Journal of Chromatography, 623 (1992) 186–190
Elsevier Science Publishers B V , Amsterdam

CHROM 24 492

Short Communication

Analysis of legume oligosaccharides by high-resolution gas chromatography

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(First received February 27th, 1992, revised manuscript received July 3rd, 1992)

ABSTRACT

The suitability of high-resolution gas chromatography (HGRC) for the analysis of the raffinose family oligosaccharides (raffinose, stachyose, verbascose) was investigated Aqueous methanol (80%) extracts of pea flour were dried and derivatized with either trimethylimidazole or N-methyl-bis(trifluoroacetamide) Separation of the sugar derivatives was achieved utilizing a 10-m DB5-60W capillary column The effects of carrier gas (He) flow-rate and split ratio on resolution and reproducibility were studied HRGC analysis was characterized by excellent resolution and satisfactory reproducibility, and proved to be a rapid, sensitive method for quantitation of oligosaccharides in pea flours

INTRODUCTION

A well known problem associated with the consumption of legume-based foods is their content of galactose-containing oligosaccharides (raffinose, stachyose, verbascose) at levels that may contribute to the development of flatulence [1] These α-galactosides escape digestion and absorption in the small intestine due to the absence of α-galactosidase activity and are consequently metabolized by bacteria in the lower intestinal tract, resulting in the production of carbon dioxide and hydrogen

A variety of chromatographic techniques have been employed for analysis of α-galactosides in legumes subsequent to an initial aqueous or alcoholic extraction and partial purification from non-carbohydrate material Tanaka *et al* [2] encountered considerable difficulties in the quantitation of α-galactosides by paper chromatography The high-performance liquid chromatographic (HPLC) methodology employed to date suffers shortcomings related to peak resolution and detector sensitivity [3,4] Anion-exchange chromatography at high pH, coupled with pulsed amperometric detection, overcomes the major shortcomings of conventional

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0021-9673/92/$05 00

HPLC for the analysis of carbohydrates [5] Its application to the analysis of legume oligosaccharides, however, has not yet been reported Packed-column gas chromatography (GC) was used by Sosulski *et al* [6] for the analysis of oligosaccharides in eleven legumes Long retention times and poor reproducibility for larger oligosaccharides were major drawbacks of their methodology

The aim of this work was to investigate the suitability of HRGC for rapid, quantitative determination of α-galactosides in pea flours

EXPERIMENTAL

Chromatographic equipment

A Hewlett-Packard 5710A gas chromatograph was equipped with a 10-m DB5-60W (0 32 mm I D , 0 25 μm film thickness) capillary column, a flame ionization detector (FID) using nitrogen as the make-up gas, and a Hewlett-Packard 3390A integrator Helium was used as the carrier gas The injector and detector temperatures were 250 and 300°C, respectively

Standards

Sorbitol, sucrose, phenyl α-D-glucoside, raffinose and stachyose were purchased from Sigma (St Louis, MO, USA)

Derivatization reagents

N-Methyl-bis(trifluoroacetamide) (MBTFA) and Tri-Sil Z reagents were purchased from Chromatographic Specialties (Brockville, Canada)

Trifluoroacetylation

Each sugar (0 1–5 0 mg) was dissolved in 0 25 ml pyridine (silylation grade, Chromatographic Specialties) in a reaction vial by shaking and heating at 70°C for 30 min Derivatization was completed by adding 0 25 ml MBTFA and heating at the same temperature for 15 min The same trifluoroacetylation procedure was applied to oligosaccharides extracted from pea flour (refer to *Extraction procedure*, below)

Silylation

Each sugar (0 1–5 0 mg) was dissolved in 0 50 ml Tri-Sil Z reagent in a reaction vial by shaking and heating at 70°C for 30 min The same silylation procedure was applied to oligosaccharides extracted from pea flour (refer to *Extraction procedure*, below)

Pea flour

High protein pea flour (air-classified pea protein concentrate), containing approximately 55% protein, was supplied by Parrheim Foods (Saskatoon, Canada)

