ELSEVIER

Journal of Chromatography A, 719 (1996) 213–219

JOURNAL OF CHROMATOGRAPHY A

Improved method for the analysis of α-galactosides in pea seeds by capillary zone electrophoresis Comparison with high-performance liquid chromatography–triple-pulsed amperometric detection

J. Frias[a], K.R. Price[b], G.R. Fenwick[b], C.L. Hedley[a], H. Sørensen[c], C. Vidal-Valverde[d,*]

[a]*Department of Applied Genetics, John Innes Centre, Colney Lane, NR4 7UH Norwich, UK*

[b]*Department of Food Molecular Biochemistry, Institute of Food Research, Colney Lane, NR4 7UA Norwich, UK*

[c]*Chemistry Department, Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871 Frederiksberg C., Denmark*

[d]*Instituto de Fermentaciones Industriales (C.S.I.C.), Juan de la Cierva 3, 28006 Madrid, Spain*

Abstract

A capillary zone electrophoretic (CZE) method is described for the analysis of the raffinose family of oligosaccharides (RFO) in pea seeds. Extraction of RFO was carried out in 80% ethanol and the extract was passed through a Sep-Pak C_{18} cartridge. This proved to be an improvement on the currently accepted purification technique. High-quality electrophoregrams were obtained which allowed the separation and quantitation of sucrose, raffinose, stachyose and verbascose. The CZE results were compared with those obtained by anion-exchange high-performance liquid chromatography coupled to a triple-pulsed amperometric detection (HPAC–PAD). The samples were obtained from four pea strains which were near-isogenic except for genes at the *r* and *rb* loci. A high degree of precision and reproducibility was obtained for the RFO compositions of all the pea strains. No statistically significant differences ($p \leq 0.05$) were found between the two analytical techniques using paired Student-*t* tests.

1. Introduction

The raffinose family of oligosaccharides (RFO) has been identified as a major cause of flatus in animals and humans [1,2]. These α-galactosides are widely distributed in legume seeds [3] and their physiological effects are one of the reasons why the consumption of legumes for human nutrition is limited. The RFO contributes to gastrointestinal problems because the intestinal mucosal enzymes are unable to hydrolyse the α(1→6) links between galactose residues, which are characteristic of these compounds. The RFOs pass, therefore, into the large intestine where they are fermented by intestinal bacteria, which results in the production of varying amounts of hydrogen, methane

* Corresponding author.

0021-9673/96/$15.00
SSDI 0021-9673(95)00236-7

and carbon dioxide [4]. Current culinary practices reduce RFO by soaking and cooking [5], or by germination [6] and fermentation [7].

Although the presence of RFO is detrimental to the nutritional acceptability and quality of the seed, these oligosaccharides do appear to have important functions within the plant. There is evidence that RFO is beneficial in cold acclimatization [8] and in conferring desiccation tolerance during seed maturation [9]. Both of these protective functions are believed to be the result of RFOs protecting membrane-bound proteins [10].

Genetic studies, which can be used to manipulate the content and composition of the RFO in breeding programmes, require that segregation patterns are followed using large numbers of single seeds, or, preferably, a small portion of one single seed, which will allow the seed to grow and produce progeny. The development of quick, reliable methods for the preparation of small amounts of seed samples is, therefore, essential.

High-performance liquid chromatography, using an anion-exchange phase column coupled to a triple-pulsed amperometric detector (HPAC–PAD), has been suggested as a simple and accurate tool for the analysis of RFO in single seeds [11]. More recently, capillary zone electrophoresis (CZE) in a borate buffer with UV detection at 195 nm has been shown to be a reproducible and accurate method for both qualitative and quantitative analysis of RFO [12]. Existing methods of sample purification, however, comprising isolation and group separation of RFO prior to CZE analysis [12], are too time-consuming for the large numbers of analyses of single seed samples required for genetic studies.

