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Note

Use of micro-bead anion-exchange resin for direct estimation of flavour nucleotides in complex solutions

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Anion-exchange column chromatography has proved particularly useful for separation and estimation of nucleotides[1–3]. However, the method may give incomplete separation of isomeric nucleotides[4] and moreover, when applied to complex solutions, it has normally been necessary first to carry out a preliminary isolation of the total nucleotide fraction[5–7]. It is also essential to remove strongly chromophoric material before final estimation of nucleotides by UV spectroscopy[8–10]. Some purification procedures lead to anomalous results[11–13], and extremes of pH, which are often employed, may cause decomposition of nucleotides or release of chemically bound nucleotides.

In the present studies mixtures of nucleotides have been conveniently separated by column chromatography using micro-bead anion-exchange resins. This method requires no initial purification step and is suitable for direct application to solutions containing other compounds such as proteins, polypeptides, amino acids, polyphenols, sugars and products from browning reactions.

The separation obtained between different nucleoside monophosphates and their isomers is shown in Fig. 1a. Resolution of 5′-nucleotides and their corresponding deoxyribonucleotides takes place without the need to use borate complexing, which has previously been found necessary to achieve this type of separation in anion-exchange chromatography[14,15]. Thus this system provides a direct and specific method for estimation of the 6-hydroxypurine riboside-5′-phosphoric acids responsible for flavour modification[16–18] in some beverages and foods.

Nucleotides are eluted using a buffer gradient of ammonium formate and formic acid giving variation in pH and ionic strength, as suggested by Hurlbert *et al.*[19]. Besides separating nucleotides due to differences in their pK_a values the lightly cross-linked (nominally 4 %) resin exploits variations in adsorption characteristics. Removal of ammonium formate buffer from eluted samples without heating[20] can be simply achieved by standing over phosphorus pentoxide and sodium hydroxide under vacuum. Regeneration of the resin with formic acid and ammonium formate solution after each run allows analysis of up to fifteen samples without repacking the column. The apparatus necessary for the present system is constructed so as to combine low cost with high serviceability. A flow diagram is given in Fig. 2.

The method has been applied successfully to the estimation of flavour nucleotides in beers. A chromatogram obtained for a beer with added adenine nucleotides

