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High-Performance Capillary Electrophoresis with Indirect UV Detection for Determination of α-Galactosides in Leguminosae and Brassicaceae

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A rapid, easy, and reproducible capillary electrophoresis method for determination of raffinose family oligosaccharides (α-galactosides) was developed. Sucrose, raffinose, stachyose, verbascose, and ajugose were determined with indirect UV detection at moderate alkaline pH 9.2, using pyridine-2,6-dicarboxylic acid as background electrolyte in a sodium tetraborate buffer with added cetyltrimethylammonium bromide. The separation efficiency measured by the number of theoretical plates (*N*) ranged from 1.4×10^5 to 2.3×10^5. The precision of the method, measured by the relative standard deviation (RSD), was less than 0.53% for the migration times and better than 3.4% for normalized areas (NA), considering all sugars except verbascose ($RSD_{NA} = 11.8\%$). Detection limits were about 110 μg/mL, corresponding to 150−320 μM. Relative response factors (RRF) were calculated on the basis of linearity studies and used for quantification of α-galactosides in a lupine sample (*Lupinus angustifolius*).

KEYWORDS: High-performance capillary electrophoresis; α-galactosides; raffinose family oligosaccharides; sucrose; raffinose; stachyose; verbascose; ajugose

INTRODUCTION

The raffinose family of oligosaccharides are composed of α-(1→6)-galactosides bound to sucrose (β-D-fructofuranosyl-(1→2)-α-D-glucopyranoside) at C6 of the glucose moiety. α-Galactosides, a common name used for these types of compounds, are dominated by raffinose, stachyose, verbascose, and ajugose, having one, two, three, and four galactosyl units, respectively (**Figure 1**). The galactosylation occurs through transfer from a myo-inositol derivative, galactinol. α-Galactosides are widespread in higher plants, especially leguminous plants, where they accumulate in high concentration in the storage organs (*1*, *2*) and their function as storage carbohydrates can easily be recognized during germination, when a distinct decline in concentration can be shown (*3*, *4*).

The raffinose family of oligosaccharides is not degraded in the upper gastrointestinal tract, as digestive enzymes there are unable to hydrolyze the α(1→6) link between the galactose residues. The oligosaccharides, together with other nondigestible food components, thus pass into the large intestine, where bacterial enzymes decompose them into short chain fatty acids and gases (*5*−*7*). This fermentation may cause negative as well as positive effects in relation to human well-being and health. It is well documented that consumption of pulses can result in flatulence and intestinal discomfort, the severity of effect being clearly dose related and depending on daily eating habits and individual differences in sensitivity with respect to intake of nondigestible oligosaccharides (*8*−*10*). Several successful attempts have been made to reduce the concentration of α-galactosides in seeds of leguminous plants by various processing techniques, including cooking, soaking, or fermentation (*3*, *4*, *11*−*15*). Another approach has been to use enzymatic degradation of α-galactosides by α-galactosidases, enzymes produced by the plant itself during germination of the leguminous seeds (*3*, *4*, *16*, *17*), or by addition of isolated α-galactosidases (*18*, *19*).

In recent years, the negative effects described above have, however, been counterbalanced by an increasing interest in nondigestible oligosaccharides as functional food ingredients. Oligosaccharides are thus stated to have great potential in improving the quality of many foods, with respect to both food flavor and physicochemical characteristics, but especially in connection with their ability to stimulate the growth of beneficial bacteria (primarily bifidobacteria) in the colon (*20*−*24*). Moreover, there is preliminary evidence that consumption of certain types of nondigestible oligosaccharides may affect mineral absorption and blood lipid variables and maybe act as chemopreventive substances in relation to carcinogenesis (*7*, *25*−*27*). Although more in-depth research and human studies are recommended to confirm the above-described effects of oligosaccharides (*7*, *28*), it is beyond dispute that oligosaccharides of various types have become increasingly popular as food

