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Determination of oligosaccharides by capillary zone electrophoresis

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ABSTRACT

Oligosaccharides occur in various biological materials, and the α-galactosides raffinose, stachyose, verbascose and ajugose are important in relation to the quality or nutritive value of legume seeds. A simple technique for the isolation and group separation of oligosaccharides was developed as an appropriate purification step prior to determination of the individual oligosaccharides by high-performance capillary electrophoresis (HPCE). The HPCE technique adapted to the separation of α-galactosides was based on capillary zone electrophoresis (CZE) in borate buffers with UV detection at 195 nm. The influence of various separation conditions, including voltage, pH, temperature and buffer composition, on the resolution, migration times, number of theoretical plates and peak areas was studied by the use of galactinol and the mono-, di-, tri- and tetra-α-galactosides of sucrose. Up to about 375 000 plates/m were obtained with the described CZE method. Tests of repeatability and linearity and the use of internal standards and relative response factors were used for evaluations of the qualitative and quantitative aspects of the method. With the combined technique of group separation, purification and CZE, a rapid and efficient method for the determination of naturally occurring oligosaccharides is now available for even complex mixtures of these carbohydrates.

INTRODUCTION

Oligosaccharides of the raffinose family are α-(1→6)-galactosides linked to C-6 of the glucose moiety of sucrose [1,2]. Raffinose is the template of this homologous series with only one galactoside unit attached. By successive binding of one, two and three additional α-galactoside units to C-6 of the terminating galactose unit of the lower homologue, the compounds stachyose, verbascose and ajugose are formed. These carbohydrates are synthesized in various plants, and appreciable amounts accumulate in legume seeds [2–4], where they seem to serve as storage compounds as found for other plants [5]. When present in too high concentrations in diets fed to animals, they may behave as anti-nutritional compounds owing to the problems they obviously can create in monogastric animals [6–9].

Methods of analysis for determination of carbohydrates require special attention as the compounds are important in many connections. In recent years, high-performance liquid chromatography (HPLC) has been the method of choice for the determination of individual oligosaccharides [2,10]. HPLC techniques for this purpose have some drawbacks, however, as they are not sufficiently rapid, cheap or efficient. The disadvantages have initiated searches for other techniques. The possibilities of using high-performance capillary electrophoresis (HPCE) for carbohydrate analysis seem promising [11–14], and for non-reducing oligosaccharides separations of borate-complexed compounds seem especially advantageous [15].

Capillary zone electrophoresis (CZE) of borate–oligosaccharide complexes was the subject for this work, with investigations of various

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important parameters affecting the separation, including buffer composition and pH, temperature and voltage. The effects of these parameters on migration time, peak shape and area, number of theoretical plates, resolution and repeatability were evaluated. A procedure for an efficient and simple sample preparation involving group separation was developed, as this was found to be a critical step in relation to the success of the CZE method for the determination of oligosaccharides occurring in plants, feed and food.

EXPERIMENTAL

Chemicals and solvents

All chemicals and solvents were of analytical-reagent grade and the water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Raffinose and stachyose were obtained from Aldrich Chemie (Steiheim, Germany), melibiose, methyl-α-galactopyranoside and fucose from Sigma (St. Louis, MO, USA) and sucrose, lactose, maltose, pentoses, hexoses, rhamnose and *myo*-inositol from the laboratory collection of reference carbohydrates. Galactinol isolated from sugar beet was avaliable and verbascose (purity 78%) and ajugose were preparatively isolated from peas using paper chromatography and preparative HPLC [10].

Sample preparation

Mature seeds of selected pea lines were ground in a coffee mill. Subsequent grinding in a mortar was occasionally necessary to obtain a uniform powder. Lactose (200 μl, 60 mg/ml) and melibiose (200 μl, 60 mg/ml) were added to 0.5 g of pea flour as internal standards prior to extraction with 3 × 5 ml of methanol–water (7:3). The pea flour was homogenized by means of an Ultra Turrax T 25 (Janke & Kunkel, Staufen, Germany) for 3 × 1.5 min. After each extraction cycle the homogenate was centrifuged in a table centrifuge (Labofuge, Heraeus Sepatech, Osterode, Germany) at 2000 *g* for 1 min. The extract was evaporated to dryness in an evaporator (Rotavapor-R; Büchi, Flawil, Switzerland) and the residue was dissolved in 1 ml of water.

