Journal of Chromatography, 479 (1989) 353–360
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 717

SEPARATION OF ALGAL ORGANIC OSMOLYTES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

N. W. KERBY*, R. H. REED and P. ROWELL
A.F.R.C. Research Group on Cyanobacteria and Department of Biological Sciences, University of Dundee, Dundee DD1 4HN (U.K.)
(Received April 20th, 1989)

SUMMARY

A method is described for the separation and quantification of a range of low-molecular-weight organic solutes, which are major osmolytes, in crude ethanolic extracts of marine algae. The method relies on the use of a silica amine modified column and refractive index detection. The acetonitrile to water ratio was varied to maximise separations. The method is suitable for the detection of simple carbohydrates, amino acids and their derivatives and is particularly suitable for the separation of the isomeric hexitols, altritol and mannitol, found in the brown alga *Himanthalia elongata*.

INTRODUCTION

Many phototrophic organisms, including algae, accumulate organic solutes as intracellular osmotica when grown under saline conditions. The accumulated organic compounds are often the major low-molecular-weight photoassimilate(s)[1] and include saccharides[2], heterosides[3], amino acids[4] or their methylated derivatives (betaines)[5] and sulphonium compounds[6]. Interest in the significance of low-molecular-weight organic solutes as osmotica in algal cells has led to the development of a range of procedures to identify and quantify these compounds. Natural abundance ^{13}C nuclear magnetic resonance (NMR) spectroscopy is the most appropriate technique to use for preliminary screening, since it identifies all organic solutes present in solution in osmotically significant amounts[7]. However, the insensitivity of ^{13}C NMR techniques necessitates either prolonged analysis times, prior concentration of algal material or access to a powerful, high-field strength spectrometer, making the technique less suitable for routine sample analysis. Consequently, such analysis and quantification of solutes is more frequently performed using one or more chromatographic procedures.

The chromatography of mono- and disaccharides has recently been reviewed[8]. Techniques have included thin-layer chromatography[9], column chromatography[10], gas–liquid chromatography[3], pyrolysis gas chromatography[11] and thin-layer electrophoresis/scanning reflectance densitometry[12]. More recently, high-performance

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liquid chromatography (HPLC) has been used to separate and quantify plant betaines and sulphonium compounds, *e.g.* β-dimethylsulphoniopropionate on a strong cation-exchange material with low-wavelength ultraviolet absorbance detection[13]. Analysis of low-molecular-weight carbohydrates by HPLC has been reviewed by Honda[14] and, unlike HPLC of amino acids and nucleosides, no general conditions have been established that allow the simultaneous analysis of all the different monosaccharides, due to the diversity of structures. Most conventional chromatographic procedures are time-consuming, requiring several purification steps prior to analysis and the organic solutes cannot always be recovered after separation and quantification. HPLC offers the potential for the non-destructive analysis of free (underivatised) solutes. However, separation of carbohydrates by normal-phase, reversed-phase and adsorption chromatography generally involves pre-column derivatisation. The need for derivatisation is usually related to the requirements of detection and not to altering the chromatographic separation of carbohydrates[8]. Reversed-phase chromatography is commonly used in the separation of simple sugars; both alkylated cyano-bonded and amino-bonded phases have been employed. Adding a small amount of a polyfunctional amine modifier to the mobile phase, to impregnate the silica column, overcomes the problems of the low solubility of carbohydrates and other solutes in the mobile phase and that of insufficient resolution[15]. Additionally, inclusion of aliphatic amines in the mobile phase ensures stability of the column by constantly renewing the surface of the stationary phase. However, silica columns modified in this way can undergo slow dissolution. Therefore, rigid walled steel columns are unsuitable for long term use, in contrast to plastic, radial compression silica columns.

The method described below combines the use of an aliphatic silica amine modifier (Waters SAM 1) with a radial compression silica cartridge and an isocratic separation system (acetonitrile–water–SAM), giving good separation of a range of algal organic osmolytes from crude ethanolic extracts, with quantification by refractometric detection. In contrast to earlier methods, the procedure is sufficiently simple and accurate to be used with large sample numbers, with automation.

MATERIALS AND METHODS

Sample collection and preparation

Prasiola stipitata Suhr in Jessen, *Enteromorpha intestinalis* (L.) Link, *Pelvetia canaliculata* (L.) Dcne et Thur. and *Himanthalia elongata* (L.) S. F. Gray were collected from Fife Ness (Nat. Grid Ref. NO639098), Fife, U.K. and were washed in filtered seawater prior to blotting dry. Samples (1 g) of algal vegetative tissue were extracted for 24 h in 5 ml 80% (v/v) ethanol at room temperature. Following freeze-drying of the ethanol extracts, the residue from each sample was dissolved in 100 μl of Milli Q water and centrifuged at 10 000 *g* for 5 min. Aliquots (10 μl) were loaded onto a Waters 10 cm × 8 mm Silica Pak cartridge (8SM HP4μ) fitted in a Waters Radial Compression Unit and were eluted at a flow-rate of 3 ml min^{-1} at room temperature. Solutes eluting from the column were detected using a refractometer, with the mobile phase as a reference. Fractions were collected, where necessary, using a Pharmacia Frac 100 fraction collector.