The aim of the present study was to compare HPAC–PAD and CZE for the analysis of RFO. In addition, a method of sample purification based on the use of a commercial Sep-Pak C_{18} cartridge is presented. The potential of these techniques to provide a simple and quick method for the semi-micro analysis of RFO in large numbers of single seeds is discussed.

2. Experimental

2.1. Samples

Dry mature pea seeds from a set of four strains, near-isogenic except for genes of the *r* and *rb* loci [13], were used for this study.

2.2. Chemicals and solvents

All chemicals and solvents were analytical-grade reagents and water was purified using a Milli-Q system (Waters Chromatography Division, Millipore Corporation, Milford, MA, USA). Sucrose, raffinose, stachyose and lactose were purchased from Sigma (Poole, Dorset, UK) and verbascose isolated from pea seeds (*Pisum sativum* var. filby), as described by Price [14].

2.3. Sample preparation

Embryos from mutant pea seeds were ground in a Glen Creston grinder (type 31-700) for 5 min and the resultant flour was used for extraction.

2.4. Extraction procedure

The samples (50 mg) were suspended in 5 ml of 80% ethanol, boiled under reflux for 15 min, cooled and then centrifuged at 3000 *g*. The residue was extracted twice more, and washed with distilled water until no carbohydrate was detected (Molisch's test [15]). The supernatants were combined, concentrated in a Buchner vortex evaporator and the sugar residue obtained. The residues were diluted in water for purification and RFO analysis. The samples were purified through a Sep-Pak C_{18} μBondapak cartridge (Waters), that had previously been conditioned with 2 ml of pure methanol and 5 ml of water. Sample extractions were performed in duplicate.

2.5. Apparatus

The HPAC–PAD analysis was performed with a Dionex (Sunnyvale, CA, USA) LC gradient pump module and a Model PAD-II detector equipped with a solvent-compatible electrode as described by Frias et al. [11]. Sample injection was via a Dionex autosampler equipped with a 25-μl sample loop. Carbohydrates were separated on a CarboPak PA-100 pellicular anion-exchange resin column (250 × 4.0 mm I.D.) and a CarboPak PA-100 guard column (25 × 3 mm I.D.) (Dionex) at a flow-rate of 1 ml/min and ambient temperature. The mobile phase consisted of 145 m*M* sodium hydroxide solution, prepared with Milli-Q water and 50% NaOH (BDH) solution. Chromatographic data were collected and plotted using Dionex AutoIon 450 software. Quantitation of each sugar was accomplished by plotting the peak areas of the samples against those of standard solutions. Recoveries of sucrose, raffinose, stachyose and verbascose from the standard solutions, obtained by comparing the stock standard solution before and after passage through the Sep-Pak C_{18} μBondapak cartridge, were 97–103%.

For CZE, a P/ACE 2210 Series capillary electrophoresis system (Beckman Instruments, Bucks, UK) was used, with a 50 cm × 50 μm I.D. fused-silica capillary. Samples were introduced from the anodic end of the capillary by 4 s vacuum injection. Detection, 43.2 cm from the injection end, was by on-column UV detection at 200 nm, and a rise time of 2.0 s. Before each run the capillary was washed with 1 *M* NaOH for 4 min followed by washing with buffer for 6 min. Data were processed with a Beckman Gold System Software Chromatopac (Beckman Instruments). Operating conditions included a temperature of 50°C and an applied field strength of 10 kV. The CZE technique adopted was based on the use of disodium tetraborate buffer (Sigma, Poole, Dorset, UK) at a concentration of 100 m*M* in water, adjusted to pH 9.9 [9]. Quantitation of each sugar was accomplished by plotting the normalized peak areas obtained from the sample against those obtained from the standard solutions. Lactose was used as a reference peak with the software normalizing the times during subsequent runs to allow for migration time variation.

3. Results

High-resolution CZE electrophoregrams were obtained when Sep-Pak C_{18} μBondapak cartridges were used for the purification of the mature pea-seed samples (Fig. 1). HPAC–PAD chromatograms obtained using this procedure were also well-resolved (Fig. 2), as previously described [11].

