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SIMULTANEOUS ANALYSIS OF BASES, NUCLEOSIDES AND NUCLEOSIDE MONO- AND POLYPHOSPHATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A single-column high-performance liquid chromatographic system for the simultaneous separation of bases, nucleosides and nucleoside mono- and polyphosphates has been developed, in which a strong porous anion-exchange resin (Aminex A-14) is used. The chromatographic run, carried out at 55° and at alkaline pH by using a linear gradient both of ionic strength and pH, takes less than 225 min. The quantitative application of the described procedure to the analysis of cell nucleotide pools is reported.

INTRODUCTION

The nucleotide pattern of cell extracts is of interest not only for defining the metabolic stage of the cell, but also for studying the compartmentation and the normal or altered metabolism of these compounds. Various chromatographic techniques can be employed to analyse bases, nucleosides and nucleotides (two-dimensional paper chromatography, paper electrophoresis, ion-exchange paper chromatography, thin-layer chromatography), but the best method, at present, for detecting, identifying and quantitatively determining such compounds is ion-exchange chromatography. In this field, the classic approach by Cohn[1], the application of a gradient elution system[2], the development of an ultraviolet effluent monitoring device[3] and the use of new efficient packing materials[4–6] have allowed the development of high-performance liquid chromatography (HPLC) ion-exchange systems. The analysis of nucleotides from cell extracts can be performed either with pellicular ion-exchange resins[4,7–11] or by reversed-phase chromatography[12]. The HPLC separation of bases or nucleosides[13–17] and of nucleosides in the presence of their bases[18–20] with porous ion-exchange resins has also been described.

More complex mixtures, in which bases, nucleosides and nucleotide monophosphates are simultaneously present, have been separated with high resolution by using either cation-[21] or anion-exchange[22,23] chromatography.

Good results, however, for the simultaneous analysis of bases, nucleosides

and nucleoside mono- and polyphosphates from cell or tissue extracts have not yet been obtained by HPLC. Few examples of the complete separation of such complex mixtures by ion-exchange chromatography have been reported. Burger[24] described the simultaneous separation of bases, nucleosides and nucleoside mono- and polyphosphates from the acid-soluble pool of different rat tissues. The conditions used by Burger (anion-exchange polystyrene resin, acetate gradient) were those described by Anderson *et al.*[22], Hori[23] and Hori and Konishi[25]. In order to solve this analytical problem, an ion-exchange chromatographic system was also proposed by Davey[26], who described the simultaneous separation of some bases, nucleosides and nucleoside mono- and polyphosphates using TEAE-cellulose. All of these chromatographic methods, however, have the disadvantages of eluting the single components in large volumes of mobile phase; in addition, long operating times are needed and tailing of the nucleoside triphosphate peaks is always observed.

The availability of an analytical system that is able to separate all of these compounds in a single chromatographic run is required in several fields, mainly in the study of the normal and pathological metabolism of purine and pyrimidine bases[27–29]. Also, during our studies on the metabolism of nucleotides and related compounds in synaptosomal preparations (to be published elsewhere), there was a need to determine simultaneously such heterogeneous groups of compounds in very small samples of purified nerve endings. In this paper, the experimental conditions for an HPLC method that is useful for the simultaneous analysis of bases, nucleosides and nucleoside mono- and polyphosphates are reported.

EXPERIMENTAL

Chemicals

Reagent-grade 2-methyl-2-amino-1-propanol (MAP), purchased from Sigma (St. Louis, Mo., U.S.A.), was used for buffer preparations without further purification. The purine and pyrimidine bases, nucleosides and nucleotides were purchased from Sigma. The other chemicals used (Carlo Erba, Milan, Italy) were of reagent grade.

Resins

Aminex A-28 ($9 \pm 2\ \mu$m), Aminex A-25 ($17 \pm 2\ \mu$m), Aminex A-14 ($20 \pm 3\ \mu$m) and AG1-X8 (—400 mesh) were purchased from Bio-Rad Labs. (Richmond, Calif., U.S.A.). All of these resins were investigated, but the chromatographic separation reported here was performed with Aminex A-14.

