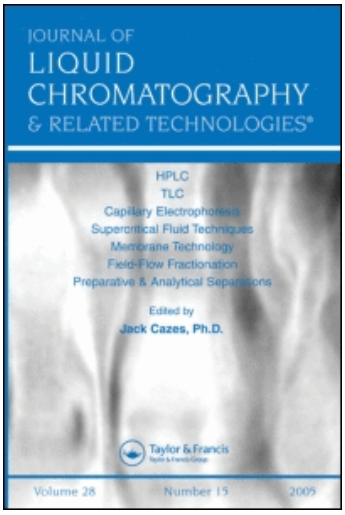

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A RAPID HPLC METHOD FOR THE DETERMINATION OF RAFFINOSE FAMILY OF OLIGOSACCHARIDES IN PEA SEEDS

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SUMMARY

Two HPLC techniques for the determination of raffinose family oligosaccharides (RFO - raffinose, stachyose and verbascose) in pea are described.. A reverse phase HPLC (RP-HPLC) on Silica C_{18} column using demineralized water as mobile phase enabled determination of the total content of RFO within less than 10 minutes. Ion Moderated Partition HPLC (IMP-HPLC) using a strong cation exchanger, in calcium form, with demineralized water as mobile phase allowed the determination of all the individual saccharides within 25 minutes. In both RP-HPLC and IMP-HPLC refractometric detection was used. The methods

were compared on a representative pea sample series, and in both techniques the lowest detectable concentration in sample solution was calculated to be 5 mg saccharide/mL, i.e., the smallest detectable amount of each saccharide was 100 ng.

INTRODUCTION

Legumes are an important input in many countries for animal and human consumption. They are an excellent source of proteins (20-40 %) and carbohydrates (50-60 %) and fairly good sources of thiamine, niacin, calcium, and iron.[1] However, per capita consumption of legumes in the US and other industrialised countries has fallen considerably in recent years.[2] This could be due to the fact that legumes have certain undesirable flavours, flatus factors, and anti-nutrients, and even toxic substances.

Flatulence is the most common symptom associated with pulse consumption, but its social implications are overshadowed by more serious accompanying consequences. Abdominal pain and diarrhoea are often experienced by susceptible individuals, especially children, causing pulses to be less readily accepted. Unfortunately those affected are often most at risk from malnutrition and pulses may be the only affordable source of good quality protein available to them. The production of flatus by monogastric animals is due to colonic fermentation of carbohydrates which escape breakdown in the stomach and small intestine. The oligosaccharides, raffinose, stachyose and verbascose, which are common in legume seeds, are thought to be the major producers of flatulence when those foods are consumed. These saccharides contain either one, two or three galactose units joined to sucrose by α - D - 1-6 linkages. Owing to the absence of an α-galactosidase enzyme capable of hydrolysing the α-1,6-galactosidic linkage, these oligosaccharides accumulate in the lower intestine and undergo anaerobic fermentation by bacteria, which may result in the production of diarrhoea, flatus gas and attendant discomfort.[3] Fibre polysaccharides and indigestible starch have also been associated with flatulence,[4] whilst the volatile fatty acids, that are produced when carbohydrates are fermented in the colon, have been suggested to have positive effects on cholesterol and carbohydrate metabolism. The decrease in faecal pH, due to production of acids, has also been suggested to be a preventive factor against the occurrence of cancer in the large bowel.[5] The benefits of oligosaccharide ingestion arise from an increased population of indigenous bifidobacteria in the colon which, by its antagonistic effect, suppresses the activity of putrefactive

bacteria and thereby reduces the formation of toxic fermentation products.[6]

Instrumental techniques for the RFO determination such as GLC, HPLC have been used.[7,8,9,10] As an alternative technique for the determinaton of such non-reducing oligosaccharides, high performance capillary electrophoresis (HPCE) has been reported[11] which is based on separation of borate complexes of the saccharides in an electric field, and has been shown to be rapid, cheap and efficient. Both chromatographic (HPAC - PAD) and electrophoretic (HPCE) techniques were compared for the RFO determination in pea[12] and the results obtained were very close. However, both techniques are expensive for those laboratories with low resources which would like to analyse these legume components in a large number of samples, for instance, in genetic studies.