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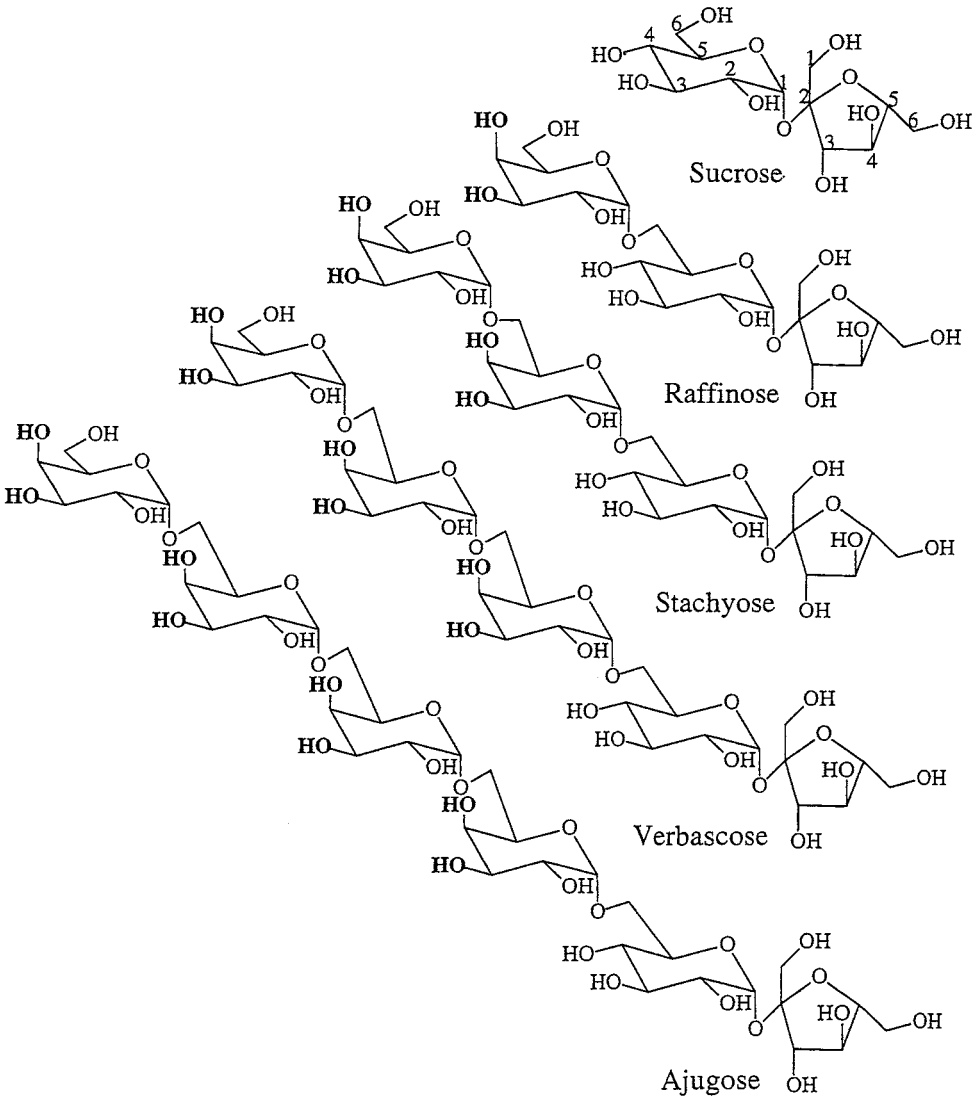

Figure 1. Structure of sucrose and the α-galactosides raffinose, stachyose, verbascose, and ajugose. Cis-diol groups are indicated in boldface type.

ingredients, and fast and reliable analysis methods for these food ingredients are required.

Analysis of α-galactosides of the raffinose family is, as for most other carbohydrates, complicated by the fact that they neither absorb ultraviolet or visible light nor fluoresce. Derivatization of carbohydrates with a suitable chromophore or fluorophore is thus an often-used technique to improve the detection limit. However, most derivatization schemes are based on the reducing property of sugars, a property that α-galactosides do not possess. Approaches to the quantitative determination of α-galactosides have included GLC and HPLC methods (*3, 29, 30*) and during recent years also high-performance capillary electrophoresis (HPCE) methods.