Apparatus

A Carlo Erba liquid chromatograph was used, fitted with a double-beam UV detector operating at 254 nm, which is equipped with a cylindrical flow cell of diameter 1 mm and path length 10 mm (volume 8 μl). The detector output is linear in absorbance units and full-scale absorbance ranges from 0.010 to 0.64. The photometer output (10 mV) is displayed on a strip-chart recorder at a chart speed of 12 cm/h. The chromatograph utilizes a 3500 p.s.i. Milton Roy Minipump. Pressure pulses, generated by the reciprocating pump, are suppressed by a pulse-damping device. A thick-walled glass column (50×0.6 cm I.D.), jacketed for temperature control,

was used for the chromatographic process. A linear gradient forming device, consisting of two glass vessels of equal cross-section (6.5 cm), was used.

Calibration mixture

The components of the standard mixture were chosen to represent more than 90% of all acid-soluble cell nucleotides.

Stock solutions of bases, nucleosides and nucleoside mono- and polyphosphates were prepared by dissolving 1–2 mg of each compound in 2.5 ml of deionized water. The exact amount of each substance was determined by UV measurements[30]. From these solutions, a working standard solution was prepared by mixing 25-μl aliquots of the stock solutions and diluting them to 1 ml with water so that the final solution contained about 10–20 μg/ml of each component. Before chromatography, an aliquot of the working solution was diluted 1:1 with the starting eluent (buffer A) and a volume of the solution (20–50 μl) used for the calibration run.

Preparation of buffers

Buffer A. This buffer was used as the starting buffer of the gradient system used for the chromatographic analysis, and consisted of 0.1 *M* MAP–0.1 *M* sodium chloride solution adjusted to pH 9.90 $\pm$ 0.02 at 25° with 3 *M* hydrochloric acid.

Buffer B. This buffer was used as the limiting buffer of the gradient system, and consisted of 0.1 *M* MAP–0.4 *M* sodium chloride solution adjusted to pH 10.00 $\pm$ 0.02 at 25° with 3 *M* hydrochloric acid.

After volume and pH adjustments, the buffer solutions were degassed under vacuum and the pH value was re-checked.

Column preparation

Aminex A-14 (Cl^-) resin was suspended in 3 *M* hydrochloric acid and kept in a water-bath at 80° for 10 min. It then was poured into a Buchner funnel and washed with water, 3 *M* sodium hydroxide solution, water, 3 *M* hydrochloric acid, water and 0.3 *M* hydrochloric acid. The resin, suspended in 0.3 *M* hydrochloric acid, after removal of air under vacuum at 55° was poured into the column and packed, by pumping 0.3 *M* hydrochloric acid at a flow-rate of 100 ml/h, to a final height of 45 cm. The column temperature was maintained at 55°. The resin was further conditioned by pumping 0.3 M hydrochloric acid for 30 min at 100 ml/h, and then equilibrated with buffer A for 20 min at the same flow-rate.

In order to prevent darkening at the top of the column and to maintain the column efficiency for a long period, a pre-column (3 $\times$ 0.6 cm I.D.) packed with AG1-X8 (Cl^-) resin was used.

Sample application

The chromatograph is equipped with a septum injector; however, with the type of column used and to have the possibility of applying large sample volumes (up to 2 ml), the injector was not used. The sample was loaded on the column with a micropipette and forced into the resin bed with nitrogen at a pressure of 50 p.s.i.

Chromatographic elution

After the application of the sample, the column was filled with buffer A

(starting eluent) and elution was carried out with a linear gradient formed by placing 200 ml of buffer A in the mixing chamber and 200 ml of buffer B in the reservoir. The chromatographic run was performed at 55° and a flow-rate of 100 ml/h and was completed within 225 min.

Regeneration of the column

Before starting a new chromatographic run, the column must be regenerated with 0.3 *M* hydrochloric acid (10 ml) and then equilibrated with buffer A (30 ml).

Peak identification

The identification of the compounds in the calibration chromatogram was based on their retention volumes. In order to determine the relative retention volume for each of the 25 compounds in the calibration mixture, we performed a set of four experiments in which six compounds with different ionic characteristics and concentrations were, in turn, chromatographed.

The substances present in the biological extracts were identified by comparing their retention volumes with those of known compounds or by repeating the chromatographic process after the addition of known compounds to the sample.

