ELSEVIER

Journal of Chromatography A, 804 (1998) 311–318

JOURNAL OF CHROMATOGRAPHY A

Ion chromatographic analysis of nutrients in seed exudate for microbial colonisation

C.E. Casey[a], O.B. O'Sullivan[b], F. O'Gara[b], J.D. Glennon[a,*]

[a]*Chemistry Department, University College Cork, Cork, Ireland*
[b]*Microbiology Department, University College Cork, Cork, Ireland*

Abstract

Information on the composition, levels, availability and microbial utilisation of the nutrients present in root and seed exudates is invaluable in harnessing selected microbes as biocontrol agents. The complex matrix that is sugar beet seed exudate is examined utilising the chromatographic modes of ion-exclusion–partition and ion-exchange. Many of the carbohydrates, organic acids and inorganic anions present there are identified. Specifically, collected seed exudate samples are analysed on an Aminex HPX-87H cation-exchange chromatography column with a dilute sulphuric acid mobile phase and refractive index detection. Improved resolution for the organic acids present (succinate, oxalate, formate, acetate) is achieved by a pre-treatment extraction on SAX cartridges prior to this chromatographic analysis. The inorganic anions fluoride, chloride, nitrate, phosphate and sulphate are directly determined alongside oxalate by ion chromatography on a Dionex IonPac AS4A column with isocratic elution and suppressed conductivity detection. In addition, the sugars (galactose, glucose, mannose, lactose, raffinose, maltose) can be analysed by high-performance anion-exchange chromatography with pulsed amperometric detection.

Keywords: Plant materials; Sugar beet; Organic acid; Carbohydrates; Inorganic anions

1. Introduction

There is increasing demand for the characterisation and quantification of substrates and metabolites in complex biological matrices. Specifically, in biotechnology, microbes can be used as soil inoculants to biocontrol plant pests and promote plant growth. In the soil, the area in the immediate vicinity of the root (rhizosphere) or seed (spermosphere) is a very nutrient-rich environment due to a range of compounds leaching from the root or seed into the surrounding soil. The composition of plant root exudate varies with plant species, and with other extrinsic factors such as soil type and available nutrients in soil. These exudates generally consist of carbohydrates, amino acids, vitamins, organic acids and other miscellaneous compounds found in minute concentrations [1–3]. It has been shown using HPLC that microbes grow relatively fast even in the presence of low environmental substrate concentrations, through the simultaneous utilisation of mixtures of carbon sources [4]. These exudates are readily available as nutrients for microorganisms and are considered to be one of the main reasons for increased microbial numbers and activity in the spermosphere or rhizosphere.

Chemotaxis, a response by soil microflora to

*Corresponding author.

0021-9673/98/$19.00
PII S0021-9673(97)01297-1

chemical stimuli, is a factor which largely influences the microbial colonisation of the rhizosphere [5]. Plant exudates have been shown to serve as chemoattractants to certain bacterial strains. Therefore, specific information about the constituents of plant exudates is invaluable in understanding the successful colonisation of the seed or root by bacteria.

Anti-microbial and secondary metabolite production by colonising bacteria has also been shown to be influenced by certain carbon sources. James and Gutterson [6] found that production of an antimicrobial metabolite by *Pseudomonas fluorescens* HV37a required the presence of glucose but that production of two other biologically active metabolites by the same strain was inhibited by glucose. The antifungal compound 2,4-diacetylphloroglucinol (DAPG) (2,4-diacetyl-1,3,5-trihydroxybenzene) [7], produced by *Pseudomonas fluorescens* F113, has been isolated in this laboratory by solid-phase extraction (SPE) and medium-pressure liquid chromatography, and the physiological parameters influencing its production investigated [8]. When Shanahan et al. [9] investigated the influence of different carbon sources on the production of DAPG, it was found that DAPG production increased in the presence of fructose, sucrose and mannitol, whereas yields of DAPG were reduced when cells were incubated in glucose or sorbose. Therefore, identification of the composition of plant exudates is important to ensure maximal production of secondary metabolites by microbial inoculants, some of which can be involved in disease suppression and growth promotion of the host plant.