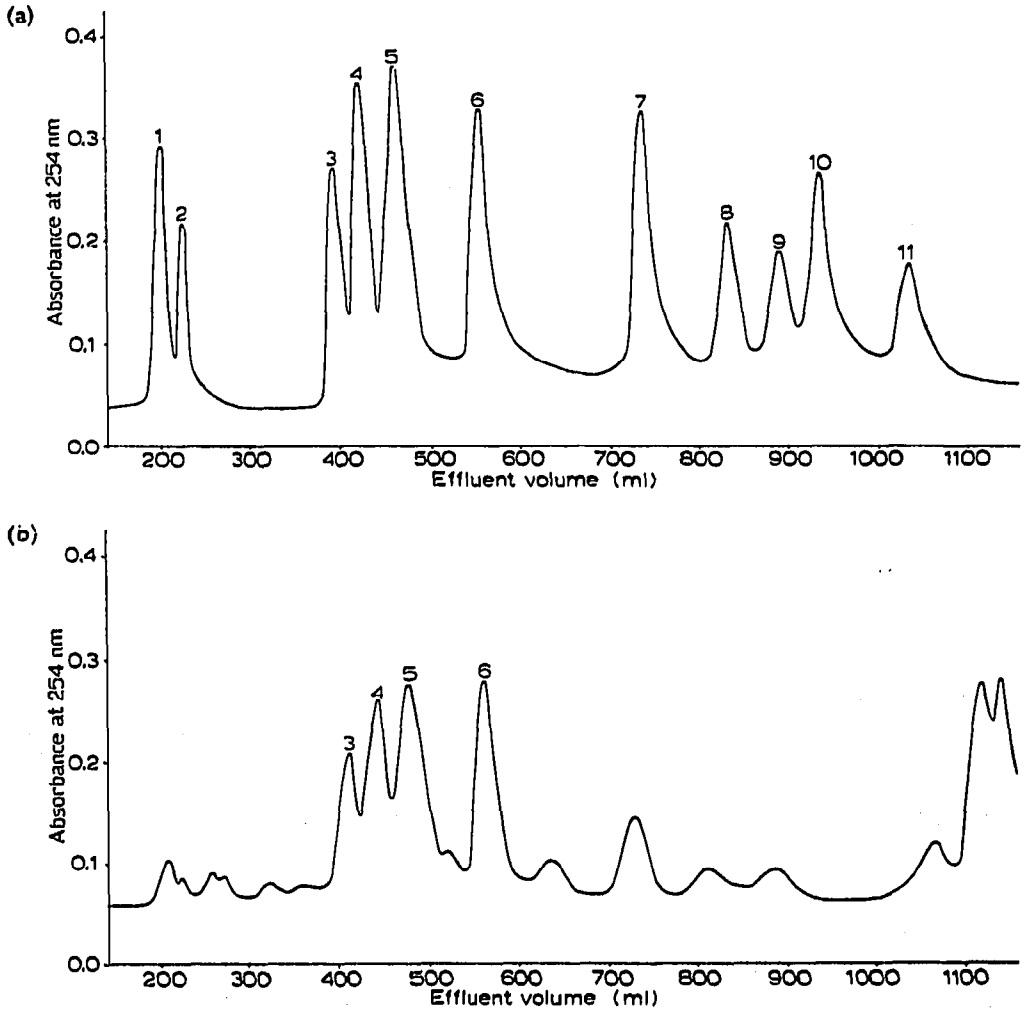

Fig. 1 (a) Chromatogram of a standard mixture of nucleotides: 1 = deoxycytidine-5′-phosphoric acid; 2 = cytidine-5′-phosphoric acid; 3 = deoxyadenosine-5′-phosphoric acid; 4 = adenosine-5′-phosphoric acid; 5 = adenosine-2′-phosphoric acid; 6 = adenosine-3′-phosphoric acid; 7 = uridine-5′-phosphoric acid; 8 = inosine-5′-phosphoric acid; 9 = deoxyguanosine-5′-phosphoric acid; 10 = guanosine-5′-phosphoric acid; 11 = guanosine-3′-phosphoric acid.
(b) Chromatogram of beer to which has been added nucleotides 3, 4, 5, and 6.

is shown in Fig. 1b. The sample of beer is placed on the resin column after adjustment of pH to 7; if beers with high ionic strength[21] are to be analysed, an initial dilution may be necessary. Nucleotides and some other compounds are retained on the resin but most non-nucleotidic material passes through the column and is removed by washing with water. Nucleoside monophosphates are separated from other chromophoric compounds with similar distribution coefficients when the sample is eluted from the column. Material remaining on the resin at the end of a run is removed during regeneration.