Direct detection of α-galactosides using HPCE generally has involved the use of borate in the separation buffer (*3, 31*). Complex formation of carbohydrates with borate gives, however, only limited improvement in UV absorbance (2−20 times) (*32*). Considerably better improvements in detection limits of underivatized carbohydrates, including α-galactosides, have been obtained by use of amperometric detection at constant potential (ADCP) (*33*), a detection scheme involving the electrochemical oxidation of carbohydrates at the surface of metallic electrodes. A third approach, which in contrast to the electrochemical detection can be used with all HPCE systems equipped with a simple UV detector, is indirect UV detection. The key principle in indirect UV detection is that the migrating analytes displace a UV-absorbing component, the background electrolyte (BGE), in the mobile phase, the separation buffer (*34, 35*). This displacement results in a negative signal, which can be reversed by monitoring a signal wavelength outside the absorption area of the BGE in addition to the reference wavelength, which are chosen to match the absorption peak for the BGE. When the signal wavelength is subtracted from the reference wavelength, the negative signal is converted to a positive signal, which can be integrated directly for quantitative analyses. Several factors have to be considered before the BGE is chosen for indirect detection systems. First of all, the BGE must have a high extinction coefficient at the detection wavelength chosen, to keep the concentration of BGE as low as possible. Another important issue is the mobility of the BGE, which has to match the mobility of analytes in order to obtain signal peaks with neither fronting nor tailing. Sodium cholate has been used for the analysis of chloride, sulfate, and nitrate (*36*), whereas pyridine-2,6-dicarboxylic acid has been shown to be successful as a BGE for both inorganic and organic anions, including ionized monosaccharides (*37−40*).

A high pH of the separation buffer (pH 12−13) has usually been used to introduce charges to the otherwise neutral carbohydrates (*38, 40−45*). A buffer pH above the pK_a' values of the carbohydrates thus improves separation efficiency;

however, this is obtained at the expense of detection sensitivity due to the effect of hydroxide anions on the transfer ratio (i.e. the number of molecules of the detectable background electrolyte displaced by each analyte molecule) (*43*, *44*). A way to overcome this problem is to introduce charges to the carbohydrates by including borate in the separation buffer at moderate alkaline pH. This was performed by Liu et al. (*42*), using sorbic acid or riboflavin as background electrolyte, and by Plocek and Chmelík (*46*), using *p*-nitrophenol.

In the present study, we have developed a HPCE method with high separation capacity toward sucrose, raffinose, stachyose, verbascose, and ajugose as well as the myo-inositol derivative, galactinol, involved in the biosynthesis of the α-galactosides. The HPCE method, using indirect UV detection based on pyridine-2,6-dicarboxylic acid as the BGE, has been optimized with respect to separation parameters, as has the method performance (precision, detection limits) as well as relative response factors determined for the individual α-galactosides. The optimized method has been applied to selected samples of leguminous seed for quantification of component α-galactosides.

MATERIALS AND METHODS

Plant Material. The lupine samples examined were from *Lupinus angustifolius* L. (blue lupine) cv. 9909/104 obtained from the plant breeding group at RVAU (Højbakkegaard, Tåstrup).

Chemicals and Reference Compounds. Pyridine-2,6-dicarboxylic acid (dipicolinic acid; $\epsilon_{192\ nm} = 43\ 680\ L\ mol^{-1}\ cm^{-1}$), maltitol, methyl-α-D-glucopyranoside, sucrose, raffinose, stachyose, and column materials for ion-exchange chromatography (Sephadex CM-25 H^+, Dowex 50W×8 H^+ 200−400 mesh, and Dowex 1×8 acetate 200−400 mesh) were obtained from Sigma Co. (St. Louis, MO). Cetyltrimethylammonium bromide was obtained from Fluka (Buchs, Switzerland). Other chemicals were of analytical reagent grade and obtained from various manufacturers. Galactinol, isolated from sugar beet, was kindly donated from Maribo Seeds A/S, Denmark, and verbascose was isolated from peas using paper chromatography and preparative HPLC. Water was purified in a Milli-Q system (Millipore, Bedford, MA).

Sample Preparation. The reference samples of oligosaccharides used for optimization of the HPCE procedure included sucrose and three α-galactosides (raffinose, stachyose, and verbascose) together with the two internal standards: maltitol and methyl-α-D-glucopyranoside. The reference compounds, between 2.7 and 3.2 mg/mL of each, were dissolved in separation buffer.