In this work, sugar beet seed exudate is selected for study over root exudate, as fungal hyphae of *Pythium ultimum*, the causative agent of damping-off, colonise sugar beet seed soon after sowing and any biocontrol protection needs to be exerted at this early seed stage. It has been shown that the first step in bacterial root colonisation is the rapid colonisation of the germinating seed which profusely exudes a wide range of amino acids, carbohydrates and organic acids as well as other compounds, during germination [10]. Ion-exclusion–partition and ion-exchange chromatographic techniques are used to profile the components of sugar beet seed exudate, focusing the search on the carbohydrate, organic acid and anion content.

2. Experimental

2.1. Chemicals

Sodium hydroxide (50%, w/v) was obtained from Reagecon (Shannon, Ireland). All stock carbohydrate standards (D-arabinose, D-fructose, D-galactose, D-glucose, D-mannose, D-xylose, L-sorbose, D-mannitol, D-ribose, sucrose, maltose monohydrate, lactose monohydrate) were obtained from Sigma (St. Louis, MO, USA). A five-anion standard containing fluoride (20 mg/l), chloride (30 mg/l), nitrate (100 mg/l), phosphate (150 mg/l) and sulphate (150 mg/l) was obtained from Dionex (UK). Sodium formate and succinic acid were obtained from BDH (Poole, UK). Tri-sodium citrate was acquired from Wardle (Cheshire, UK).

2.2. Preparation of sugar beet seed exudate

Sugar beet seeds (500 g, *Beta vulgaris* cv. ''Rex'') were added to 1 l of deionised water and shaken for 3 h at room temperature. The resultant liquid was gravity filtered through Whatman filter paper, followed by successive filtration through Whatman filters of 5.0-μm, 3.0-μm, 1.2-μm, 0.45-μm and 0.2-μm pore size, under vacuum to avoid filter blocking. This seed exudate was then filter-sterilised through Whatman 0.45-μm and 0.2-μm filters under vacuum using autoclaved filters and apparatus, and stored at −20°C. In addition, a lyophilised seed exudate sample was prepared for CHN analysis. It should be noted that seeds were not sterilised prior to exudate preparation to avoid sterilisation washes with methanol and hypochlorite, which would potentially remove components from the exudate.

2.3. Measurement of free amino acids

A modified Cd–ninhydrin method [11] was used. A 100-μl sugar beet seed exudate sample was diluted to 1 ml with deionised water. Cd–ninhydrin reagent (2 ml) was added to this and the mixture was heated at 84°C for 5 min, cooled and the absorbance measured at 507 nm. Concentrations were derived from a standard curve of D-alanine and analyses were carried out in triplicate.

2.4. Osmolarity and pH

Osmolarity was measured on an Advanced Instruments osmometer (Model 3MO) and pH was measured on a Corning pH meter (Model 220).

2.5. SPE on ODS cartridges

Sep-Pak C_{18} (360 mg) cartridges (Waters–Millipore, Millford, MA, USA) were preconditioned by passing 10 ml methanol through, followed by 10 ml deionised water. Aliquots of collected sugar beet seed exudate samples (1 ml), were applied to the cartridge where necessary and the eluents collected for chromatographic analysis. The cartridges were washed with 1-ml quantities of deionised water and any retained solutes eluted with methanol.

2.6. Ion-exchange SPE

Bond Elut SAX (100 mg) cartridges (Varian, CA, USA) containing trimethylaminopropyl chloride were wetted by passing 10 ml methanol through followed by 10 ml deionised water. Aliquots of sugar beet seed exudate (1 ml) were applied to the cartridges and the unretained components including carbohydrates collected. The cartridges were each washed with 1 ml deionised water and the retained organic acids and anions eluted with 1 ml 1 *M* hydrochloric acid. This ion-exchange SPE pre-treatment step was only of benefit prior to analysis by ion-exclusion–partition chromatography on the Aminex HPX-87H column.

2.7. Instrumentation

Collected seed exudate samples were analysed on an Aminex HPX-87H cation-exchange column (30 cm×7.8 mm I.D., Bio-Rad Labs., Richmond, CA, USA) using a LKB Bromma 2150 pump, a 20-μl Rheodyne injection valve and a Shodex RI-71 refractive index detector. The column was operated at 60°C and 0.005 *M* sulphuric acid was used as the mobile phase at a flow-rate of 0.6 ml/min.