The samples for analysis by HPAC–PAD were diluted 200-fold compared with the CZE samples, mainly because of the high sensitivity and selectivity of the PAD detector to the sugars [16]. Calibration curves for each RFO and sucrose obtained by HPAC–PAD and CZE were plotted, and the correlation coefficients for all sugars were always higher than 0.99, showing good linearity for both methods (Tables 1 and 2, respectively).

The correlation between HPAC–PAD and CZE for the determination of sucrose, raffinose, stachyose and verbascose is shown in Fig. 3. In the figure, all points are very close to the line representing a correlation of 1, which indicates that the accuracy in the determination of these sugars is good for both techniques.

Quantitation of sucrose, raffinose, stachyose and verbascose for the four pea strains was carried out using both HPAC–PAD and CZE (Table 3). The Student-*t* test was applied to each of the carbohydrates on a paired basis. No significant differences between the methods for the analysis of sucrose and the α-galactosides were observed ($p \leq 0.05$). In addition, when the ratio %RFO in the %total soluble carbohydrates was statistically analysed, no significant differences were obtained ($p \leq 0.05$), confirming a high degree of precision for both techniques.

Differences in the soluble carbohydrate content were found for the pea strains studied, mainly due to variation in individual RFOs.

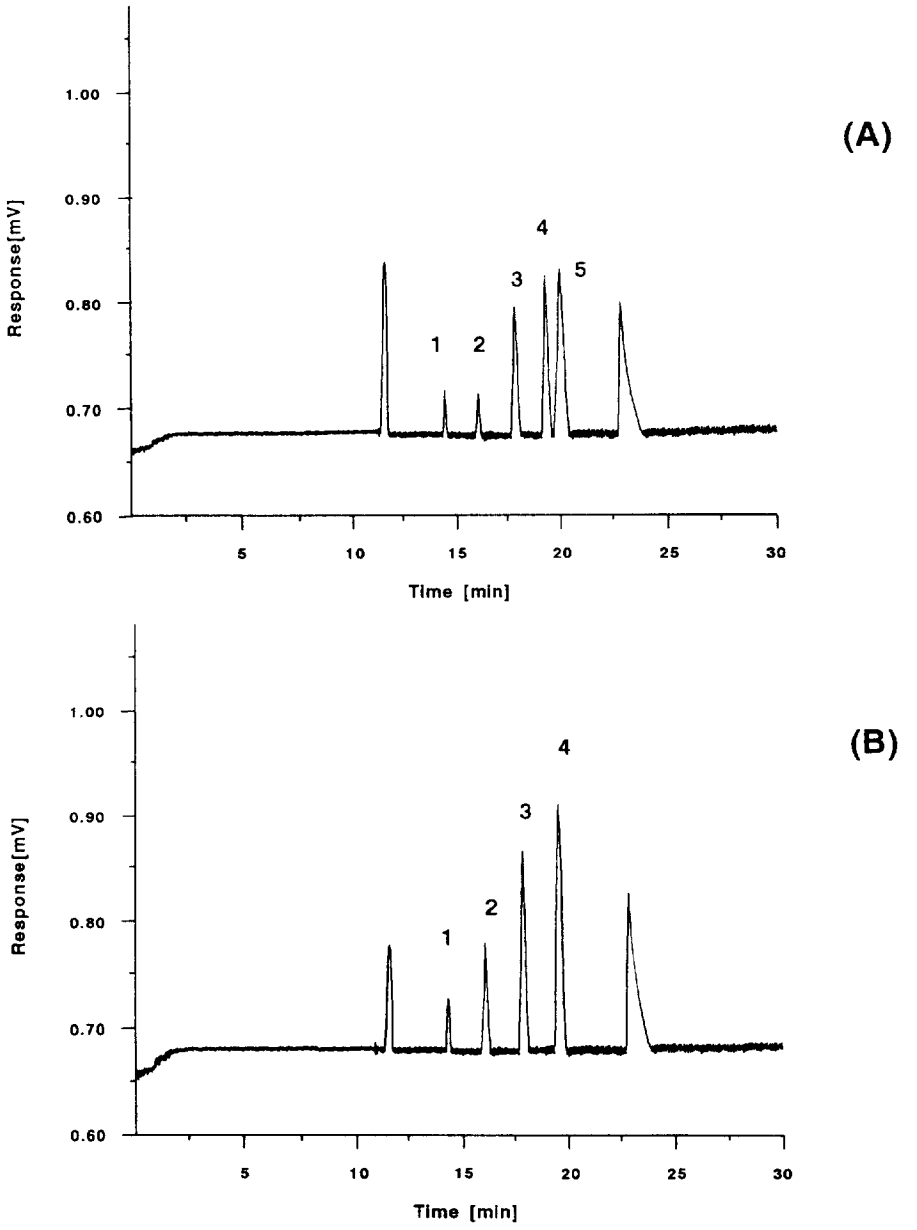