Identification of compounds giving rise to peaks in chromatograms has been

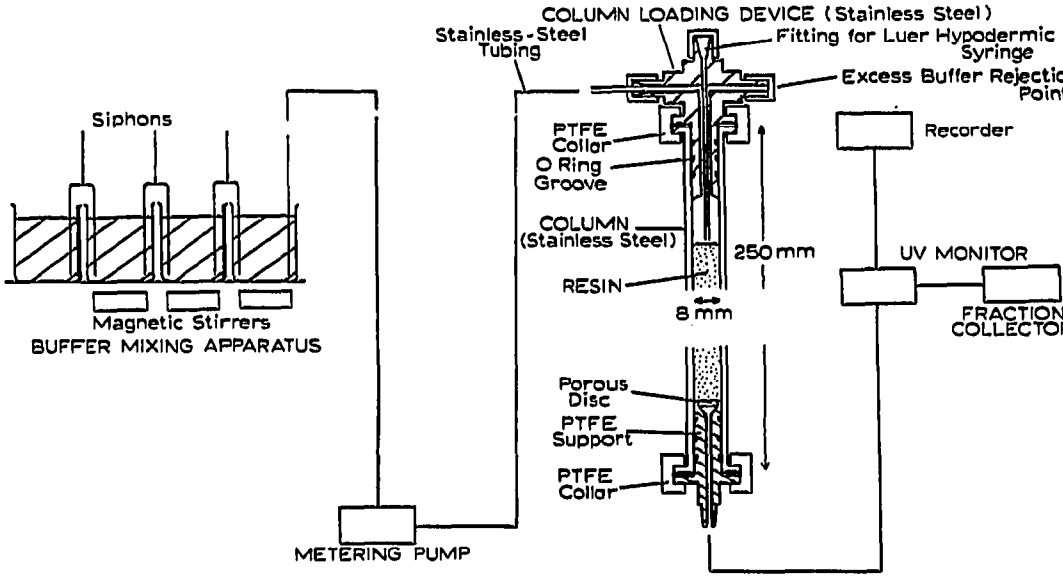

Fig. 2. Flow diagram of micro-bead anion-exchange system.

made by comparison of elution order and retention volumes for nucleotide standards, and confirmation of peak enhancement by addition of reference nucleotides. Nucleotide concentrations are estimated by comparison of peak areas with those for internal standards. Recoveries for added nucleotides are around 90%, as estimated by UV spectroscopy, and levels of guanosine-5'-phosphoric acid as low as 0.1 p.p.m. can be detected by this procedure. Identity of eluted nucleotide samples has been confirmed by anion-exchange thin-layer chromatography on DEAE-cellulose.

EXPERIMENTAL

Preparation of column

Strongly basic anion-exchange resin (DeAcidite FF, 4% crosslinked, Permutit Co. Ltd.) in the form of micro-beads (average diameter 17 μ) was converted into the formate form and packed into a stainless-steel tube (see Fig. 2). The bottom of the tube was closed by a PTFE insert supporting a porous PVC disc (Porvic M, Porous Plastics Ltd.). A stainless-steel column-loading device[22] was fitted into the top of the column and connected to a metering pump (Micro Pump Series II, 100 strokes/min, Head Size No. 2, Metering Pumps Ltd.) using 0.125-in. stainless-steel tubing and compression fittings (Kromlok Compression Fittings, Alenco Industrial Components Ltd.). The pump was connected to a reservoir of degassed water and the resin bedded down to give a column of *ca.* 190 mm height. The flow-rate was adjusted to 25 ml/h.

Sample application and chromatography

Standard mixtures containing about 0.5 mg of each nucleotide were applied to the column as solutions in 50% sucrose. The sample (1 ml) was injected onto the surface of the resin using a Luer hypodermic syringe in conjunction with the column loading device.

For analysis of nucleotides in complex solutions samples were pumped directly onto the resin. Thus, a beer (50 ml), to which reference nucleotides were added where appropriate, was adjusted to pH 7 with saturated sodium carbonate solution, degassed by vacuum filtration, and then loaded onto the resin. The column effluent was monitored by measuring the light absorption at 254 nm using a single-beam photometer (Uvicord, LKB Produkter AB) connected to a 10-mV recorder (Speedomax W, Leeds Northrup Ltd.). The column was washed with degassed water until the UV absorption remained constant.

A simple gradient buffer mixing apparatus, see Fig. 2, was constructed from four 600-ml beakers and three syphons made of glass tubing (4 mm I.D.) fitted with rubber teats. After filtering and degassing, the following solutions were placed in the beakers: (i) 0.5 *M* formic acid–0.5 *M* ammonium formate (500 ml); (ii) 0.5 *M* formic acid (500 ml); (iii) water (500 ml); (iv) water (500 ml).

Siphons were formed using the rubber teats, and sample elution initiated after placing the pump inlet in the fourth beaker. After completion of a run the resin was regenerated by pumping a solution containing 5 *M* formic acid and 1 *M* ammonium formate (250 ml) through the resin. Only slight loss in resolution was found on halving the volumes of the eluate solutions.

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