Extraction of oligosaccharides from seed material was performed as follows. Seed materials were ground in a coffee mill. Samples of 0.5 g of ground seed material were added to 100 μL of a reference mixture consisting of 125 mM maltitol and 125 mM methyl-α-D-glucopyranoside. Extractions of oligosaccharides were performed by homogenization with an Ultra Turrax T 25 (Janke & Kunkel, Staufen, Germany) in 2 × 3 mL of boiling methanol−water (7:3) twice for 2 min. After each extraction cycle, the homogenate was centrifuged in a benchtop centrifuge at 2000*g* for 2 min. The supernatants obtained were pooled and evaporated to dryness and redissolved in 5 mL of water. A 3.0 mL portion was subjected to group separation by ion-exchange chromatography according to the principles described by Bjerg et al. (*47*) and Sørensen et al. (*48*). Aqueous suspensions (1 mL; 1:1) of (A) Sephadex CM-25 H^+, (B) Dowex 50WX8 H^+ 200−400 mesh, and (C) Dowex 1X8 acetate 200−400 mesh were packed into 1 mL plastic columns supplied with disks of silica material at the bottom. The columns were regenerated by use of 15 mL of 2 M acetic acid (A), 1 M HCl (B), and 2 M sodium acetate, respectively, followed by washing with water to neutral pH. Column A was placed at the top of column B, which was placed at the top of column C, and the assembled columns were placed in a vacuum manifold (Supelco, Bellefonte, PA). After application of the sample (6 × 0.5 mL), the material was allowed to pass into the column material and the columns were then washed with 2 × 5 mL of water. The aqueous eluate was evaporated to dryness, redissolved in 200 μL of water, and 100 μL of the solution was evaporated to dryness and redissolved in separation buffer before HPCE analysis.

HPCE Analysis. The apparatus used was a Hewlett-Packard HP^{3D} CE capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany) equipped with a diode-array detector and with data processing carried out with an HP Chemstation V. 6.01. The capillary used for analyses was an 800 mm × 0.05 mm i.d. fused silica capillary. UV detection was performed on-column at a position 700 mm from the injection end. The signal wavelength was set at 350 nm with a reference at 275 nm (*40*). Samples were introduced from the cathodic end of the capillary by vacuum injection for 5 s at 5 kPa. The specific HPCE conditions were varied during optimization of the method. The separation buffer was basically composed of the background electrolyte pyridine-2,6-dicarboxylic acid, sodium borate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$), and hexadecyltrimethylammonium bromide adjusted to varying pH values (8.0−10.0). The separation buffer was filtered through a 0.20 μm membrane filter before use, and the standard procedure for capillary wash between analyses included a flush with 1.0 M NaOH for 2 min, water for 1 min, and separation buffer for 5 min.

Relative Response Factors. Relative response factors (RRF) were used to determine the absolute amounts of the individual α-galactosides in the samples. RRF (relative to the internal standard maltitol) were determined from the slope (α) of the calibration curves for the α-galactosides and the internal standard according to the equation $RRF_x = \alpha_{maltitol}/\alpha_x$.

RESULTS AND DISCUSSION

The buffer system used in the present study was based on borate at moderate alkaline pH, as compared to the method of Soga and Serwe (*40*), who used highly alkaline pH conditions for determination of carbohydrates in food samples. However, the two HPCE systems had in common that separation was carried out in the counter-electroosmotic migration mode by including cetyltrimethylammonium bromide in a concentration below the critical micellar concentration (CMC), resulting in reversal of the electroosmotic flow (EOF). Tsuda (*49*) has demonstrated the effect of cetyltrimethylammonium bromide concentration on the direction of EOF. This approach, combined with reversal of the polarity for sample injection, results in codirectional movement of the α-galactosides and the EOF, ensuring fast analysis and increased separation efficiency (*45*, *50*). In the present study, the concentration of cetyltrimethylammonium bromide in the buffer was 0.5 mM. Changes in concentration below the CMC value were shown to have only limited effect on the separation of α-galactosides.