Fig. 1. Electrophoregrams obtained using CZE: total capillary length 50 cm; detection at 432 mm from the injection end at a wavelength of 200 nm; 4-s vacuum injection; buffer consisting of 100 m*M* sodium tetraborate adjusted to pH 9.9; temperature 50°C; voltage 10 kV; current 98 μA; (A) standard solution; (B) RR RbRb pea seed sample; peaks: 1 = sucrose; 2 = raffinose; 3 = stachyose; 4 = verbascose; 5 = lactose.

Verbascose was present in the highest concentration in all of the strains, ranging from 2% to 4.2%, followed by stachyose, which ranged from 1.5% to 3.5%. The lowest levels were found for raffinose (0.5–1.5%). Sucrose was also present at high concentrations (1.5–3.5%) in all samples. In determination of the ratios of RFO to total soluble carbohydrates for each strain, HPAC–PAD and CZE also gave very similar results. It may be stated, therefore, that, in the context of

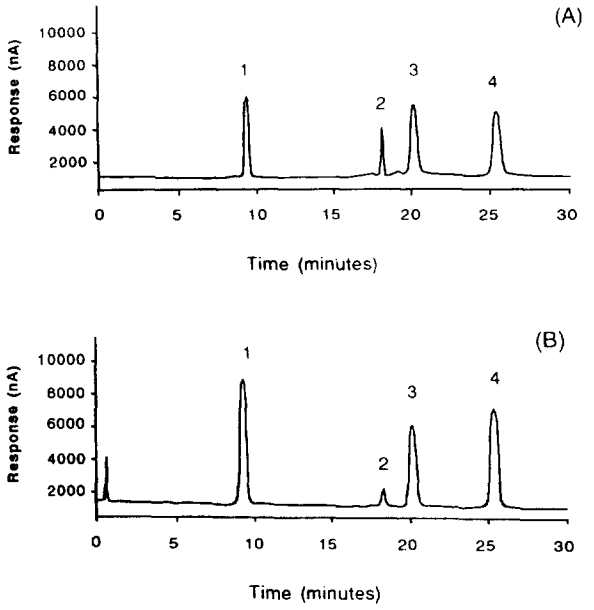

Fig. 2. Chromatograms obtained using HPAC–PAD: CarboPak PA 100 (250 × 4.0 mm I.D.); eluent 145 m*M* NaOH; (A) standard solution; (B) RR RbRb pea seed sample; peaks: 1 = sucrose; 2 = raffinose; 3 = stachyose; 4 = verbascose.

the present study, no difference in performance between HPAC–PAD and CZE was evident.

4. Discussion

HPAC–PAD and CZE are modern techniques than can be used for the analysis of soluble carbohydrates. The former has been reported to be a simple and useful tool for single seed analysis or when the amount of sample is a limiting factor [11]. CZE has been used for the analysis of RFO [12], but a purification stage involving cation–anion exchange is required in order to obtain high-resolution electrophoregrams suitable for quantitation. This sample clean-up procedure is time-consuming and is not always convenient for the analysis of large numbers of samples, such as those needed in seed development and genetic studies.