The crude extract was centrifuged and subjected to group separation according to the principles described by Bjerg *et al.* [16]. Aqueous suspensions (1:1) of (A) Dowex 50W-X8, 200–400 mesh (H^+) and (B) Dowex 1-X8, 200–400 mesh (OH^-), (Sigma) were prepared. Portions of 1 ml of A or B were packed into 1-ml syringes (columns) supplied with discs of silica material as the bottom. Column A was placed above column B in a vacuum manifold (Supelco, Bellefonte, PA, USA), and 1 ml of crude extract was transferred to column A. The sample was allowed to pass into the column material, which subsequently was washed with 3 × 3 ml of water. The aqueous effluent was concentrated to an appropriate volume, kept at −20°C until use and analysed by CZE. Volumes of *ca.* 50 μl were transferred into vials (0.5 ml, Model 1298; Kartell, Milan, Italy), which were supplied with rubber caps (Model 4E1634; Applied Biosystems, Foster City, CA, USA) supplied with a slit to diminish evaporation.

Apparatus

The capillary electrophoresis instrument used was an ABI Model 270 A-HT (Applied Biosystems). The fused-silica capillary had the dimensions 720 mm × 50 μm I.D. × 360 μm O.D., including coating material. The detector window was placed 500 mm from the injection end (anode). On-column UV detection was performed at 195 nm and data processing was effected on a Shimadzu (Kyoto, Japan) Chromatopac C-R3A.

Separation conditions

The influence of separation parameters, including concentration of borate electrolyte (20–100 m*M* $Na_2B_4O_7$; Merck, Darmstadt, Germany), pH (9.2–10.3), temperature (30–60°C), voltage (10–20 kV) and concentration of 2-propanol modifier (0–15%, v/v), were investigated. Fresh solutions of borate were prepared every day and stored at room temperature. Unless specified otherwise, the injection time was 2.0 s, the detector rise time was 0.5 s and the range was 0.01. Between each run the capillary was flushed with 1 *M* NaOH for 3 min and with borate buffer for 5 min. The buffer at the

injection end (anodic) was changed each ten runs.

The influence of the running conditions on the separation was described for individual compounds by the migration time (*MT*, min), the normalized area (*NA* = absolute peak area/*MT*) and the number of theoretical plates per metre of capillary (plates/m), and for pairs of compounds by the resolution (R_s) [17].

Concentrations of individual sugars were calculated primarily based on lactose as internal standard. In situations where lactose co-eluted with ajugose, or where the peak was followed by a trough, the melibiose peak was used for correction. Calibration graphs were made by determining increasing concentrations (20, 40, 60, 80 and 100%) of a standard solution consisting of sucrose, raffinose, stachyose, verbascose, lactose and melibiose. Suitable detector signals were obtained for a stock solution (100%) with a concentration of each carbohydrate of about 6 μmol/ml.

RESULTS AND DISCUSSION

The overall method for the determination of sucrose and oligosaccharides in mature peas and other plant products consists of extraction, sample preparation by group separation and analysis by CZE.

Efficient extraction of low-molecular-mass carbohydrates from dietary fibres and other high-molecular-mass compounds in mature peas was achieved by repeated homogenizations in boiling methanol–water (7:3). This extraction medium was preferred to other methanol–water proportions, to cold methanol–water and to ethanol–water (7:3). Extraction with hot water was avoided because of the gelling properties of the pectin fraction, which can trap extractable compounds. Gel formation was disrupted in the presence of methanol. Moreover, effective inactivation of glycosidases was obtained, and no products of oligosaccharide or starch hydrolysis were observed such as melibiose, galactose and maltotriose or larger glucose oligomers. The enzyme inactivation during the initial extraction is important as products of enzymatic hydrolysis have been observed with extraction in water at temperatures below 60°C [18,19].

The group separation procedure developed for sample preparation appeared to be essential for the attainment of high-quality electropherograms (Fig. 1). The samples were passed directly from a strongly acidic cation exchanger to a strongly basic anion exchanger in order to remove compounds that had a positive or negative net charge under the conditions specified. The neutral carbohydrates were recovered in the aqueous effluent. The method is a modification of the procedure used for the preparation of oligosaccharide samples for HPLC [2,10]. The major improvement was the substitution of weakly basic anion-exchange material with strongly basic material. Substances interfering with the separation and with detection at 195 nm, which is within the absorbance range of a wide group of compounds, was efficiently avoided with the modified method.