The aim of this work was to develop and validate suitable analytical methods for the RFO screening in a large pea collection, i.e., hundreds of samples. The present study had two main purposes: firstly to find a rapid and relatively cheap HPLC method for RFO determination, and secondly to replace the toxic and relatively expensive mobile phase ($MeCN:H_2O$) which is commonly used in conventional IR-HPLC carbohydrate determination. The method developed will be used for the screening of pea varieties for flatulence-causing saccharides and will be a factor in:

- finding pea varieties with low RFO levels
- finding parental lines suitable for the breeding of new varieties
- searching for genetic dependence

MATERIAL AND METHODS

Samples, Chemicals and Instrumentation

Dry pea seeds were obtained from the Research Institute of Technical Crops and Legumes (Šumperk, Czech Republic). Four pea samples of different genotypes (iso-line 1 to 4) were provided from John Innes Centre (Norwich, UK). Sugar standards (stachyose, raffinose, sucrose, maltose, glucose, galactose and fructose) were obtained from SIGMA-ALDRICH, Ltd. (Prague, Czech Republic). The stainless steel (250x8 mm ID) chromatographic column with a strong cation exchanger OSTION LG KS 0803, 4.2 % DVB (Spolek pro chemickou a hutní výrobu, Czech Republic) in calcium form was filled in our laboratory. Reverse phase chromatography columns (stainless steel 250x4 mm

ID) Separon SGX RPS (high carbon loaded ODS), 7μm and Separon SGX C_{18}, 5 μm were purchased from Tessek (Prague, Czech Republic). A desalting guard column served strong cation exchanger HEMA BIO SB, 10 μm (30x3 mm ID) and strong anion exchanger HEMA BIO Q, 10 μm (30x3 mm ID) from Tessek. Other chemical products were of analytical grade.

The HPLC analysis was performed on a modular chromatograph from Thermo Separation Products (Watrex Praha, Czech Republic) consisting of isocratic pump ConstaMetric 3200, sample injector Rheodyne 7725i (20 μl loop) and differential refractometer RefractoMonitor IV. The chromatograms were evaluated with a PC system using CSW software (DataApex, Prague, Czech Republic).

HPLC Conditions

These chromatographic methods were employed for RFO determination in pea extracts:

1) RP-HPLC: An analytical column Separon SGX RPS 7 μm or Separon SGX C_{18} 5 μm with guard columns (Separon RPS or Separon C_{18}) at ambient temperature were eluted with demineralized water at a flow rate of 1 mL/min or 0.7 mL/min, respectively. The injection volume was 20 μl.

2) IMP-HPLC: Analytical column filled with strong cation exchanger OSTION LG KS 0403, 17 - 20 μm in Ca^{++} form heated to 80°C was fitted with desalting guard columns and eluted by degassed demineralized water at a flow rate of 0.4 mL/min. The injection volume was 20 μl. Refractometric (sensitivity $1x10^{-6}$ RIU/10 mV) detection was employed in both RP-HPLC and IMP-HPLC methods.

3) HPAC-PAD analysis of oligosaccharides in representative pea samples was performed on a DIONEX system as described by Frias et al.[10]

Calibration

An external standard calibration method was used. In the case of IMP-HPLC six concentration levels of sucrose, raffinose and stachyose were measured (30 - 1000 mg/l) and four levels in the case of RP-HPLC.

Coefficients of calibration equations describing the relationship between concentration of sugar and peak area were found by linear regression.

Sample Treatment

An extract of RFO from ground pea sample was obtained, using a 80% ethanol-water mixture, by two ways, i.e., boiling under reflux and by sonication.

Boiling under reflux: Two g of ground pea sample was weighed into a 50mL Erlenmeyer flask, 20 mL of 80% ethanol added and boiled under reflux for 60 minutes. After cooling the mixture was transferred into a 200mL volumetric flask and made up to volume with demineralized water. The extract was then filtrered through a membrane (0.45 μm) and analyzed by HPLC.

Sonication: One hundred mg of ground pea sample was placed in a 5 mL vial, 2 mL of 80% ethanol added, vial stoppered and placed in a sonic bath for 20 minutes. The suspension was transferred into a 25mL volumetric flask and made up to volume with demineralized water. After filtration through a membrane filter (0.45 μm) the extract was analyzed by HPLC.