High-performance anion-exchange chromatography–pulsed amperometric detection (HPAEC–PAD) analysis was performed on a Dionex system (Sunnyvale, CA, USA) utilising a gradient pump module and PAD with a gold working electrode. Neutral monosaccharides were separated on a Dionex CarboPac PA1 pellicular anion-exchange resin (25 cm×4 mm I.D.) and guard column at a flow-rate of 1.0 ml/min at ambient temperature. The pulse potential (E) and duration (t) used for detection were: $E_1=+0.2$ V ($t_1=440$ ms); $E_2=+0.8$ V ($t_2=180$ ms); $E_3=-0.3$ V ($t_3=360$ ms) [12]. The mobile phase employed was 50 m*M* sodium hydroxide prepared by dilution of 50% (w/v) sodium hydroxide solution with deionised water (unless otherwise stated).

Ion chromatography was performed using a Dionex IonPac AG4A-SC guard column (5 cm×4 mm I.D.) and a Dionex IonPac AS4A analytical column (25 cm×4 mm I.D.) in series for the separation of inorganic anions. A Dionex anion self-regenerating suppresser (ASRS-I, 4 mm) was used to reduce background conductivity. A Dionex gradient pump module was used in conjunction with a Dionex series 4000I conductivity detector. The mobile phase was 1.7 m*M* Na_2CO_3, 1.8 m*M* $NaHCO_3$ prepared from dissolving sodium carbonate and sodium bicarbonate in 1 l deionised water. Calibrations were performed by analysis of different concentration levels of standard mixtures, each injected six times. Calibration functions were calculated using curve fitting by means of linear regression analysis.

Flame atomic absorption spectroscopy (FAAS) was carried out on a Pye Unicam SP9 atomic absorption spectrometer using air/acetylene to determine the iron content in the seed exudate.

3. Results and discussion

The general properties of the seed exudate are of interest to biotechnologists, as they would influence its ability to act as a growth medium for bacteria. In addition, it is helpful to know the nature of the sample matrix being analysed. The seed exudate is a low-iron media, as verified by atomic absorption spectroscopy (AAS) to be 0.096 ppm. It is yellow–brown in colour, with a pH of 6.46±0.02 and an osmolarity of 117.7±1.2 mosmols. The amino acid concentration was estimated using the Cd–ninhydrin

test to be 0.38± 0.06 mg/ml. Carbon, nitrogen and hydrogen content of lyophilised sugar beet seed exudate was determined to be 19.67, 5.45 and 2.87% (w/w), respectively.

It was observed that when sugar beet seed exudate solutions were left to stand for long periods, there was an increase in exudate colour to dark brown and on long standing some precipitation occurred. This browning reaction is likely due to carbonyl amino acid-type reactions, which include the reaction of aldehydes, ketones and reducing sugars with amines, amino acids, peptides and proteins (termed the Maillard reaction), and/or oxidative type reactions in which ascorbic acid and polyphenols are converted into di- or polycarbonyl compounds [13]. Experiments carried out in this laboratory show that an increase in pigment colour occurs only when oxygen is present but that the role of light is minimal. As it is recognised that some deterioration inevitably occurs during sample collection and that such deterioration of the sample will change sample composition, solution standing time was minimised and frozen storage introduced. Any pigmentation present in the exudate samples could be removed using SPE pre-treatment on ODS cartridges. These cartridges retain coloured lipophilic and aromatic compounds [14], allowing polar compounds such as carbohydrates, organic acids and anions to pass through. This pre-treatment step proved to be useful in removing pigmentation prior to the determination of bacterial cell growth curves from optical density measurements, in follow-up studies on microbial utilisation of exudate constituents. The pre-treatment step was also used initially in this work prior to chromatographic analysis to protect the column from contamination by impurities and reduce baseline drift. Subsequently, it was deemed redundant in the chromatographic analysis as pigmentation was kept to a minimum.

Seed exudate samples were first analysed on the Aminex cation-exchange column with a dilute sulphuric acid mobile phase and refractive index detection. Large molecules such as proteins or polysaccharides are excluded from the pores and elute with the void volume. For organic acids, the degree of retention is determined by the pK_a values of the acids and the proton concentration in the mobile phase, with mechanisms of retention involving, to varying degrees, ion-exclusion, hydrophobic interactions and Van der Waals forces. The degree of retardation increases with decreasing degree of ionisation. The relevant pK_a values are given in Table 1, alongside retention data obtained on injection of standard solutions of a variety of solutes. Members of a homologous series, such as formic, acetic and propionic acids elute in order of decreasing acid strength. Dibasic acids elute sooner than monobasic acids of the same carbon number e.g., oxalic and succinic acids emerge ahead of acetic and propionic acids (Table 1). A typical chromatogram is that obtained for a synthetic mixture of citrate, succinate and lactate in the presence of glucose and fructose, as shown in Fig. 1a.