A simple and inexpensive CZE method was adapted from Hoffstetter-Kuhn *et al.* [15] for the determination of oligosaccharides in extracts of

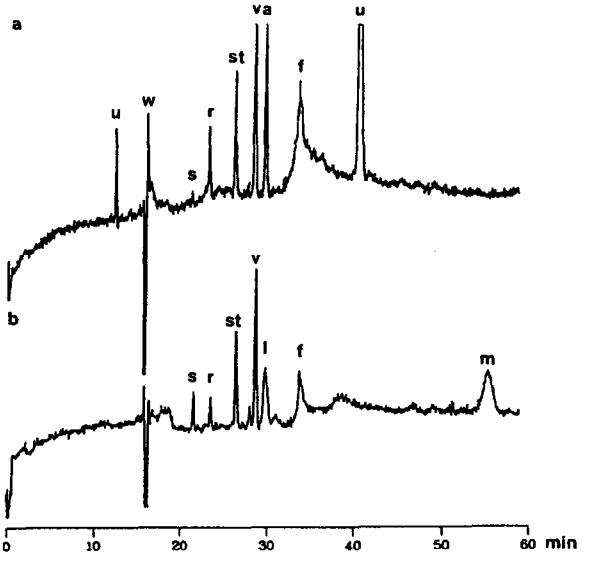

Fig. 1. (a) Electropherogram of a mixture of oligosaccharides. Peaks labelled u are impurities from the ajugose preparation. (b) Electropherogram of a pea extract purified by group separation. Separation conditions: 100 m*M* $Na_2B_4O_7$, pH 9.9, 50°C, 10 kV. s = Sucrose; r = raffinose; st = stachyose; v = verbascose; a = ajugose; l = lactose; w = water front; m = melibiose; u = unknown; f = "false peak".

mature peas. Separation and UV detection of carbohydrates by this method were based on the formation of borate–polyol complexes. The separation parameters borate concentration, pH, temperature and voltage were varied in order to find the best compromise that complied with the demands for high separation efficiency (plates/m and R_s), short durations of analyses (MT) and sufficiently high detector responses (NA). With the described system, the MT of oligosaccharides increased as a function of the molecular mass, whereas reducing sugars and especially monosaccharides migrated more slowly compared with their molecular mass (Fig. 1).

The formation of borate–polyol complexes was favoured with increasing concentrations of sodium tetraborate, which positively affected the electrophoretic mobility of the carbohydrates. As a consequence, the MT increased by a factor of 2 for raffinose, 2.2 for stachyose and 2.3 for verbascose when the borate concentration was increased from 20 to 100 mM. Simultaneously the number of plates/m increased by a factor of 1.7–2.0, while the R_s of raffinose and stachyose rose 3.9 times and that of stachyose and verbascose 3.3 times. The carbohydrate signals were hardly detectable at 10 mM borate and the absolute peak areas increased considerably with increasing borate concentrations. The NA values were also positively affected by increasing borate concentration, and increased by a factor of about 1.5 over the whole concentration range (20–100 mM). Hence borate complexation is important for both separation and detection [15]. At concentrations ≤60 mM verbascose co-eluted with a false peak that appeared in every electropherogram. Therefore, either 80 or 100 mM sodium tetraborate was used. At higher concentrations the analyses proceeded too slowly.