Sep-Pak C_{18} μBondapak cartridges have found widespread application for the purification of samples from different sources prior to HPLC analysis. This paper shows that sample purification Sep-Pak C_{18} μBondapak cartridges can provide a quick, easy route to obtaining not only well-resolved chromatograms but also equally

Table 1
Linearity of the method for CZE conditions: correlation coefficients (r) from linear regression analyses of normalized areas and use of different concentrations of standard carbohydrates

Compound	Migration time[a]	Concentration range (μg/ml)	r
Sucrose	14.5 ± 0.2	560–2260	0.9977
Raffinose	16.2 ± 0.5	150–610	0.9985
Stachyose	18.0 ± 0.2	300–1200	0.9993
Verbascose	19.5 ± 0.5	286–1155	0.9988
Lactose	20.2 ± 0.5	144–582	0.9963

[a] Values are the mean ± standard deviation of 10 determinations.

Table 2
Linearity of the method for HPAC–PAC conditions: correlation coefficients (r) from linear regression analyses of normalized areas and use of different concentrations of standard carbohydrates

Compound	Retention time[a]	Concentration range (ng/ml)	r
Sucrose	9.5 ± 0.5	220–4400	0.9994
Raffinose	18.0 ± 0.5	61–1220	0.9983
Stachyose	20.2 ± 0.7	100–2000	0.9980
Verbascose	25.5 ± 0.8	240–4800	0.9981

[a] Values are the mean ± standard deviation of 10 determinations.

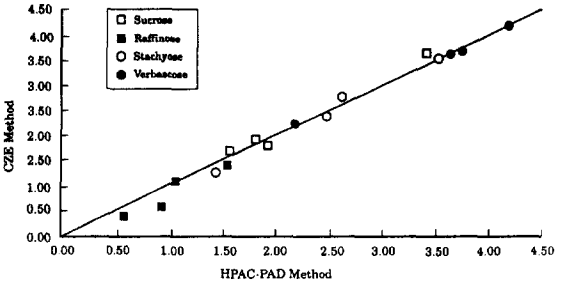

Fig. 3. Comparison between HPAC–PAD and CZE methods for the quantitation of sucrose, raffinose, stachyose and verbascose. The line represents a correlation of 1.

Table 3
Sucrose and RFO content of seeds from mature near-isogenic peas analysed by HPAC–PAD and CZE

Isoline	Sucrose	Raffinose	Stachyose	Verbascose	RFO	Total	%RFO/Total
HPAC–PAD							
RR RbRb	1.81 ± 0.03	0.56 ± 0.05	1.45 ± 0.03	2.16 ± 0.03	4.17 ± 0.05	6.02 ± 0.06	69
RR rbrb	1.89 ± 0.05	0.92 ± 0.05	2.48 ± 0.03	3.63 ± 0.05	7.03 ± 0.05	8.98 ± 0.01	78
rr RbRb	1.54 ± 0.08	1.05 ± 0.05	2.62 ± 0.08	3.74 ± 0.05	7.41 ± 0.09	9.01 ± 0.10	82
rr rbrb	3.44 ± 0.08	1.51 ± 0.08	3.54 ± 0.09	4.21 ± 0.07	9.26 ± 0.15	12.77 ± 0.22	73
CZE							
RR RbRb	1.91 ± 0.10	0.45 ± 0.07	1.32 ± 0.09	2.22 ± 0.08	4.00 ± 0.09	5.90 ± 0.14	68
RR rbrb	1.80 ± 0.03	0.96 ± 0.07	2.35 ± 0.12	3.56 ± 0.09	6.87 ± 0.10	8.67 ± 0.08	79
rr RbRb	1.66 ± 0.10	1.07 ± 0.04	2.73 ± 0.10	3.60 ± 0.13	7.39 ± 0.12	9.18 ± 0.09	81
rr rbrb	3.52 ± 0.05	1.38 ± 0.02	3.49 ± 0.06	4.08 ± 0.07	8.95 ± 0.13	12.46 ± 0.15	72

Data are expressed as percentage dry matter. Values are the mean of four determinations ± standard deviation. Pairwise t test between HPAC–PAD and CZE (degrees of freedom = 4). Sucrose $t = 5.10$; raffinose $t = -1.03$; stachyose $t = -0.88$; verbascose $t = -1.52$.

good CZE electrophoregrams from which the concentrations of the sugars in seed samples can be quantified.