Optimization of the separation buffer system was performed with respect to concentration of borate (5−150 mM) and pH (8.0−10.0), which were shown to be the major determinants of separation in the present method. As expected, no borate complexes with α-galactosides were seen at pH values below 9, and the three oligosaccharides and sucrose migrated as a single peak in the electropherogram. Higher buffer pH was therefore needed, and the pH 9.2 value chosen combined successful complex formation with a stable baseline. Borate concentration was of importance for both migration time and signal response. **Figure 2** shows how the migration time for sucrose and raffinose decreased at increasing concentration of borate in the separation buffer up to a certain level, after which the migration time starts to increase. The initial decrease in migration time can be explained by α-galactoside complex formation with borate, introducing a negative charge to the otherwise neutral oligosaccharides and thereby increasing their mobility in the electric field. At the same time, however, the increasing ionic strength of the buffer reduces the electroosmotic flow, and this is possibly the explanation for the prolonged migration time at high borate concentration.

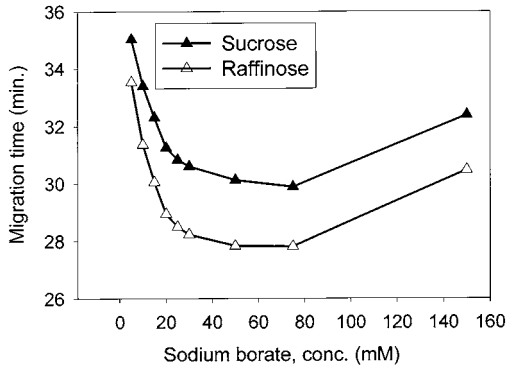

Figure 2. Migration time for sucrose and raffinose as a function of sodium borate concentration in the separation buffer.

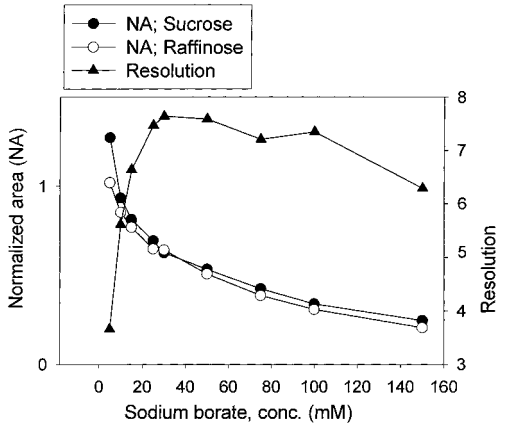

Figure 3. Normalized peak area (NA) and resolution (R_s) for sucrose and raffinose as a function of sodium borate concentration in the separation buffer.

The observed reduction in signal response (normalized peak area (NA)) at increasing borate concentration (**Figure 3**) is in accordance with results obtained by Plocek and Chmelík (*46*). The phenomenon is due to the effect of borate anions on the transfer ratio, equivalent to the effect seen with hydroxide ions in highly alkaline buffers for carbohydrate separation with indirect UV detection (*51*). In the present study, the relative response at 150 mM borate concentration was 46% (sucrose) of that at 50 mM, whereas the corresponding figure was 40% for raffinose. These results are comparable to data from the 50% reduction reported by Plocek and Chmelík (*46*). As the resolution between the peaks of sucrose and raffinose was found to be at its maximum at borate concentrations from 30 to 50 mM, it was thus decided to continue with 50 mM borate in the separation buffer. A concentration of 50 mM was preferred to 30 mM in order to ensure sufficient complex formation, even at high concentrations of α-galactosides in the sample. Increments of separation temperature and voltage generally lowered the time of analysis but resulted also in a disturbed baseline at too high values, and the best compromise was found to be −10 kV and 30 °C.

An electropherogram showing separation of sucrose, raffinose, stachyose, and verbascose in the optimized HPCE system is shown in **Figure 4**. The separation efficiency measured by the number of theoretical plates (N) ranged from 135 000 to 229 000, with resolutions (R_s) from 3.8 to 9.8. The elution order, with sucrose migrating with the lowest mobility and verbascose eluting first of the α-galactosides, can be explained by differences in the influence of borate complex formation on the electrophoretic behavior of the carbohydrates in the standard mixture. It is well-known that the magnitude of charge for the