Electropherograms of sufficiently high quality were only obtained within the pH range 9.2–10.3. This pH was required for sufficient complex formation, and at lower and higher pH the noise of the baseline increased unacceptably. Two series of measurements were performed, where the pH was increased within the ranges 9.2–9.9 and 9.7–10.3. MT (Fig. 2a), NA and R_s (Fig. 2b) increased when the pH of the electrolyte was increased. The number of theoretical

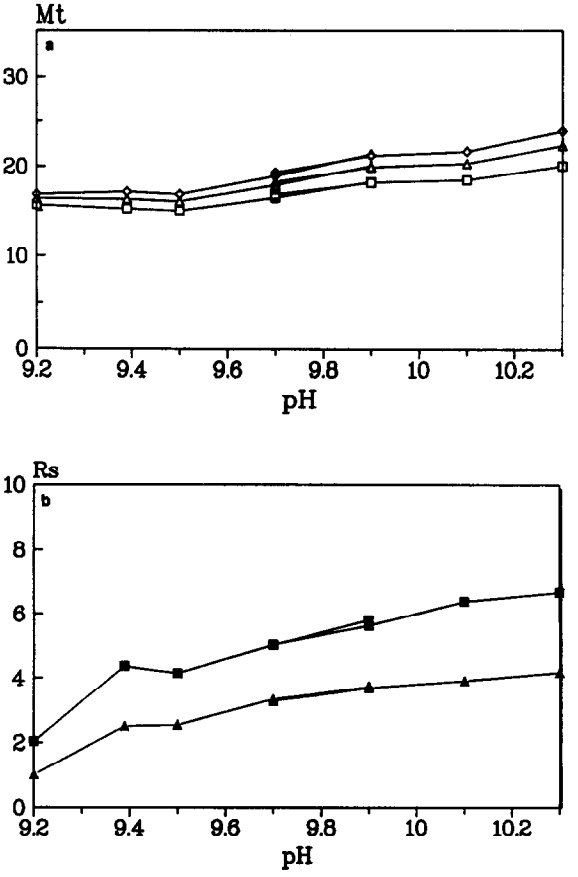

Fig. 2. (a) Migration times (MT, min) for (□) raffinose, (△) stachyose and (◇) verbascose and (b) resolution (R_s) of (■) raffinose and stachyose and (▲) stachyose and verbascose as a function of pH of the borate electrolyte (80 mM $Na_2B_4O_7$, 10 kV, 60°C).

plates/m also rose with increase in pH, but only small effects were observed within the pH range 9.4–10.1, and at pH 10.3 there was a tendency for a decline. The electropherogram at pH 9.2 was of poor quality because of noise. At pH 9.4, which was the pH of the dissolved $Na_4B_4O_7$, the baseline was planar. The baseline started to slant with increase in pH, especially when the voltage was also increased. A convenient compromise between the utilization of the advantageous effect of higher pH on separation and detection on the one hand and acceptable migration times and slope of the baseline on the other was found to be pH 9.9.

The migration times decreased by a factor of

about 1.5 when the temperature was increased from 30 to 60°C (Fig. 3a). Within the same temperature range *NA* increased by a factor of 1.4–2.0 owing to the injection of larger sample volumes because of the reduced viscosity of the electrolyte (Fig. 3b). The number of theoretical plates/m and the resolution between the oligosaccharides decreased with increasing temperature (Fig. 3c and d). In contrast, an increase in the resolution of borate–monosaccharide complexes as a function of the temperature was reported in another study [15]. Usually band broadening becomes more pronounced at higher temperatures owing to diffusion, but it was argued that the enhanced rates of complex formation made the compounds move in narrower zones, which compensated for the diffusion effect. This observation could not be verified in this study. The formation of hemiacetals seemed to be strongly involved in the formation of borate–monosaccharide complexes [15]. For non-reducing sugars such as sucrose and oligosaccharides of the raffinose family, however, this reaction can be excluded. The rate of ring opening may depend more on the temperature than the reaction rate for borate–polyol complex formation. Hence the optimum temperature for the CZE analysis of reducing sugars seems to be higher than for non-reducing sugars.

An increased voltage resulted in decreased *MT* and higher *NA* (especially for verbascose), whereas almost constant plate numbers were observed within the range 10–15 kV, with a decline at 18–20 kV (Fig. 4). The resolution was relatively constant, with a declining tendency, when the voltage was increased from 10 to 20 kV.