Injection of the seed exudate yields a complex chromatogram containing a number of peaks in which inorganic anions elute at the void volume, sugars elute between 8 and 12 min, with disaccharides emerging first, followed by monosac-

Table 1
Retention times (t_r) and ionisation constants, pK_a, of carbohydrates and organic acids on Aminex HPX-87H column

No.	Compound	t_r (min)	pK_a^a
1	Oxalate	6.93	1.25
2	Citrate	9.00	3.13
3	Succinate	12.66	4.21
4	Lactate	13.09	3.79
5	Formate	15.25	3.74
6	Acetate	16.07	4.76
7	Fluoride	6.34	–
8	Chloride	6.32	−2.20
9	Nitrate	6.37	−1.30
10	Phosphate	9.19	2.15
11	Sulphate	n.d.	–
12	Glycerol	14.98	–
13	Mannitol	11.22	–
14	Rhamnose	11.34	–
15	Arabinose	11.98	12.43
16	Galactose	10.82	12.39
17	Glucose	9.93	12.28
18	Mannose	10.77	12.08
19	Xylose	10.80	12.15
20	Fructose	10.81	12.03
21	Sorbose	10.22	–
22	Ribose	12.27	12.21
23	Lactose	8.32	11.98
24	Raffinose	8.03	–
25	Maltose	8.11	11.94

[a] pK_1 values only.