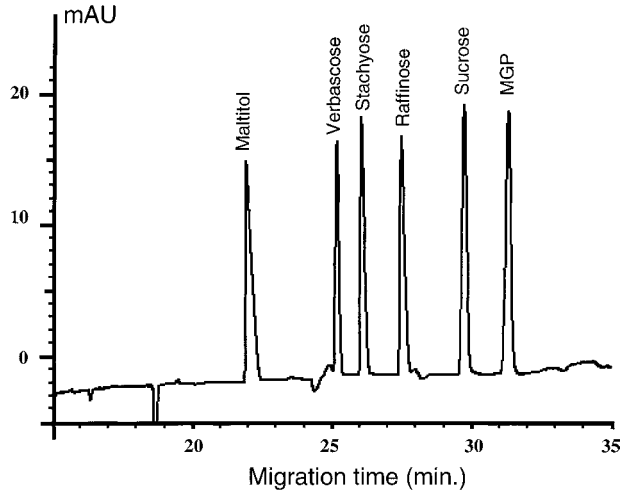

Figure 4. Electropherogram from HPCE of sucrose and the α-galactosides raffinose, stachyose, and verbascose. Maltitol and methyl-α-D-glucopyranoside are both internal standards.

Table 1. Correlation Coefficients Obtained from Linearity Studies of Sucrose and α-Galactosides Analyzed by HPCE with Indirect Detection

compd	concn range for injected sample, μg/mL	concn range for injected sample, μM	r^2
sucrose	110–5500	320–16070	0.9997
raffinose	110–5450	180–9170	0.9997
stachyose	110–5600	160–8400	0.9998
verbascose	130–6400	150–7730	0.9998

[a] For separation conditions, see text.

borate−carbohydrate complex is dependent on the stability of the complex and that the stability of the complex depends strongly on the configuration of hydroxyl groups in the carbohydrate (*32*). As shown in **Figure 1**, sucrose has no cis-diol group and will therefore form a weak complex compared to the α-galactosides with their galactose units, having a *cis*-3,4-diol group. The electrophoretic mobilities of sucrose (0.06×10^{-3} cm^2/(V s)), raffinose (0.13×10^{-3} cm^2/(V s)), and stachyose (0.16×10^{-3} cm^2/(V s)) as measured by Hofstetter-Kuhn et al. (*32*), in a 60 mM sodium tetraborate buffer, thus confirm the migration order found in the present study. Furthermore, the electroosmotic flow will increase the migration velocity with increasing molecular size.

The robustness of the optimized method was checked in a repeatability trial with 15 consecutive injections of the standard mixture. The repeatability of migration time for the α-galactosides was good, with values of relative standard deviation below 0.6%. The repeatability with respect to normalized area was in the range of about 3−4%, except for verbascose having a RSD value above 10%. This figure could probably be improved, however, as the reason for this poor repeatability is a very low content of verbascose in the standard mixture used for the repeatability study. The linearity study thus confirmed a high precision with respect to normalized areas for the optimized method (**Table 1**).

The detector response was shown to be linear within the concentration range shown in **Table 1**. The lowest concentration in this interval represents the detection limit (signal-to-noise ratio 3). It should, however, be possible to increase the concentration in the upper range even further, as no decline in response could be observed at the highest concentrations for any of the compounds in the standard mixture. The two internal

standards (maltitol and methyl-α-D-glucopyranoside) tested in this system also showed satisfying linearity (maltitol, concentration range 0.30−15.54 mM, $r^2 = 0.9999$; methyl-α-D-glucopyranoside, concentration range 0.54−27.80 mM, $r^2 = 0.9998$).

The absolute detection limits calculated from data obtained in the linearity studies were found to be in the picomole range: sucrose (1.89 pmol), raffinose (1.08 pmol), stachyose (0.98 pmol), verbascose (0.91 pmol), maltitol (1.80 pmol), and methyl-α-D-glucopyranoside (3.24 pmol). These correspond to a sample concentration of 110 μg/mL for sucrose, raffinose, and stachyose and 130 μg/mL for verbascose (injection ~6 nL). The detection limits of this study are approximately 5 times higher than the detection limits for various monosaccharides analyzed by Soga and Heiger (38), using a similar CE system, but at higher pH and without sodium borate. Compared to other studies of α-galactosides, the method presented here is approximately 3 times more sensitive than the CE method presented by Frias et al. (*3*) but is still 500 times less sensitive compared to analysis using high-performance anion exchange chromatography with triple-pulsed amperometric detection (HPAC-PAD).