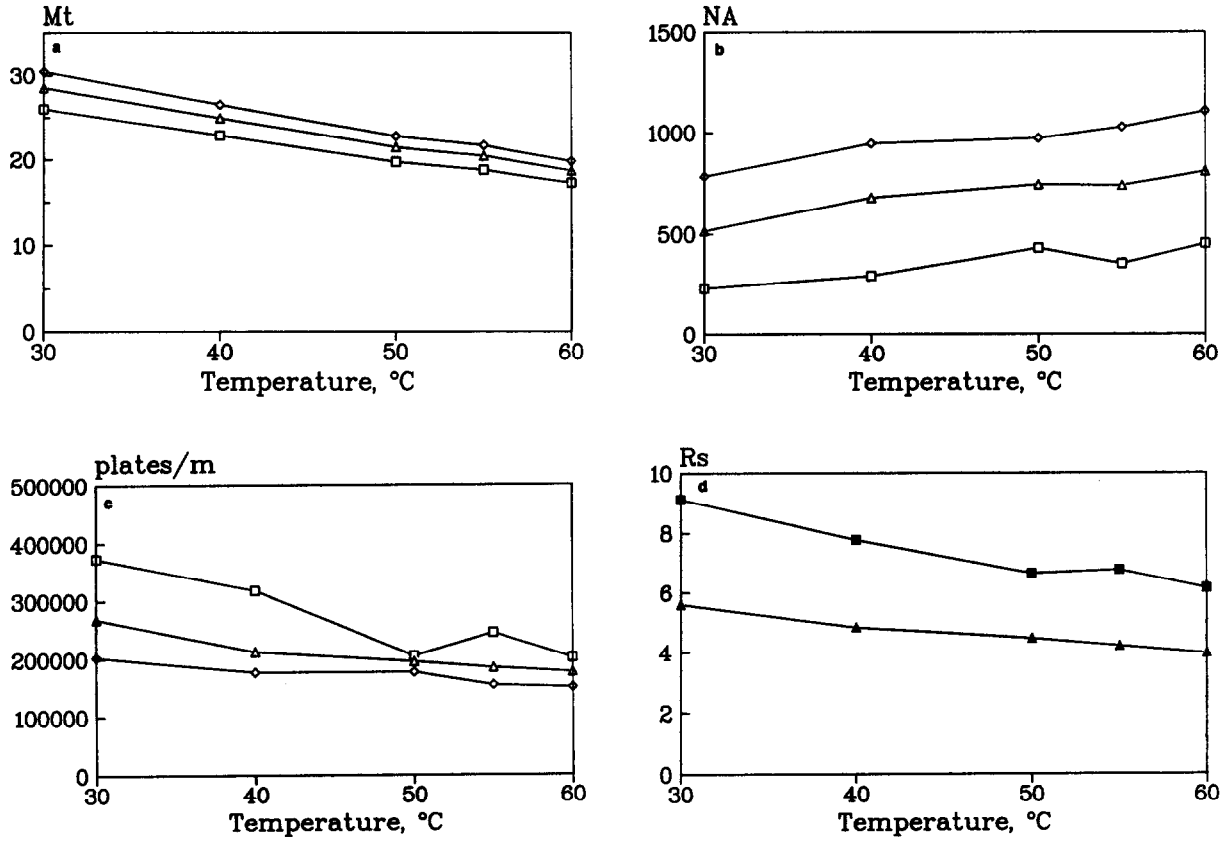

Fig. 3. (a) Migration times (*MT*, min), (b) normalized areas (*NA*) and (c) number of theoretical plates per metre (plates/m) for (□) raffinose, (△) stachyose and (◇) verbascose and (d) resolution (R_s) of (■) raffinose and stachyose and (▲) stachyose and verbascose as a function of temperature (100 m*M* $Na_2B_4O_7$, pH 9.4, 10 kV).