Extraction procedure

Extraction of α-galactosides from pea flour was carried out according to the procedure of Sosulski *et al* [6] with modifications Pea flour (2 0 g) was homogenized for 2 min in 30 ml 80% aqueous methanol Samples were then centrifuged at 1000 *g* for 4 min The supernatant was treated with saturated lead acetate solution (20 drops/5 ml) to precipitate soluble proteins, and clarified using a hydrophobic (0 22-μm) membrane filter (Acrodisc PTFE-Gelman, Montreal, Canada) A 2-ml volume of the filtrate was treated with 2 drops of saturated monopotassium phosphate to remove excess lead and filtered again Finally, 1 ml of clear filtrate plus 1 ml of phenyl α-D-glucoside solution (1 0 mg/ml in 80% methanol) were evaporated to dryness in a reaction vial at 50°C under nitrogen Refluxing of pea flour (2 0 g) for 1 h in 30 ml 80% aqueous methanol was an equally effective extraction procedure

RESULTS AND DISCUSSION

Effect of carrier gas flow-rate on elution profile

As expected, the flow-rate of the carrier gas (He) had a pronounced effect on the elution profiles of the α-galactoside derivatives Retention times decreased significantly with increasing flow-rates (Fig 1) The excellent resolution obtained for trimethylsilyl (TMS) derivatives permitted the use of relatively high carrier flows, which markedly shortened analysis times

MBTFA derivatives exhibited greater volatility than did the corresponding TMS derivatives, which led to considerably shorter retention times (Fig 2) These findings were in agreement with results obtained earlier by Selosse and Reilly [7] for MBTFA derivatives of several trisaccharides, including raffinose However, we observed poor resolution between the peaks corresponding to sucrose and

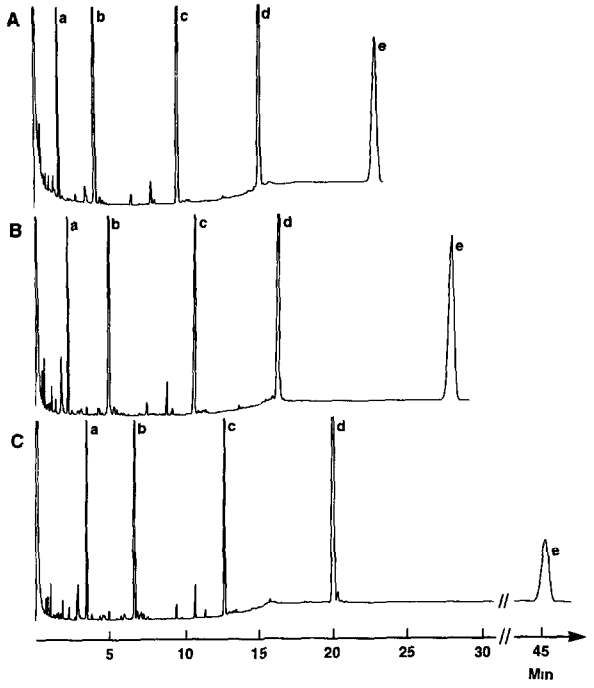

Fig 1 The effect of carrier gas (He) flow-rate on the elution profiles of TMS derivatives of legume oligosaccharides extracted from air-classified pea protein concentrate (A) 6 7 ml/min, (B) 3 7 ml/min, (C) 1 6 ml/min Chromatographic conditions split ratio 1 50, temperature programming initial temperature, 188°C, temperature gradient, 8°C/min, final temperature, 316°C, hold time at final temperature, 8–32 min, column DB5-60W (10 m × 0 32 mm I D , 0 25 μm film thickness) Components identified a = phenyl α-D-glucoside, b = sucrose, c = raffinose, d = stachyose, e = verbascose