RESULTS AND DISCUSSION

Column characteristics are summarised in Tables 1 and 2. Chromatograms of the model mixture of saccharides and a pea sample extract are shown in Figures 1 and 2, respectively. It is clear that using RP-HPLC the RFO were not sufficiently resolved for the saccharides, i.e., raffinose, verbascose and stachyose could not be quantified individually but only as their sum. On the other hand the IMP chromatography allows good separation of the individual α-galactosides (see Figures 1 and 2).

In spite of the higher efficiency of the RP-HPLC column Separon C_{18} compared with that of the Separon RPS column, the latter column was used for routine analyses due to a larger retention time gap between raffinose and ethanol resulting in better quantitative results of RFO. The column life of the Separon RPS was more than 100 analyses without regeneration (column rinsing with 80% ethanol overnight) and more than 500 analyses on the same column could be carried out without significant loss of column efficiency. The column life of the OSTION column was more than 200 analyses without regeneration

Table 1

Reverse Phase Column Performance

Saccharide	Retention Time (min)		Column Efficiency (N/m)		Resolution	
	Separon SGX RPS 7μm	Separon SGX C_{18}, 5μm	Separon SGX RPS, 7μm	Separon SGX C_{18}, 5μm	Separon SGX RPS, 7μm	Separon SGX C_{18}, 5μm
Glucose	2.11	3.15	3050	7600	-	-
Galactose	2.10	3.11	3080	9500	-	-
Fructose	2.17	3.23	3100	9400	-	-
Maltose	2.45	3.63	3300	11400	1.03	1.70
Sucrose	2.62	4.24	3800	13800	0.52	2.18
Stachyose	3.18	5.40	4800	14700	1..51	3.59
Raffinose	3.39	5.85	4600	15700	0.55	1.23

Table 2

Ion Moderated Partition Column Performance

Saccharide	Retention Time (min)	Column Efficiency (N/m)	Resolution
Stachyose	11.97	13600	-
Raffinose	13.25	15600	1.54
Sucrose	14.82	17100	1.79
Maltose	14.65	17300	-
Glucose	17.47	11500	2.40
Galactose	19.42	20800	1.64
Fructose	21.18	21300	1.38

(column rinsing with 0.1 M - $Ca(NO_3)_2$ overnight). Desalting guard columns withstood approximately 50 analyses, whilst more than 800 analyses are possible using the same analytical column.

The external standard calibration method was used for quantitative analysis. The calibration analyses were carried out using stachyose, raffinose and sucrose. Since the standard of verbascose was not available the qualitative analysis of verbascose was based on the published data of elution order of saccharides on cation exchanger (IMP-HPLC);[14] the higher degree of polymerization of saccharide the shorter retention time. Hence, verbascose as a pentasaccharide was assumed to elute before a tetrasaccharide, i.e., stachyose. The identity of the verbascose peak was also confirmed from previous studies.[10,12] The calibration results obtained are shown in Tables 3 and 4.

