mg samples the amount found was at the limit of the detection level. Previous reports have shown excellent resolution and quantification of low molecular weight carbohydrates in legumes by HPLC-RI techniques working with large amounts of sample. Knudsen (1986) determined the content of the raffinose oligosaccharide family in soya beans, chickpeas, garden peas and kidney beans using 10 g of sample. Arentoft and Sørensen (1992) and Muzquiz *et al.* (1992) analyzed the content of raffinose, stachyose and verbascose in pea and lupin species working with 0.5 g of initial sample, and Vidal-Valverde *et al.* (1992, 1993a) with 2.0 g and 10 g

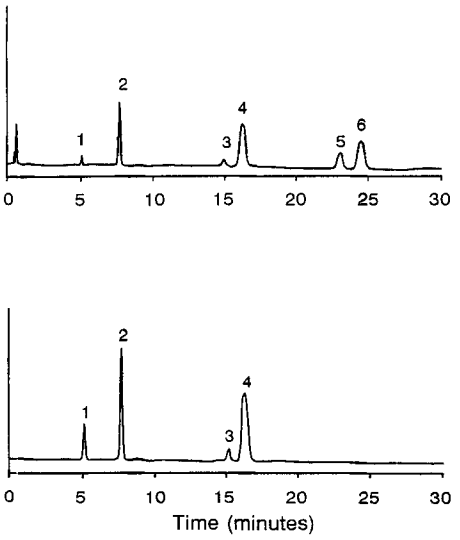

FIGURE 2.- Chromatograms obtained using HPLC-PAD system: CarboPac PA100 (4.0 x 250 mm) (Dionex); eluent, 145 mM NaOH. (a) Standard solution; (b) SLL cotyledon lentil sample, where: 1.- Fructose; 2.- Sucrose; 3.- Raffinose; 4.- Stachyose; 5.- Ciceritol; 6.- Verbascose.

samples, respectively. The conditions that we have established here, with the HPLC-RI system, allowed the determination of a very small amount of sample. In the case of fructose and verbascose their contents were quantified totally when the sample size was 20 mg. For sucrose, raffinose and ciceritol the amount of sample required was higher (30 mg), and 40 mg of lentil flour was required to accurately determine the stachyose content (Figure 3). These results, therefore, demonstrate that the limit of the amount of sample required to quantify all of the low molecular weight sugars in lentil meal using the HPLC-RI method is 40 mg.

Figure 4a and 4b show the content of monosaccharides, disaccharides and α-galactosides using high performance anion-exchange chromatography with the pulsed amperometric detector (HPAC-PAD) system. In this case, all the sugars were accurately analysed using sample sizes from 10 - 100 mg (Figure 4a). In addition, sample weights between 1 and 10 mg were also successfully analyzed and quantified using this method (Figure 4b). We are not aware of any other data regarding the analysis of soluble carbohydrates in legumes using such small amounts of sample. Several workers have

TABLE 2

Chromatographic constants of HPLC-RI system

Sugar	equation y = a + bx		r	Range (μg/mL)	Detection limit (μg/mL)
Fructose	5469	17981	0.999	4100-680	1300
Sucrose	22576	24517	0.999	50600-6325	450
Raffinose	22015	10092	0.999	5900-980	170
Stachyose	86237	10981	0.999	23900-3980	1380

TABLE 3

Chromatographic constants of HPAC-PAD system

Sugar	equation y= a + bx		r	Range (ng/mL)	Detection limit (ng/mL)
Fructose	-13607	11342	0.994	17-170	28
Sucrose	656750	4943	0.997	126-1260	138
Raffinose	-45919	4413	0.999	23-295	16
Stachyose	-235627	8517	0.996	30-230	43

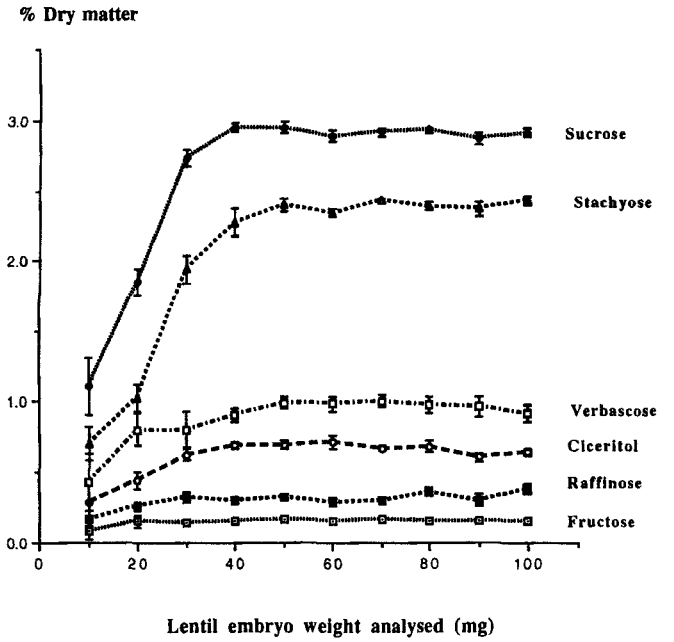

FIGURE 3.- Monosaccharide, disaccharide and α-galactoside content in g/100g dry weight of SLL lentil embryos using HPLC-RI system: μBondapack/ Carbohydrate column (3.9 x 300 mm) (Waters Associates) at 35°C; eluent acetonitrile/water (75:25). Values are the mean of four determinations and the bars record the standard deviation.