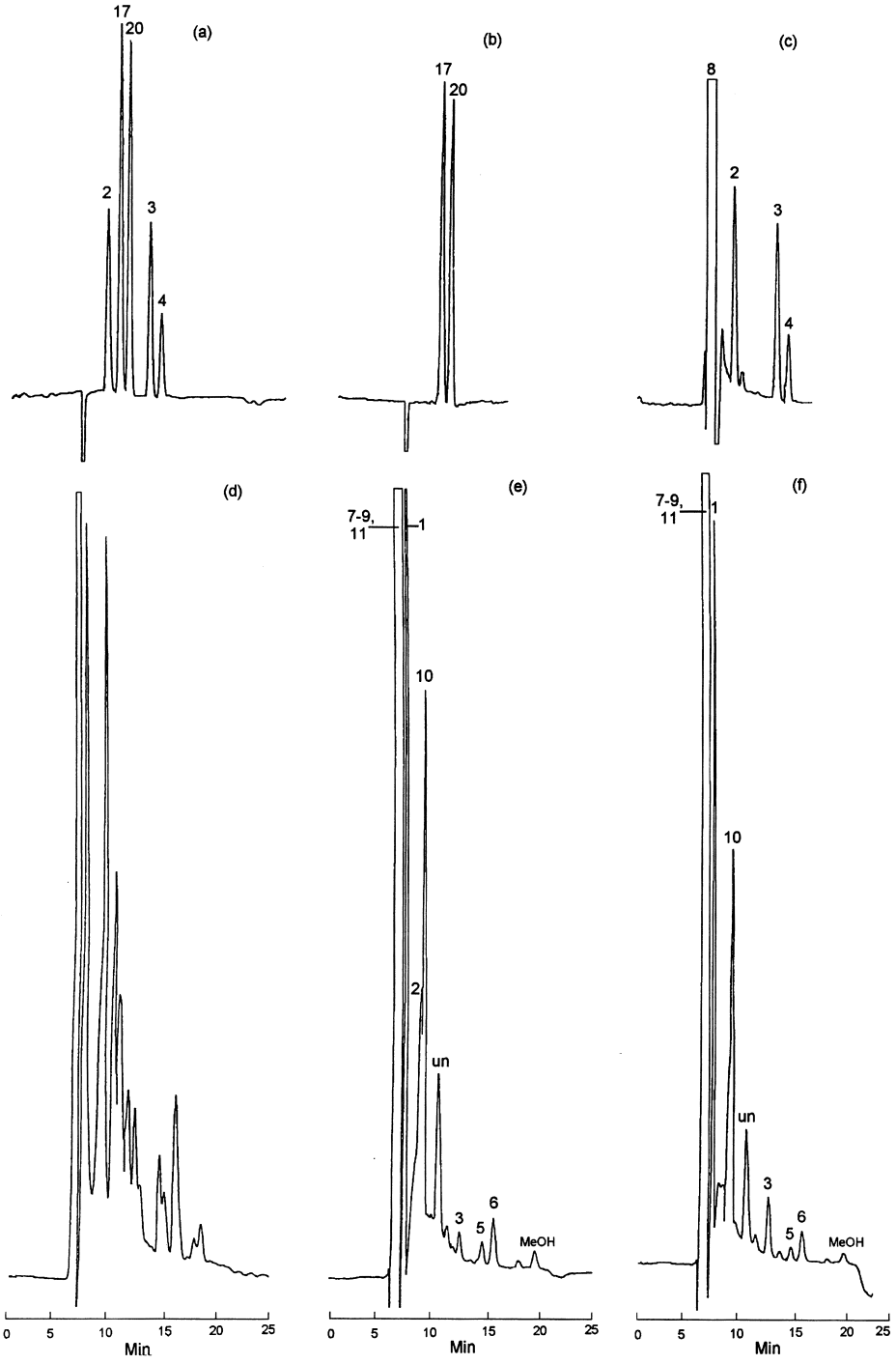

Fig. 1. Chromatographic analysis on an Aminex HPX-87H column: (a–c) use of SAX cartridge for organic acid trapping, (a) standard mixture before SPE, (b) unextracted sugar standards, (c) organic acids eluted with HCl; (d) seed exudate, (e) seed exudate eluted from SAX cartridge with HCl, (f) eluate (e) spiked with succinate. Conditions: eluent 0.005 *M* H_2SO_4, flow-rate 0.6 ml/min, temperature 60°C, refractive index detection. Peak numbers refer to Table 1.

charides, and weak acids eluting between 7 and 19 min. As can be seen from Fig. 1d, the chromatogram is quite complicated between 8 and 20 min with poor resolution due to the co-elution or overlap of a number of matrix components interfering with the identification and quantification of the peaks of interest. Improved selectivity for organic acids can however be achieved by first passing the exudate sample through an SAX cartridge. SAX cartridges retain such solutes as carboxylic acids, phosphates and inorganic anions, whereas carbohydrates pass through unretained (Fig. 1a–c). This SPE pre-treatment of the exudate samples increases the selectivity of the chromatographic method for organic acids (Fig. 1e) and in particular, oxalate, succinate, formate and acetate are identified. Subsequent ion chromatographic analysis of collected fractions confirms the presence of phosphate. The benefit of the pre-treatment is particularly well illustrated for succinate, which appeared only as a shoulder in Fig. 1d. These identifications were confirmed by careful spiking experiments, as shown for succinate in Fig. 1f.

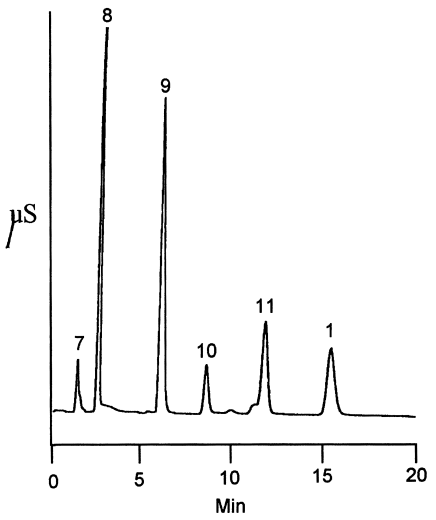

Fig. 2. Anion analysis of injected seed exudate by ion chromatography. Conditions: Dionex IonPac AS4A column and AG4A-SC guard column, eluent 1.7 m*M* Na_2CO_3, 1.8 m*M* $NaHCO_3$, flow-rate 1.0 ml/min, ASRS-I conductivity detection. Peak numbers refer to Table 1.