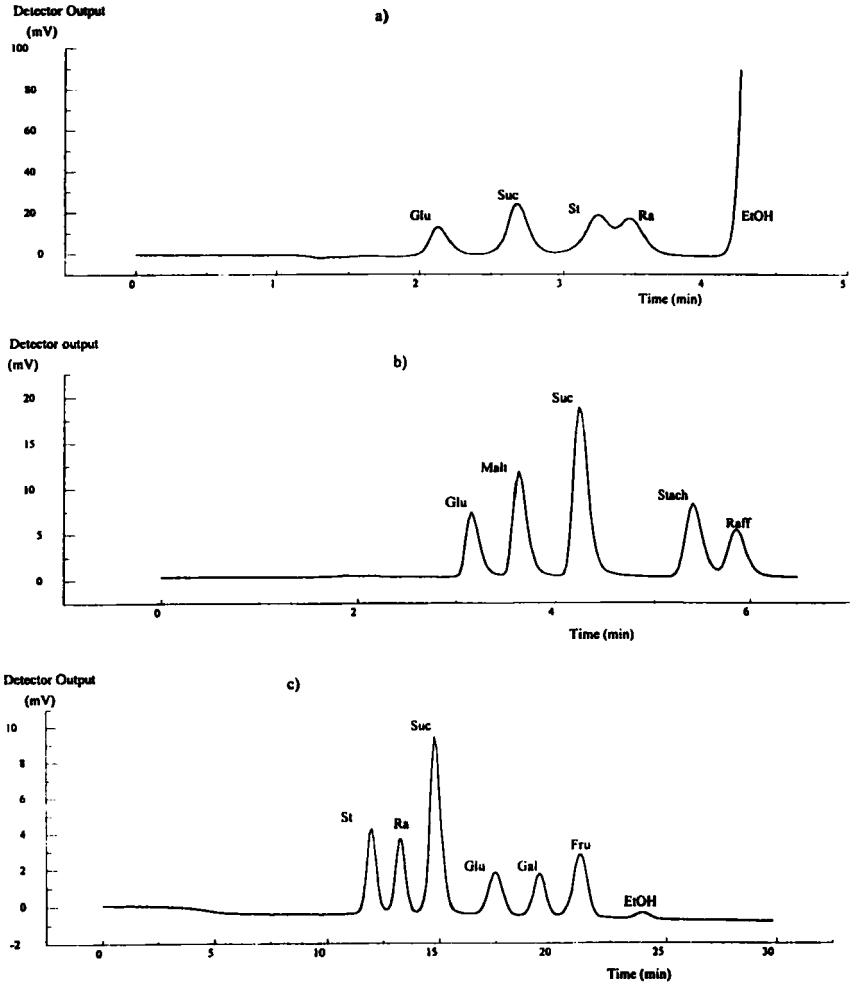

Figure 1. Chromatograms of standard mixture of saccharides; (a) RP-HPLC on Separon SGX RPS of 100 mg/mL glucose (Glu), 200 mg/mL of sucrose (Suc), 200 mg/mL of Stachyose (St) and 200 mg/mL of raffinose (Ra); (b) RP-HPLC on Separon SGX C_{18} of 104 mg/mL glucose (Glu), 93 mg/mL of maltose (Malt), 194 mg/mL of sucrose (Suc), 118 mg/mL of Stachyose (St) and 99 mg/mL of raffinose (Ra); (c) IMP-HPLC of 120 mg/mL of stachyose (St), 100 mg/mL of raffinose (Ra), 250 mg/mL of sucrose (Suc), 100 mg/mL of glucose (Glu), 100 mg/mL of galactose (Gal) and 120 mg/mL of fructose (Fru).