HPLC equipment

A Waters Maxima 820 chromatography workstation was used to collect data and control a Waters 510 pump. Low-molecular-weight organic compounds were detected with a Waters differential refractometer (R401) and the eluent was also monitored at 254 nm using a Waters LC spectrophotometer. Aliquots (10 μl) were injected onto the column via a Rheodyne injector.

Column conditioning and the mobile phase

Prior to analysis, columns were conditioned with 500 ml of a column conditioning solution, acetonitrile–water–SAM 1 reagent (Waters) (77:3:20, v/v/v) at a flow-rate of 1 ml min^{-1}. Following the first 50 ml, the solution was recycled.

The acetonitrile–water–SAM 1 ratio in the mobile phase was varied from 77:21:2 to 85:13:2 (v/v/v). Milli Q (Millipore) water was used throughout, the mobile phase was filtered through 0.22-μm Durapore filters and degassed by sonication in a water-bath (Cole Parmer) prior to use.

Chemicals

All chemicals were of the highest grade available and were purchased either from BDH (Poole, Dorset, U.K.), Millipore (U.K.) (Watford, U.K.) (for Waters products) or Rathburn Chemicals (Walkerburn, U.K.).

RESULTS

Various low-molecular-weight organic solutes, including carbohydrates, amino acids and betaine, are found in algae and, therefore, an HPLC method for the separation of such compounds is required. To investigate the effect of increasing the acetonitrile concentration in the mobile phase on the separation of a number of low-molecular-weight organic solutes (Table I) the acetonitrile concentration was varied from 77 to 85% (v/v) (Fig. 1). Increasing the acetonitrile concentration delayed the elution of individual solutes. Retention times for the individual components of the standard mixture are given in Table I. Mannitol and proline were incompletely resolved at 77% (v/v) acetonitrile. However, baseline separation was achieved when the acetonitrile concentration was increased to 82% (v/v). A further rise in acetonitrile concentration to 85% (v/v) resulted in inferior separation of these two solutes due to peak spreading. It should be noted that trehalose was not detected with a mobile phase containing 82 or 85% (v/v) acetonitrile and sucrose was also absent when 85% acetonitrile was employed (Fig. 1c, d; Table I). Therefore, optimal separation of diverse low-molecular-weight solutes, similar to those found as intracellular osmotica in algae, can be achieved by altering the water content of the mobile phase. A linear response for mannitol over the range 75–7500 nmol was found (data not shown), confirming that the method is suitable for quantitative analysis.

Fig. 2 shows the elution profile of an 80% (v/v) ethanolic extract of the green alga *Enteromorpha intestinalis* using 77% (v/v) acetonitrile in the mobile phase. The profile was characterised by a peak (retention time 3.8 min) which also had a correspondingly high UV absorption at 254 nm (data not shown) with identical chromatographic features to NaCl. This peak was detected in all marine algal samples tested and is due presumably to carry over of (sea)salt in marine samples. Ethanolic extracts

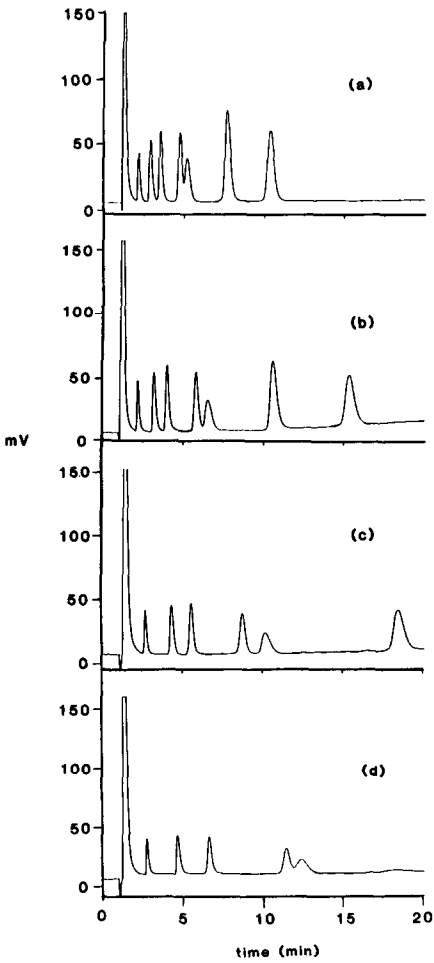

Fig. 1. Standards chromatographed using a mobile phase with increasing acetonitrile concentration. Organic solutes were detected by refractive index. (a) 77% (v/v), (b) 80% (v/v), (c) 82% (v/v), (d) 85% (v/v) acetonitrile. 500 nmol of each standard (see Table I) were applied in 10 μl of Milli Q water.