Comparing the two techniques, CZE has the advantages of quicker run time, cheaper capillary and eluent cost and less mechanical parts than HPAC–PAD, although it should be remembered that time for rinsing and conditioning has to be allowed between all CZE runs. On the other hand, the UV detector, used in the CZE technique, is less sensitive, requiring concentrations of μg sugar/ml for analysis; the PAD detector, approximately 1000 × more sensitive, e.g. allows the carbohydrates to be analysed in more dilute form (ng sugar/ml).

5. Conclusions

Two modern techniques for the analysis of RFO have been compared using near-isogenic pea strains, and the following conclusions have been reached:

(1) The use of Sep-Pak C_{18} μBondapak cartridges provides quick, easy sample purification and is the key to high-quality electrophoregrams being obtained.

(2) High-performance liquid chromatography using an anion-exchange column coupled to a triple-pulsed amperometric detection (HPAC–PAD) and capillary zone electrophoresis (CZE) with a borate buffer and a UV detector set at 200 nm showed linearity and reproducibility in the detection and determination of sucrose and RFO.

Acknowledgements

The authors gratefully acknowledge support from the EC PEA ECLAIR Programme (AGRE No. 0073). J. Frias acknowledges support from the European Union through an individual bursary (AIR3-BM93-1118).

References

[1] E. Cristofaro, F. Mottu and J.J. Wuhrmann, in H.L. Sipple and K.W. McNutt (Editors), Nutrition Foundation Monograph Series, Sugar in Nutrition, International Conference, Nashville, TN, Academic Press, New York, London, 1974, p. 313.
[2] K.R. Price, J. Lewis, G.M. Wyatt and G.R. Fenwick, Nährung, 32 (1988) 609.
[3] N.R. Reddy and D.K. Salunkhe, Cereal Chem., 57 (1985) 356.
[4] J.R. Wagner, R. Becker, M.R. Gumbmann and A.C. Olson, J. Nutr., 48 (1976) 1650.
[5] C. Vidal-Valverde, J. Frias and S. Valverde, J. Food Protect., 55 (1993) 301.
[6] C. Vidal-Valverde and J. Frias, Z. Lebensm. Unters. Forsch., 194 (1992) 461.

[7] C. Vidal-Valverde, J. Frias, M. Prodanov, J. Tabera, R. Ruiz and J. Bacon, J. Agric. Food Chem., 197 (1994) 449.
[8] E.M. Castillo, B.O. de Lumen, P.S. Reyes and H.Z. de Lumen, J. Agric. Food Chem., 38 (1990) 351.
[9] S.A. Blackman, R.L. Obendorf and A.C. Leopold, Plant Physiol., 100 (1992) 225.
[10] K.A. Santarious, Planta, 113 (1973) 105.
[11] J. Frias, C.L. Hedley, K.R. Price, G.R. Fenwick and C. Vidal-Valverde, J. Liq. Chromatogr., 17 (1994) 2469.
[12] A.M. Arentoft, S. Michaelsen and H. Sørensen, J. Chromatogr. A, 652 (1993) 517.
[13] T. Wang and C.L. Hedley, Seed Sci. Res., 1 (1991) 1.
[14] K.R. Price, Chem. Ind., 3 (1986) 180.
[15] D. Pearson, in C. Livingstone (Editor), The Chemical Analysis of Food, London, 1975, p. 107.
[16] R.D. Rocklin and A. Pohl, J. Liq. Chromatogr., 6 (1983) 1577.