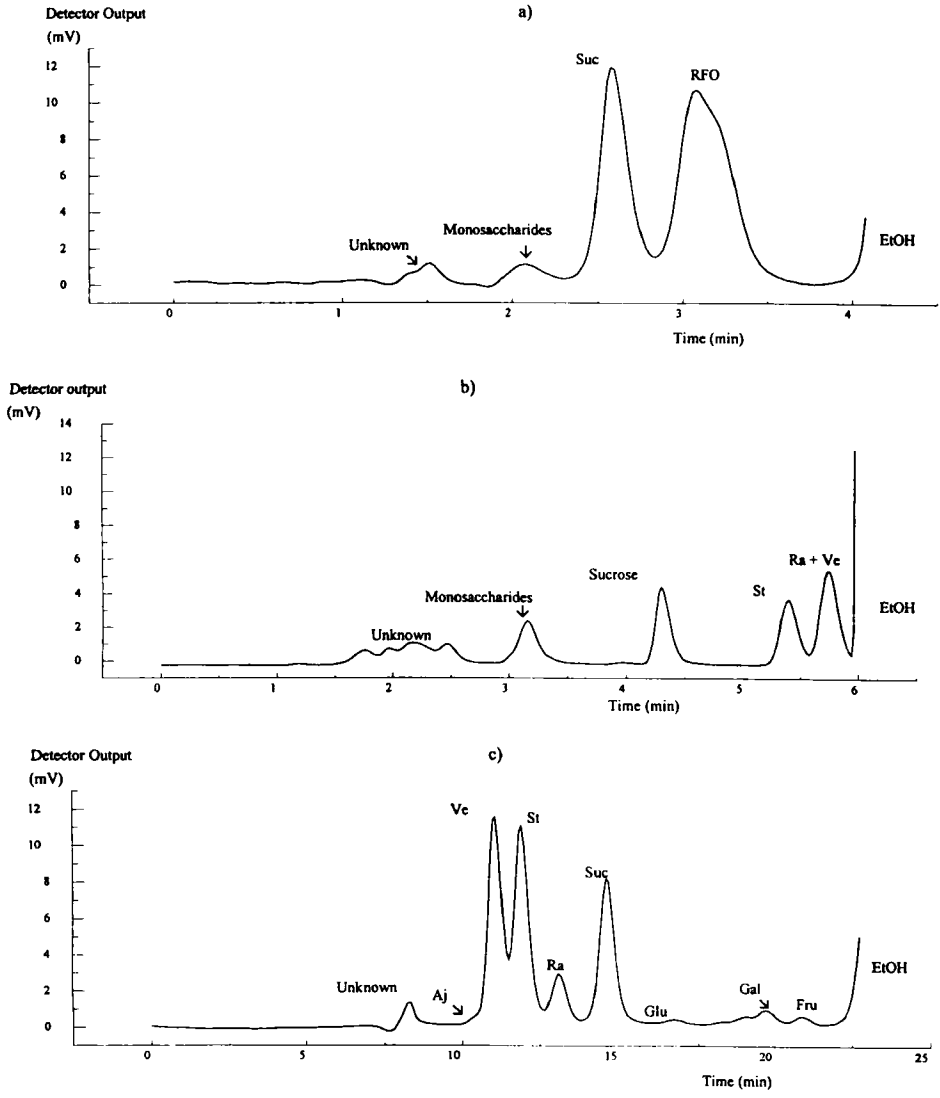

Figure 2. Chromatograms of pea sample extracts (pea variety Uladovskij 387); (a) RP HPLC on Separon RPS; (b) RP HPLC on Separon C_{18}; (c) IMP HPLC on OSTION LG KS 0403 in Ca form.

Table 3

Results of Calibration Analysis (RP-HPLC); Coefficients of Calibration Equations Y = a x X + b; Y = Peak Area (mV.s) and X = Concentration (μg/mL); Four Calibration Levels

Saccharide	a	b	Correlation Coefficient	Concentration range (μg/mL)
Stachyose	1.59	2.3	0.9987	50 - 500
Raffinose	1.68	5.8	0.9997	25 - 250
Sucrose	1.62	3.8	0.9995	50 - 500

Table 4

Results of Calibration Analysis (IMP-HPLC); Coefficients of Calibration Equations Y = a x X + b; Y = Peak Area (mV.s) and X = Concentration (μg/mL); Six Calibration Levels

Saccharide	a	b	Correlation Coefficient	Concentration range (μg/mL)
Stachyose	0.756	30.8	0.9990	100 - 1000
Raffinose	0.604	12.1	0.9982	20 - 200
Sucrose	0.744	38.0	0.9997	100 - 1000

From these data it is clear that both chromatographic methods showed good linearity between peak area and concentration for each saccharide. An average response factor (ratio of peak area and saccharide concentration) of stachyose and raffinose was used for the quantitative analysis of RFO determined by RP-HPLC. In the case of IMP-HPLC the quantitative analysis of individual saccharides was based on their own calibration equation (stachyose, raffinose and sucrose) and quantitative analysis of verbascose was based on calibration results of stachyose. For both HPLC techniques the lowest detectable concentration in sample solution analysed was found to be 5 μg of saccharide/mL, i.e., the smallest detectable amount of saccharide was 100 ng.

A comparison between the extraction efficiency of a sonication technique and boiling under reflux was made. It was found that the latter

technique was twice as efficient as the sonication technique (Figure 3) and the optimum extraction time for boiling was found to be 60 minutes (Figure 4).