The quantification of α-galactosides requires the use of relative response factors (RRF), which were determined relative to the internal standard maltitol from the slope (α) of the calibration curves for the α-galactosides and the internal standard, according to the equation $RRF_x = \alpha_{maltitol}/\alpha_x$. The relative response factors determined on a millimole basis were as follows: sucrose (1.48), raffinose (0.91), stachyose (0.81), and verbascose (0.94). Variations in the RRF values reflect differences with respect to the charged analyte molecule capabilities to displace the BGE molecules in the buffer in order of maintaining constant conductance throughout the entire capillary, as described by Garner and Yeung (*52*). The volume changes caused by structural and possible conformational changes as well as the net charge define the displaced BGE and thereby may explain the observed effect on the RRF. The net charge of the carbohydrate−borate complex is influenced both by variations in pK_a' values of the individual sugars and by differences in their affinity toward borate. The α-galactosides are expected to have $pK_a' > 12.5$ (sucrose 12.51, raffinose 12.74) (*38*, *41*). As the run buffer pH was 9.2, pK_a' variations of the sugars are expected to have only limited effects on the net charge of the borate complexes. Increasing the galactosyl chain length from sucrose ($n = 0$) to raffinose ($n = 1$) and further to stachyose ($n = 2$) increases the net charge and thus decreases the respective RRF values. The increase in RRF value from stachyose (0.81) to verbascose (0.94) can be explained by the expected change in the conformation of the galactosyl chain, making borate complex formation more difficult. The lower net charge and the expected more compact conformation of verbascose results in less capability to displace the BGE molecules.

An example of an electropherogram from analyses of *Lupinus angustifolius* is given in **Figure 5**. On the basis of the RRF values, the concentrations of α-galactosides in *Lupinus angustifolius* L. cv. 9909/104 were calculated as 18.6 μmol/g (ajugose), 21.4 μmol/g (verbascose), 37.5 μmol/g (stachyose), and 10.4 μmol/g (raffinose), corresponding to 6.6% (w/w) α-galactosides in the lupine seeds. In addition, the lupine seed contained 56.3 μmol/g seed of sucrose equal to 1.9% (w/w).

In conclusion, the work presented here provides a fast and reliable analytical method for quantification of α-galactosides, a method being of general interest considering the ongoing research focus on nondigestible oligosaccharides in food and feed.

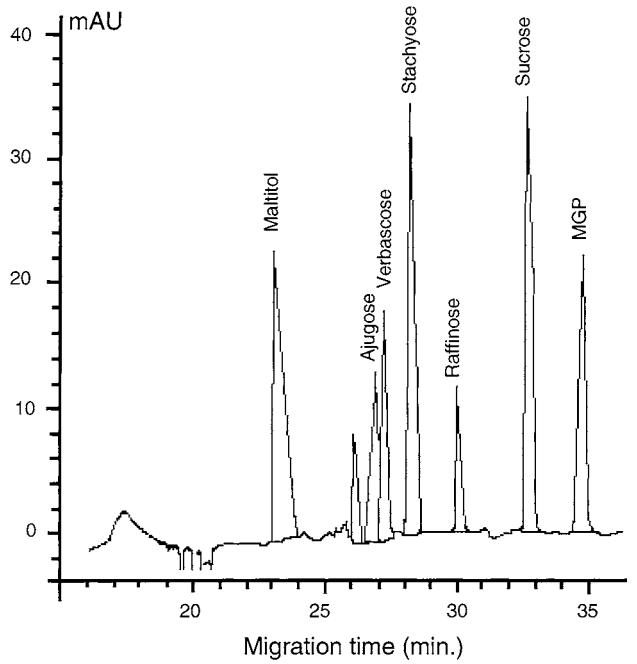

Figure 5. Electropherograms of α-galactosides isolated from *Lupinus angustifolius* L. (9909/104) obtained by HPCE with indirect detection. The peaks are identified as follows: MT (migration time) 23.18 min (maltitol; internal standard); MT 26.96 min (ajugose); MT 27.21 min (verbascose); MT 28.14 min (stachyose); MT 30.01 min (raffinose); MT 32.63 min (sucrose); MT 34.55 min (methyl-α-D-glucopyranoside; internal standard).

ABBREVIATIONS USED

α, linearity curve slope; BGE, background electrolyte; EOF, electroosmotic flow; *N*, number of theoretical plates; NA, normalized areas; RSD, relative standard deviation; RRF, relative response factors; R_s, resolution

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