of fresh wateralgae did not contain this peak (data not shown). The peak eluting at 4.9 min was shown to be β-dimethylsulphoniopropionate (DMSP) by alkaline hydrolysis using 17% (w/v) NaOH and also using purified DMSP as a standard[16]. A small, broad peak which eluted at 7.6 min corresponded to sucrose. DMSP had previously been shown to be the major intracellular organic solute in *E. intestinalis*, with sucrose present as a trace constituent[17].

The separation of four intracellular organic solutes from ethanolic extracts of *Prasiola stipitata* using 80% (v/v) acetonitrile is shown in Fig. 2b. The peak at 4.9 min corresponded to salt and those at 4.2, 5.9, 7.0 and 11.6 min to ribitol, sorbitol, proline and sucrose, respectively. These solutes have previously been identified in marine algae by NMR (ref. 17, and our unpublished data). The two polyols and the amino acid proline appeared to be the major low-molecular-weight organic solutes and

TABLE I

RETENTION TIMES FOR LOW-MOLECULAR-WEIGHT ORGANIC SOLUTES AS A FUNCTION OF THE PERCENT ACETONITRILE IN THE MOBILE PHASE.

The elution profiles for these low-molecular-weight organic solutes are shown in Fig. 1. NR = not resolved within 20 min.

Solute	*Retention time (min)*			
	% acetonitrile in the mobile phase			
	77	*80*	*82*	*85*
Glycerol	2.16	2.20	2.70	2.79
Betaine	2.89	3.19	4.31	4.70
Ribitol	3.52	4.00	5.55	6.68
Mannitol	4.73	5.80	8.75	11.50
Proline	5.19	6.52	10.15	12.44
Sucrose	7.67	10.58	18.43	NR
Trehalose	10.35	15.33	NR	NR

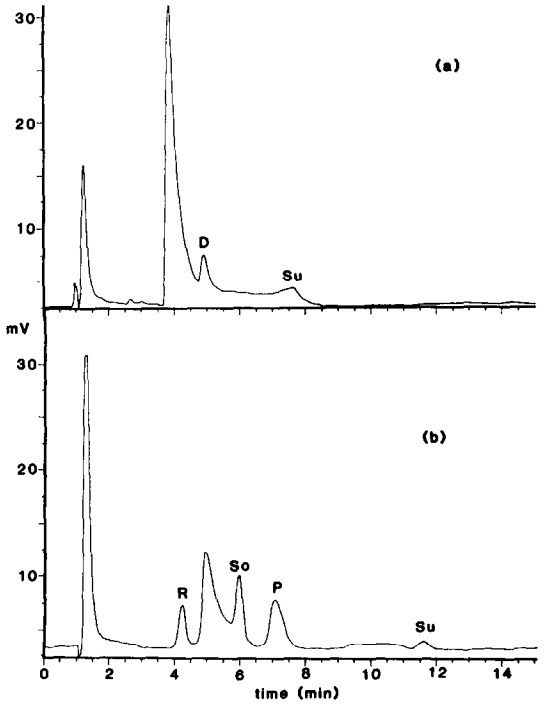

Fig. 2. Elution profile of an ethanolic extract of (a) *Enteromorpha intestinalis* using 77% (v/v) acetonitrile, and (b) *Prasiola stipitata* using 80% (v/v) acetonitrile. D = DMSP; P = proline; R = ribitol; So = sorbitol; Su = sucrose.

sucrose was identified as a minor component in *P. stipitata*, as in *E. intestinalis* (Fig. 2a). A mobile phase of 80% (v/v) acetonitrile was required to maximise the separation of these components.

The hexitol mannitol is the major photoassimilate in members of the Phaeophyta[18] and accumulates in response to osmotic shock[19]. A single peak of mannitol is detected in the majority of brown algae, *e.g., Fucus* spp., *Ascophyllum nodosum* and *Laminaria* spp. However, the isomeric hexitol, altritol, has been shown to accumulate to osmotically significant amounts in *Himanthalia elongata* and in several related Australian species using natural abundance ^{13}C NMR spectroscopy[20,21]. Fig. 3a