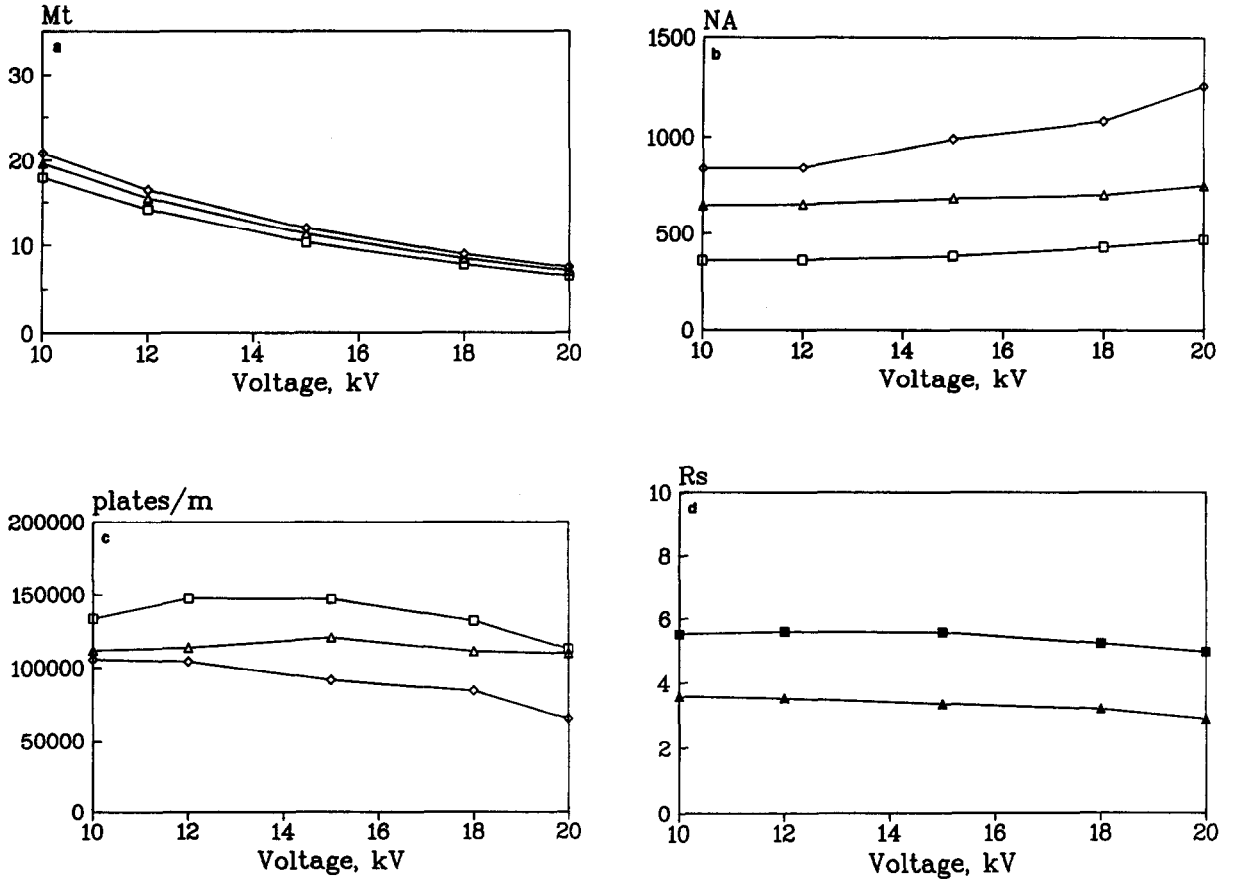

Fig. 4. (a) Migration times (*MT*, min), (b) normalized areas (*NA*), (c) number of theoretical plates per metre (plates/m) for (□) raffinose, (△) stachyose and (◇) verbascose and (d) resolution (R_s) of (■) raffinose and stachyose and (▲) stachyose and verbascose as a function of voltage (80 m*M* $Na_2B_4O_7$, pH 9.9, 60°C).

The baseline became inclined and more noise was observed when the voltage was increased. The most appropriate voltage was found to be 10 kV.

2-Propanol (0–15%) was added to the electrolyte as a modifier. Improved resolution was obtained, but at the same time *NA* and the number of plates/m decreased and MT increased. The reduced number of theoretical plates was a result of asymmetric peaks (fronting) with a broad base. The modifier could be used at concentrations up to about 8% without affecting the peak shape seriously.

Linear calibration graphs were obtained for sucrose and individual oligosaccharides with correlation coefficients (r) between 0.99 and 1.00. Lactose was eluted as a broader peak than sucrose and oligosaccharides of the raffinose family, and the correlation coefficients were generally lower for this compound (0.97–1.00).