Peak quantitation

The chromatographic system described here is suitable for quantitative analysis. Owing to the differences in the molar extinction coefficients of the compounds tested, a calibration run should be performed with exactly known amounts of each, in order to obtain the relative calibration factors. These factors were obtained by dividing the amount of each analyte by its peak area. The area (A) of a symmetrical peak was calculated according to the equation $A = HW$, where H is the peak height and W is the width at half-height.

Preparation of biological samples

The acid-soluble nucleotides from biological samples were prepared by perchloric acid (PCA) extraction. Weighed (1–1.5 g) rat liver samples, obtained immediately after decapitation of the animals, were homogenized in a glass homogenizer fitted with a PTFE pestle in 10 volumes of 5% PCA at 4°. After 10 min on ice, the suspension was centrifuged and the residue was re-extracted with 5% PCA (2 ml per gram of tissue). The combined supernatants were placed on ice and neutralized with 10 *M* potassium hydroxide solution. The potassium perchlorate formed was separated by centrifugation. A 2.5-ml volume of 1 *M* MAP–1 *M* sodium chloride buffer (pH 9.9) was added to the supernatant and the volume was adjusted to 25 ml with deionized water. An aliquot of this solution was used for chromatographic analysis. The samples were stored at −20° if not used immediately. Yeast extracts were prepared by the same procedure as for rat liver.

RESULTS AND DISCUSSION

Standard chromatographic resolution

The simultaneous chromatographic separation of bases, nucleosides and nucleoside mono- and polyphosphates is shown in Fig. 1, and the resolution factors

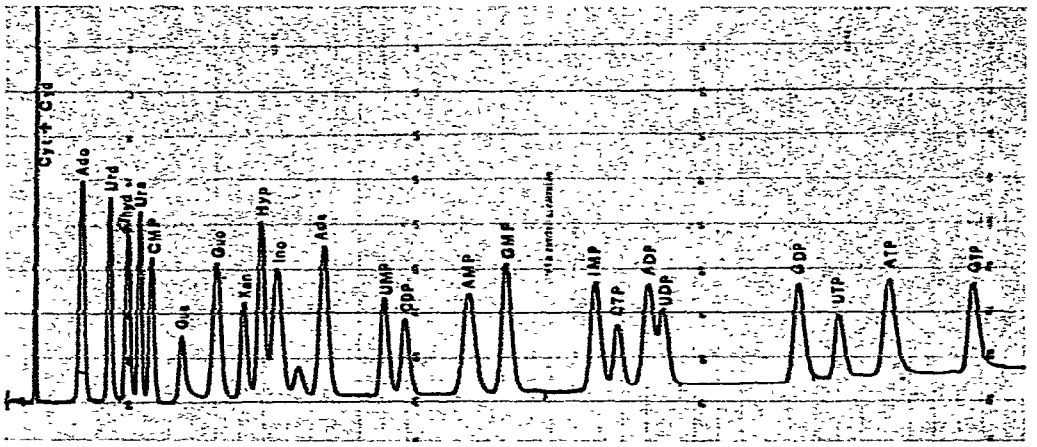

Fig. 1. Separation of bases, nucleosides and nucleoside mono- and polyphosphates from a standard solution. Mobile phase, ionic strength and pH gradient from buffer A to buffer B (see Experimental); column temperature, 55°; flow-rate, 100 ml/h; column pressure, 150 p.s.i.; UV output, 0.080 a.u.f.s.; sample size, 0.25–0.5 μg of each compound.

(R_w), heights equivalent to a theoretical plate (HETP) and widths of the peaks at half-height (W) are reported in Table I. The resolution factor (R_w) is equal to $\Delta \bar{V}/\Sigma W$, where $\bar{V}$ and W are the elution volumes of the peaks and widths at half-height (in volume units) of two neighbouring elution peaks, and $\Delta \bar{V}$ and ΣW are their difference and sum, respectively[17]. The HETP value is derived according to the equation HETP (mm) $= 0.18\, L\, (W/V_x)^2$, where V_x is the eluent volume of the peak, W is the width (in volume units) at half height and L (mm) is the column length [12]. It can be seen in Table I that, with the exceptions of the pair Cyt–Cyd which is not resolved ($R_w = 0$) and the pair ADP–UDP which shows overlapping ($R_w = 0.6$), each component appears in the chromatogram as a sharp peak that is sufficiently well separated to be accurately determined. HETP values range from 0.07 to 0.24 mm for bases and nucleosides and from 0.02 to 0.1 mm for nucleoside phosphates. These values, which are better than those reported for chromatographic separations on porous resins[12,13,17,31,32], indicate the high efficiency of Aminex A-14 under the conditions used.