Furthermore, with the small aliphatic organic acids, acetate and formate already analysable on the Aminex column and in order to avoid the use of gradient elution [15], the direct ion chromatographic analysis of the inorganic anion content of the seed exudate using isocratic elution with a carbonate–hydrogencarbonate eluent and suppressed conductivity detection was carried out (Fig. 2). Seed exudate was shown to contain five anions identified as fluoride (50 ppm), chloride (733 ppm), nitrate (1014 ppm), phosphate (388 ppm), sulphate (334 ppm) and one organic acid, oxalate (514 ppm). This resolution is achieved without any added selectivity from SPE pre-treatments, as required above.

In addition, the carbohydrates present in the seed exudate are directly analysable by HPAEC–PAD. The carbohydrates are weak acids and at high pH, are partially ionised and thus, can be separated by anion-exchange methods [16]. The general elution order is sugar alcohols, monosaccharides, disaccharides and oligosaccharides. Fig. 3a shows the elution profile of sugar beet seed exudate obtained at 50 m*M* NaOH. Although a number of sugars are identified at this eluent concentration (namely rhamnose, lactose, raffinose and maltose), many of the monosaccharides co-elute namely, glucose/mannose/xylose and fructose/sorbose. In addition, galactose and arabinose are overlapping. To improve resolution, the eluent concentration was decreased to 5 m*M* NaOH. From Fig. 3b and c, it can be seen that decreasing NaOH concentration leads to increased capacity factors for the monosaccharides. At this concentration, galactose, glucose and mannose are now resolved, xylose is partially resolved but fructose and sorbose still co-elute. Also, the order of elution for arabinose and rhamnose is reversed at this concentration. The carbohydrate peaks were identified by spiking a sample with standard solutions.

4. Conclusion

The incorporation of selective SPE pre-treatment prior to analysis by ion-exclusion–partition chromatography, and the use of selective electrochemical detection with ion-exchange chromatographic techniques, allows the carbohydrate, organic acid and anion content of seed exudate to be determined. The information provided is of particular interest to biotechnologists involved in the area of biocontrol of