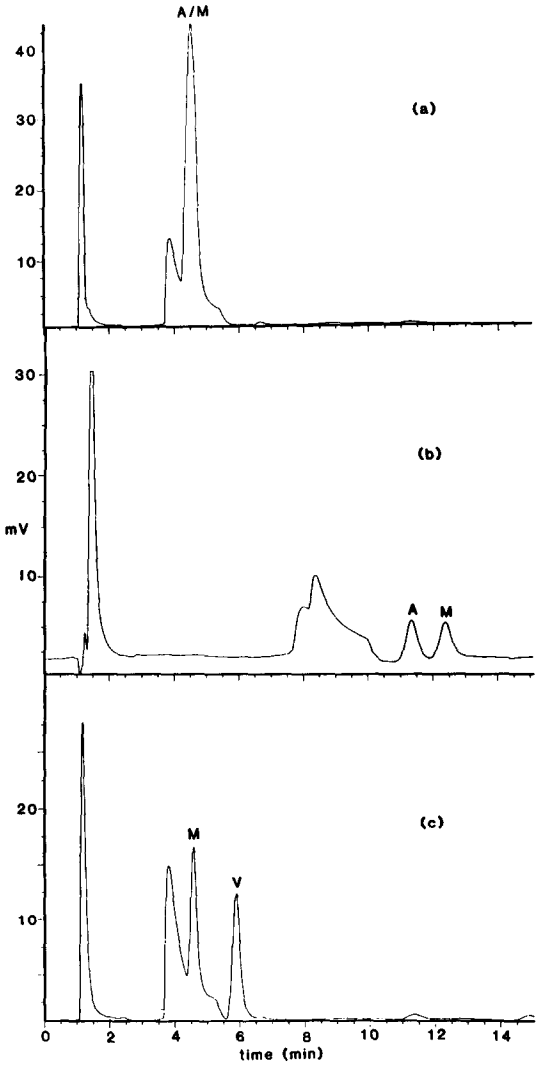

Fig. 3. Elution profile of an ethanolic extract of (a) *Himanthalia elongata* using 77% (v/v) acetonitrile, (b) *H. elongata* using 85% (v/v) acetonitrile and (c) *Pelvetia canaliculata* using 77% (v/v) acetonitrile. A/M = altritol/mannitol; A = altritol; M = mannitol; V = volemitol.

shows an elution profile for *H. elongata* using 77% (v/v) acetonitrile in the mobile phase. In addition to the salt peak at 3.9 min, a single large peak eluted with a retention time of 4.5 min which had similar chromatographic properties to mannitol and is due to both hexitols. Similarly, these isomers are not resolved by conventional gas–liquid chromatography[22] and paper chromatography[23]. Increasing the acetonitrile concentration to 85% (v/v) in the mobile phase resulted in the complete resolution of both hexitols (altritol 11.3 min and mannitol 12.4 min; Fig. 3b). The mannitol peak was confirmed using authentic mannitol whereas the identity of altritol was confirmed, following collection of the fractions, by gas–liquid chromatography[24]. Gas–liquid chromatographic separation of these hexitols requires long run times of over 1 h and gives broad peaks which are incompletely resolved. However, it is an adequate method for identification. Fig. 3c shows the separation of a hexitol (mannitol, retention time 4.6 min) and a heptitol (volemitol, retention time 5.9 min) in ethanolic extracts from the brown alga *Pelvetia canaliculata*. Volemitol is restricted to this species and shows a smaller response than mannitol to changes in salinity[19].

DISCUSSION

The method described here provides a reliable and rapid means of separating and quantifying a range of low-molecular-weight organic solutes and is especially suitable for algal samples containing compounds of diverse structure, *e.g.*, polyols, sugars, amino acids, quaternary ammonium and tertiary sulphonium compounds, which are present in osmotically significant amounts. Sample preparation is minimal, with no requirement for derivatisation. Good separation is achieved by varying the acetonitrile concentration, with short run times. The method is non-destructive and fractions can be collected with good recovery.

In all marine algal samples tested a peak due to the carry over of seasalt, during sample preparation, was present. This was unavoidable since rinsing in distilled water to remove adhering seawater would result in the loss of low-molecular-weight intracellular constituents[25]. However, by changing the amount of acetonitrile in the mobile phase the salt may be resolved from the intracellular components.

This method is particularly suitable for the separation of the two isomeric hexitols, altritol and mannitol, found in the brown alga *Himanthalia elongata*. Baseline separation can be achieved with an acetonitrile concentration of 85% (v/v) in the mobile phase within 15 min, providing significant advantages over techniques previously employed.

The separations reported here rely on the use of an amino-bonded silica column, modified with an aliphatic silica amine. Increasing the water content of the mobile phase speeds up elution on amino-bonded columns. Such separations have been assumed to be due to normal-phase partition chromatography. However, controversy over the mechanism of separation exists; some prefer to regard this as reversed-phase adsorption while others consider that three mechanisms are involved, *i.e.*, adsorption, partition and surface tension (see ref. 14).

ACKNOWLEDGEMENTS

We thank the Agricultural and Food Research Council for financial support and Gail Alexander for her helpful comments.

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