separated different sugar standards previously with an HPAC-PAD analytical system. Rocklin and Pohl (1983) separated alcohols, monosaccharides, disaccharides and other oligosaccharides such as maltose oligomers and the detection limits were found to be as low as 30 ppb for sugar alcohol and monosaccharides and about 100 ppb for maltose oligomers. They determined the lactose content of flavoured potato chips, containing a high concentration of potentially interfering salts, and found this to be about 70 ppm. Similar results were obtained by Townsend *et al.* (1988), who used the same method to separate a variety of neutral, silylated and phosphorylated oligosaccharides. Other authors for example Hardy *et al.* (1989), have analyzed different carbohydrates in biological samples and quantified at the subnanomole level neutral and amino sugars using pulsed amperometric detection. Wang and Zopf (1989), separated and quantified different lacto-N-fucopentoses in

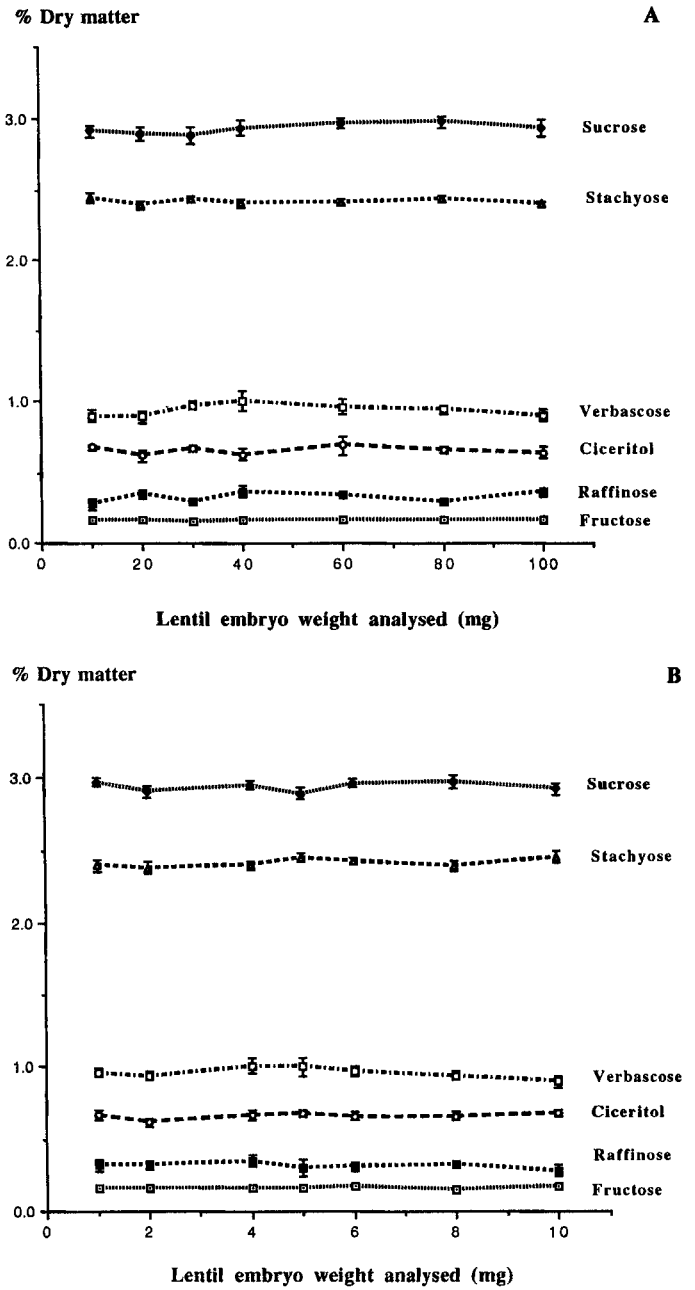

FIGURE 4.- Monosaccharide, disaccharide and α-galactoside content in g/100g dry weight of SLL lentil embryos [(a) between 10 - 100 mg; (b) between 1 - 10 mg] using HPLC-PAD system: CarboPac PA100 (4.0 x 250 mm) (Dionex); eluent, 145 mM NaOH. Values are the mean of four determinations and the bars record the standard deviation.

50 μl of human milk. The method presented here allowed us to analyse 1mg of lentil flour and to detect 8ng of fructose, 150 ng of sucrose, 34 ng of raffinose, 120 ng of ciceritol, 120 ng of stachyose and 48 ng of verbascose.

CONCLUSION

The HPLC-IR and HPAC-PAD analytical procedures described here for the quantification of low molecular weight carbohydrates allow the separation and detection of these sugars in legume seeds. This study shows that both methods are reliable when the seed size is not a limiting factor. The anion exchange column with triple-pulsed amperometric detection, however, results in a higher sensitivity for detecting carbohydrates and will allow parts of seeds, embryos and testas of lentils to be analysed individually, which is a prerequisite for genetic studies.

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