Lactose and melibiose, which were both absent from the plant material studied, were chosen as internal standards. For samples containing considerable amounts of ajugose the choice of lactose is not ideal, as the compounds migrated with almost identical velocities through the capillary (Fig. 1). However, the ajugose content in peas is usually negligible, and the inclusion of the second internal standard melibiose in the samples allowed correction in those few instances where a contribution to the peak area from ajugose could not be excluded. Disadvantages of the use of melibiose are its higher migration time compared with the

oligosaccharides, prolonging the time of analysis, and its relatively broad peak shape. The peak is well defined, however, and does not co-elute with sample carbohydrates or any of the reference compounds tested. Suitable alternatives to the chosen internal standards with good absorbance properties at 195 nm and with *MT*s lower than that of sucrose are required.

Relative response factors (*RRF*) were calculated by dividing the slope of the calibration graph for lactose by the corresponding slope for individual analytes. Mean *RRF* values calculated on the basis of thirteen calibration graphs are given in Table I. Decreasing *RRF* values were observed when the number of galactose units per molecule increased, *i.e.*, higher detector signals were recorded per molecule as a function of the length of the α-galactoside chain in a non-linear manner. Thus, a *ca.* fourfold decrease was observed on going from sucrose to raffinose. The second and third galactose units caused smaller decreases in the *RRF*.

The relative standard deviation (R.S.D.) of the relative response factors describes the accuracy of determinations of individual compounds (Table I). The highest R.S.D. was found for sucrose (7.5%), which also has the lowest UV absorption and electrophoretic mobility, because the structure of sucrose does not favour complex formation with borate. The R.S.D.s for raffinose, stachyose and verbascose ranged from 3.5 to 5.4%.

TABLE I

MEANS AND VARIATION OF RELATIVE RESPONSE FACTORS (*RRF*) FOR SUCROSE AND OLIGOSACCHARIDES USING CAPILLARY ZONE ELECTROPHORESIS

The slopes of calibration graphs ($n = 13$) were used for the calculation of *RRF* [=slope of graph for the internal standard (lactose)/slope for the analyte]. Separation conditions: 100 m*M* $Na_2B_4O_7$, pH 9.9, 50°C, 10 kV. S.D. = standard deviation; R.S.D. = relative standard deviation = S.D./mean *RRF*.

Analyte	*RRF*	S.D.	R.S.D. (%)
Sucrose	5.15	0.39	7.5
Raffinose	1.23	0.05	4.1
Stachyose	0.76	0.03	3.5
Verbascose	0.60	0.03	5.5

CONCLUSIONS

Repeated homogenizations in boiling methanol–water (7:3) ensured efficient extraction of oligosaccharides and other low-molecular-mass sugars from mature peas. Moreover, no artifact formation was observed with the procedure owing to immediate inactivation of glycosidases. The introduction of the internal standards lactose and melibiose prior to extraction allowed corrections for differences among samples in injection volume.

Compounds with positive or negative net charges at relatively extreme pH values were removed by group separation, as the seed extracts were purified using a strongly acidic cation exchanger, which was connected with a strongly basic anion exchanger. This procedure was essential to obtain high-quality electropherograms by CZE analysis.

The determination of oligosaccharides by CZE was based on the formation of borate–carbohydrate complexes. Increasing borate concentration (20–100 m*M* $Na_2B_4O_7$) and pH favoured the complex formation, which improved the UV absorption at 195 nm and increased the electrophoretic mobility of the compounds, leading to improved separation and longer *MT*s. The running conditions that were found to provide the best compromise between acceptable separation and detection efficiency and duration of analysis were 100 m*M* $Na_2B_4O_7$, pH 9.9, 50°C, 10 kV and omission of 2-propanol modifier. Under these conditions about 144 000, 105 000, 84 000 and 74 000 plate/m for sucrose, raffinose, stachyose and verbascose, respectively, were obtained. With other combinations of running conditions up to about 375 000 plates/m were obtained for raffinose. The number of theoretical plates/m that can be obtained by HPLC using amino-bonded silica was at least one order of magnitude lower for sucrose and raffinose compared with the CZE method. Other advantages of the CZE procedure over the widely used HPLC methods based on refractive index detection are

the use of on-column UV detection, low cost per analysis, ease of operation and the use of non-toxic chemicals. Moreover, rapid analyses are possible because of the relatively short migration times of the non-reducing oligosaccharides. Hence the CZE method presented provides a good alternative to existing methods for the determination of oligosaccharides of the raffinose family, and it can be adapted for the determination of other low-molecular-mass carbohydrates.

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