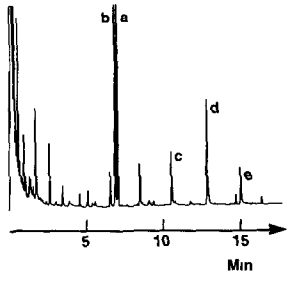

Fig 2 HRGC analysis of MBTFA derivatives of oligosaccharides extracted from air-classified pea protein concentrate Chromatographic conditions carrier (He) flow, 2 7 ml/min, split ratio, 1 100, temperature programming initial temperature, 80°C, temperature, gradient 8°C/min, final temperature, 250°C, column DB5-60W (10 m × 0 32 mm I D , 0 25 μm film thickness) Components identified a = phenyl α-D-glucoside, b = sucrose, c = raffinose, d = stachyose, e = verbascose

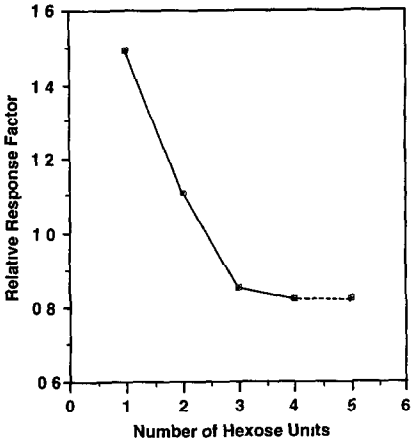

Fig 3 Curve depicting relative response factors for sorbitol (1 hexose unit), sucrose (2 hexose units), raffinose (3 hexose units), and stachyose (4 hexose units) Phenyl α-D-glucoside was employed as internal standard Dashed line indicates extrapolation required to obtain a response factor for verbascose (5 hexose units) Flow rate 6 7 ml/min, split ratio 1 50

phenyl α-D-glucoside, the most suitable internal standard identified At a carrier gas flow of 2 7 ml/min, the retention times for sucrose and phenyl α-D-glucoside were nearly identical at 7 0 and 7 2 min, respectively Resolution was not improved at carrier gas flows lower than 2 7 ml/min (data not shown)

Quantitation of oligosaccharide TMS derivatives

The relationship between relative response factor (mass basis) and the number of hexose units is shown in Fig 3 for sorbitol, sucrose, raffinose and stachyose TMS derivatives as determined at a flow-rate of 6 7 ml/min and a split ratio of 1 50 No standard was available for direct determination of a response factor for verbascose However, extrapolation from the response factor curve indicated that it was very similar to that of stachyose Quantitation of verbascose was based on this assumption The marked decrease in relative response for larger oligosaccharides was attributed to discrimination against higher mass/less volatile derivatives in the injector of the chromatograph, and to on-column breakdown of derivatives, particularly at higher column temperatures (larger oligosaccharides) Injector discrimination was particularly evident at higher split ratios, as shown in Fig 4, where the verbascose peak, in particular, became progressiv-