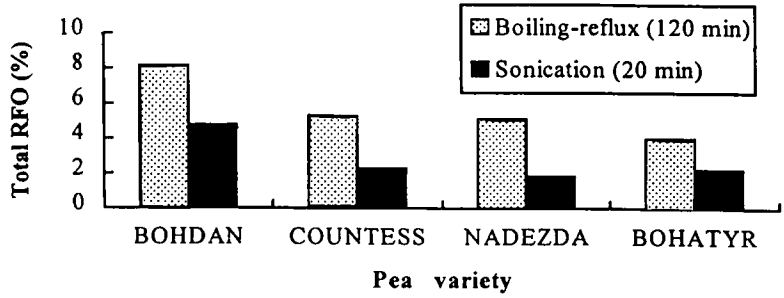

Figure 3 Extraction efficiency - comparison of two techniques

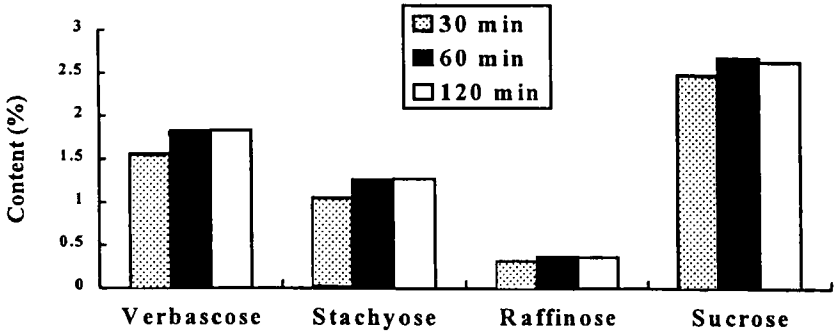

Figure 4 - Time course of extraction efficiency of boiling under reflux technique (pea sample Uladovskij 387)

The relative standard deviation and repeatability of the HPLC analyses and recoveries of RFO are summarised in Table 5. The RSD of HPLC analyses were calculated on the basis of results of 10 repetitive injections of the same sample extract. The repeatability of the HPLC techniques was obtained on the basis of 10 repetitive analyses of the same pea sample (Uladovskij 387). Recovery figures are an average of two different standard additions at levels corresponding to 50 % and 100 % of the actual saccharide content.

The IMP HPLC was compared with HPAC-PAD method (High

Performance Anion-exchange Chromatography with Pulsed Amperometric Detection) developed at the John Innes Centre and the Institute of Food Research in Norwich (UK).[10] On the basis of the figures shown in Table 6 it can be stated that the IMP HPLC results are in good agreement with those obtained with HPAC-PAD.

Table 5

Statistics of HPLC Analyses

	RP HPLC			IMP HPLC
	Verbascose	Stachyose	Raffinose	Sum of RFO
RSD of injection (%)	1.2	1.3	2.8	2.8
Repeatability (%)	2.6	3.8	5.8	4.2
Recovery (%)	-	93	94.5	95.2

Table 6

Comparison of IMP HPLC (I)* with HPAC-PAD (II)

Sample	Verbascose		Stachyose		Raffinose		Total RFO	
	I	II	I	II	I	II	I	II
iso-line 1	3.12	2.16[b]	2.03	1.45[b]	0.69	0.56[b]	5.84	4.17[b]
iso-line 2	5.47	3.74[b]	3.48	2.62[b]	1.15	1.05[b]	10.10	7.41[b]
iso-line 3	4.71	3.63[b]	4.86	2.48[b]	1.26	0.92[b]	10.83	7.03[b]
iso-line 4	3.99	4.21[b]	4.32	3.54[b]	1.28	1.51[b]	9.59	9.26[b]
BOHDAN	4.03	3.37	3.11	2.87	0.98	0.88	8.12	7.12
COUNTESS	1.25	1.34	2.22	2.38	0.55	0.64	5.31	4.97
NADEZDA	1.49	1.63	2.84	2.93	0.81	0.78	5.14	5.34
BOHATYR	1.97	1.73	2.35	2.21	0.99	1.03	4.02	4.36

*average of three replicates (for RSD see Table 5)

b) data from Frias et al., 1995[10] (with permission).

CONCLUSION

Both RP and IMP chromatography methods give acceptable results for quantitative analyses of the RFO in pea. The RP HPLC is a rapid method suitable for the determination of total RFO, especially in cases of large sample sets. The IMP HPLC enables the determination of the individual RFO, i.e., verbascose, stachyose and raffinose in pea samples within 30 minutes. Similar repeatability (< 5%) and recovery of RFO (93 - 95 %) was found for both methods.

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