The peak widths at half-height (W) range from 0.83 to 4.4 ml for bases and nucleosides and from 1.9 to 4.98 ml for nucleoside phosphates.

The elution order of bases is Cyt, Ura, Gua, Xan, Hyp, Ade, which is different from that expected from the relative pK_a' values which determine their anionic character at pH 9.90. The order should be Cyt, Ade, Ura, Gua, Hyp and Xan because the pK_a' values for the enol groups are 12.2, 9.8, 9.5, 9.2, 8.0 and 7.4, respectively[33]. The chromatographic behaviour to be expected from ion-exchange equilibrium considerations is not observed owing to the non-polar interaction between the purine ring and the benzenoid matrix of the resin. This interaction is more marked for monosubstituted purine bases (Ade, Hyp) than for purines with a second substituent at the C_2 position (Gua, Xan). The hydrophobic effect gives rise to a considerable increase in the retention time of Ade, which proved to be the most strongly retained base, and in the reversed elution of the pair Hyp–Xan.

The elution order of nucleosides conforms with that predicted from the pK_a'

TABLE I

CHROMATOGRAPHIC PARAMETERS FOR THE CALIBRATION MIXTURE

Compound	*W (ml)*	R_w	*HETP (mm)*
Cyd	0.83		0.24
		0	
Cyt	0.83		0.24
		5.8	
Ado	1.83		0.28
		2.9	
Urd	1.66		0.11
		1.7	
dThyd	2.08		0.16
		1.1	
Ura	1.66		0.07
		1.1	
CMP	1.90		0.10
		2.3	
Gua	2.49		0.12
		2.2	
Guo	3.33		0.15
		1.5	
Xan	2.57		0.07
		1.3	
Hyp	2.49		0.05
		0.9	
Ino	3.33		0.10
		1.7	
Ade	3.33		0.07
		3.4	
UMP	2.82		0.03
		1.1	
CDP	3.33		0.04
		2.9	
AMP	4.15		0.06
		1.7	
GMP	3.82		0.04
		3.8	
IMP	3.98		0.03
		1.0	
CTP	3.33		0.02
		1.2	
ADP	4.98		0.04
		0.6	
UDP	4.15		0.03
		5.8	
GDP	3.98		0.02
		1.7	
UTP	4.15		0.02
		2.0	
ATP	4.98		0.02
		3.2	
GTP	4.15		0.02

values. However, the lipophilicity of adenosine is still evident and determines its relative position in the elution pattern. In fact, although no net anionic charge is shown by Ado and Cyt, their separation is achieved owing to the more lipophilic nature of the purine nucleoside, which is more strongly retained by the resin.

The elution order of the nucleoside monophosphates is CMP, UMP, AMP, GMP, IMP, which is different from that observed for the corresponding bases and nucleosides. According to the pK_a' values[33], the elution order at pH *ca.* 9.9–10 should be CMP, AMP, UMP, GMP, IMP; however, owing to the interaction with the matrix of the resin, AMP appears after UMP in the chromatographic pattern. This non-ionic interaction is particularly evident at acidic pH. In fact, with the same type of exchanger, the expected elution order according to the net anionic charge[34] at pH 4.4 should be CMP, AMP, GMP and UMP. It was found instead that both purine nucleotides are eluted after UMP[8,12,23]. Moreover, with the stationary phase used in this work, it was observed that, at acidic pH, the degree of non-ionic adsorption of ATP and GTP is such that they cannot be eluted at ionic concentrations higher than those used for their elution at alkaline pH.