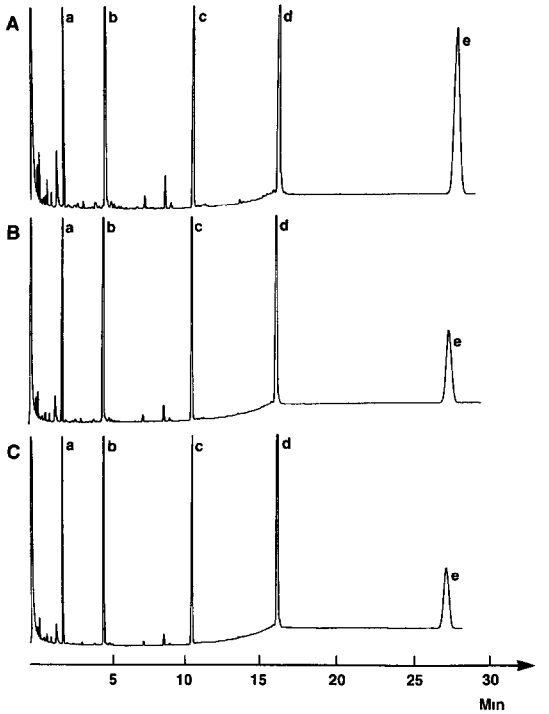

Fig 4 The effect of split ratio on the elution profiles of TMS derivatives of legume oligosaccharides extracted from air-classified pea protein concentrate (A) 1 50, (B) 1 100, (C) 1 150 Chromatographic conditions carrier gas (He) flow-rate, 3 7 ml/min, temperature programming - initial temperature, 188°C, temperature gradient, 8°C/min, final temperature, 316°C, hold time at final temperature, 16 min, column DB5-60W (10 m × 0 32 mm I D , 0 25 μm film thickness) Components identified a = phenyl α-D-glucoside, b = sucrose, c = raffinose, d = stachyose, e = verbascose

ely smaller relative to other component peaks as the split ratio was increased from 1 50 to 1 150 Sosulski *et al* [5] reported a similar, but essentially linear in their case, inverse relationship between relative response and mass using packed-column GC methodology

Reproducibility of analyses

Although TMS derivatives generally gave satisfactory reproducibility at all carrier gas flow-rates (Table I), results were most reproducible at the highest flow-rate tested, 6 7 ml/min of He Verbascose was an exception, reproducibility of analysis being poorest at 1 6 ml/min and best at 3 7 ml/min Reproducibility improved (smaller coefficients of variation) with decreasing split ratio at all flow-rates tested (Table I) with the exception of raffinose at 1 6 ml/min and sucrose and stachyose at 3 7 ml/min

Results using MBTFA as derivatizing agent were poorly reproducible, which we attributed to more severe and variable discrimination at the injector with the more volatile MBTFA derivatives For example, the coefficients of variation for triplicate analyses of raffinose, stachyose and verbascose were 13, 16 and 25%, respectively, at 2 7 ml/min and a split ratio of 1 50 This was not the case in an earlier packed-column GC study [8] using MBTFA derivatives, where reproducibility for raffinose and stachyose was satisfactory

TABLE I

THE EFFECT OF FLOW-RATE AND SPLIT RATIO ON REPRODUCIBILITY OF ANALYSIS OF α-GALACTOSIDE TMS DERIVATIVES

Flow-rate (ml/min)	Coefficient of variation (%)				Split ratio
	Sucrose	Raffinose	Stachyose	Verbascose	
1 6	2 4	5 8	4 2	10 2	1 50
	2 5	5 9	5 1	11 2	1 100
	2 7	5 7	6 2	11 6	1 150
3 7	2 5	2 2	0 4	2 2	1 50
	4 7	5 3	5 9	3 5	1 100
	4 6	6 1	4 9	7 6	1 150
6 7	1 4	0 8	0 3	5 2	1 50
	2 2	2 9	3 1	7 1	1 100
	2 8	3 1	4 5	8 3	1 150

CONCLUSIONS

The aim of the present study was to establish the efficacy of HRGC as a chromatographic technique for the analysis of legume oligosaccharides (raffinose, stachyose, verbascose) Research was focussed on the effect of analysis parameters such as carrier flow-rate, split ratio and the nature of the derivatizing agent on peak broadening and resolution, reproducibility, and analysis time

TMS derivatives gave satisfactory results over a wide range of carrier gas (He) flow-rates (1 6–6 7 ml/min) with the fastest analysis (retention time of 23 min for verbascose, the largest oligosaccharide) and the least peak broadening at 6 7 ml/min Resolution was excellent at all flow-rates and split ratios investigated Reproducibility, in general, was satisfactory, with the best results at a flow-rate of 3 7 ml/min and a split ratio of 1 50 (coefficients of variation were 2 5% or less for all oligosaccharides) The more volatile MBTFA derivatives gave much lower elution temperatures and shorter analysis times than did TMS derivatives, but discrimination during splitting of the sample in the injector of the gas chromatograph caused serious reproducibility problems

In summary, HRGC proved to be a rapid, sensitive method for quantitation of oligosaccharides in pea flour Compared to packed-column GC, HRGC offered superior resolution and reproducibility, shorter analysis times and reduced peak broadening for larger oligosaccharides, verbascose in particular

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