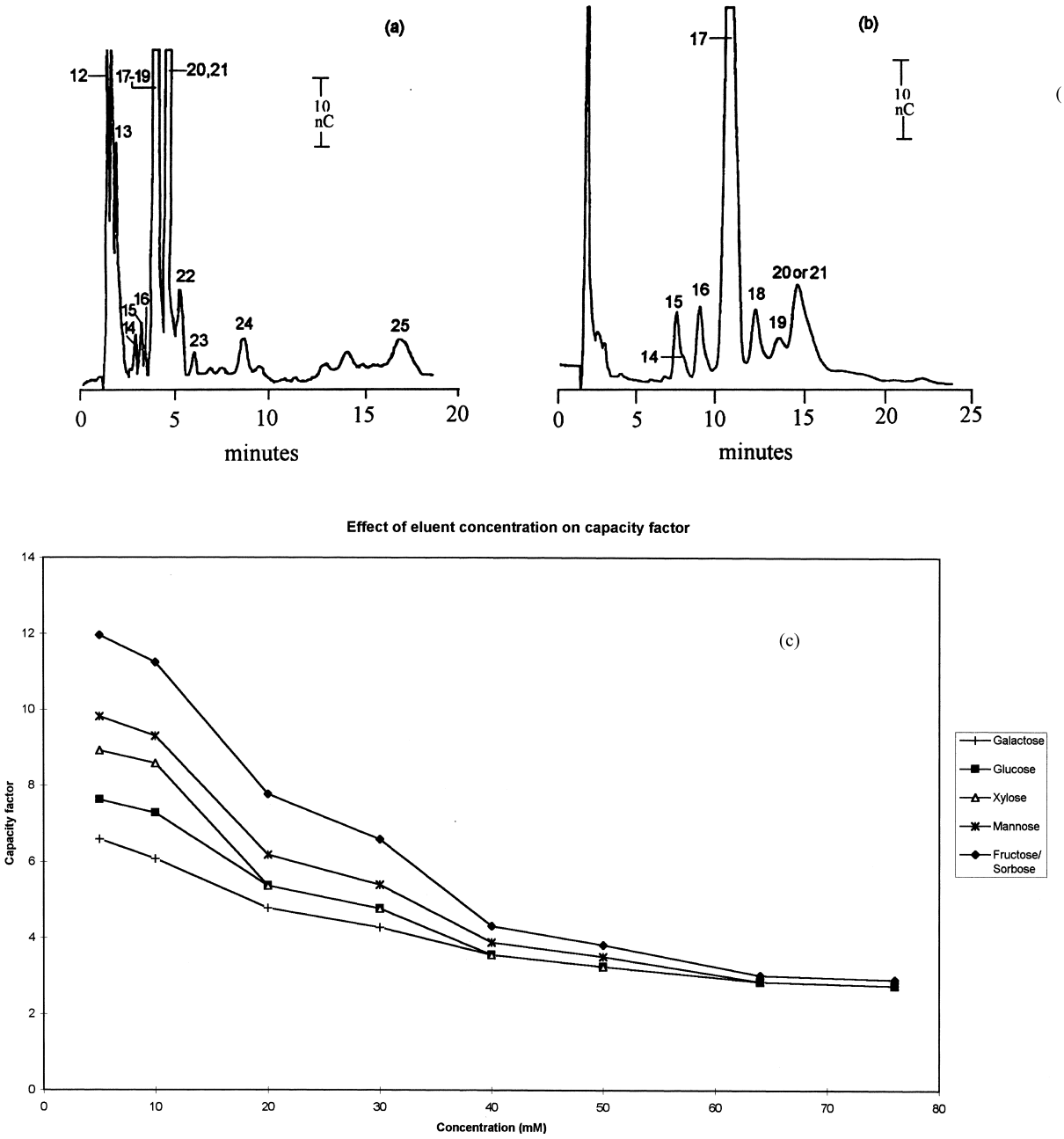

Fig. 3. Chromatographic profile (HPAEC–PAD) of seed exudate sugars, unretained on the SAX cartridge, obtained using: (a) 50 m*M* NaOH, (b) 5 m*M* NaOH and (c) capacity factors plotted against eluent NaOH concentration. Conditions: Dionex CarboPac PA1 column, eluent NaOH, flow-rate of 1.0 ml/min, PAD with a gold working electrode. Peak numbers refer to Table 1.

plant pathogens by bacterial inoculation in the rhizosphere or spermosphere, allowing the levels of nutrients and the bacterial consumption of carbon sources to be monitored.

Acknowledgements

This work was supported in part by grants from the European Commission (BIO2-CT93-0053, BIO4-CT96-0027, BIO2-CT93-0196, BIO2-CT92-0084, BIO4-CT97-2227, BIO4-CT96-0181, FMRX-CT96-0039).

References

[1] A.D. Rovira, Bot. Rev. 35 (1969) 35.
[2] V. Vancura, A. Hovadik, Plant Soil XXII 1 (1965) 21.
[3] S. Mehta, S.A. Sharma, G.S. Sindhan, Indian J. Mycol. Pl. Pathol. 22 (1992) 227.
[4] U. Lendenmann, M. Snozzi, T. Egli, Appl. Environ. Microbiol. 62 (1996) 1493.
[5] G.L. Hazelbaeur, Can. J. Microbiol. 34 (1988) 466.
[6] D.W. James, N.I. Gutterson, Appl. Environ. Microbiol. 52 (1986) 1183.
[7] C. Keel, P.H. Wirthner, T.H. Oberhansli, C. Voisard, U. Burger, D. Haas, G. Defago, Symbiosis 9 (1990) 327.
[8] P. Shanahan, A. Borro, F. O'Gara, J.D. Glennon, J. Chromatogr. 606 (1992) 171.
[9] P. Shanahan, D.J. O'Sullivan, P. Simpson, J.D. Glennon, F. O'Gara, Appl. Environ. Microbiol. 58 (1992) 353.
[10] W.H. Lynch, M. Franklin, Can. J. Microbiol. 24 (1978) 56.
[11] B. Folkertsma, P.F. Fox, J. Dairy Res. 59 (1992) 217.
[12] W.A. Lacourse, D.C. Johnson, Anal. Chem. 65 (1993) 50.
[13] J.E. Hodge, J. Agric. Food Chem. 1 (1953) 928.
[14] N.S. Noura, J.A. Kuhnle, H.W. Hilton, Int. Sugar J. 86 (1984) 244.
[15] G. Saccani, S. Gherardi, A. Trifiro, C. Soresi Borbini, M. Calza, C. Freddi, J. Chromatogr. A 706 (1995) 395–403.
[16] A. Henshall (Editor), Analysis of Carbohydrates by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC–PAD), LPN 032857-02, Dionex, Sunnyvale, CA, 1995.