The elution order of nucleoside polyphosphates is similar to that for the corresponding monophosphate compounds, with the exception of the pair ADP–UDP, which shows a reversed order. The chromatographic behaviour of these nucleoside diphosphates can be explained by considering that UDP is eluted at a point on the gradient at which the pH is about 9.97, which is 0.5 pH unit higher than the elution pH of UMP, and by considering that the pK_a' value of UDP is 9.4 compared with 9.5 for UMP. This situation involves an increase in the anionic charge of UDP such as to allow its elution after ADP. Temperature also influences the separation of the pair ADP–UDP (see below).

Optimal operating conditions

The operating conditions described are the result of a series of trials in which the different chromatographic parameters were varied so as to obtain the best separation of bases, nucleosides and nucleoside mono- and polyphosphates.

The choice of the ion exchanger was suggested by the following considerations. Pellicular anion-exchange resins give good separations of nucleoside mono- and polyphosphates, particularly at acidic pH, but they give inadequate separations of bases and nucleosides owing to their low capacity (about 10 μequiv./g), which results in an insufficient retention power. The use of porous resins to solve this problem is necessary because of their high capacity (about 3.5 mequiv./g). Of the resins tested (AG1-X8, Aminex A-28, Aminex A-25 and Aminex A-14), the best results were obtained with Aminex A-14, and this resin was subsequently used in this work. Good results for the simultaneous separation of bases and nucleosides were also given by Aminex A-28 (CH_3COO^-), as already observed by Brown *et al.*[18]. However, the acetate modifier was inadequate for the elution of nucleoside polyphosphates. To achieve the elution of these strongly anionic substances in relatively short times and with moderate ion concentrations, the use of Cl^- as an ionic modifier is more suitable. This choice requires a buffer of pH higher than 9, so that bases and nucleosides are suitably charged to be retained by the exchanger. The optimal pH appears to be *ca.* 9.90 with this anion-exchange resin. At this pH, all of the bases and nucleosides, except Cyt, Cyd and Ado, show anionic character and can be separated with an

anion-exchange mechanism because of the different pK'_a values of their enolic groups. At pH lower than 9.90 the chromatographic pattern shows several changes of V_R, with overlapping between several peaks. In the optimal pH range, the separation of the pair ADP–UDP proved to be critical; it is possible only by using a pH gradient from 9.9 to 10.0 in addition to the ionic strength gradient and operating at 55° (see below). Excellent separations with the exception of these nucleoside diphosphates can be achieved at constant pH at all values between 9.90 and 9.95.

The choice of the buffer was made on the basis of several factors. MAP shows a good buffer efficiency in the pH range 9–10.5, minimal absorbance at 254 nm, and the commercial product is sufficiently pure to require no further purification. The optimal ionic strength gradient was from 0.1 to 0.4 *M* sodium chloride in a total volume of 400 ml for the type of column and at the flow conditions selected. Changes in these ionic conditions influence the chromatographic separation more than changes in pH from 9.90 to 9.95.

As regards the optimal temperature for the chromatographic separation, after a series of tests at 40–70° we chose 55° as a compromise. In fact, the optimal temperature is 50°, which gives the best R_w values for all of the compounds, especially Xan, Hyp and Ino, but the pair ADP–UDP is not resolved. If a temperature of 55° is used, the good separation of Xan, Hyp and Ino is slightly perturbed, but the two nucleoside diphosphates are separated. Another practical advantage obtained by using the higher temperature is a shorter analysis time.

Under the conditions described, the stability of the resin was satisfactory. We have used Aminex A-14 for 1 year and no degeneration in retention time or separation was observed. Finally, during the chromatographic process no significative degradation of the tested analytes was observed.

Performance of chromatographic system

The precision of a chromatographic analysis is chiefly determined by the sensitivity and the reproducibility of the system. Under the chromatographic conditions described, the minimal size of the nucleotide samples is $5 \cdot 10^{-8}$ g, which is lower than the value (10^{-9} g) reported for chromatographic systems in which columns of smaller diameters are used[7], but it is sufficient for the usual analytical purposes. In fact, if samples contain amounts of each nucleotide less than 10^{-8} g, aliquots 10–40 times larger than 20–50 μl can be chromatographed without a decrease in resolution.

The reproducibility of the performance of the chromatographic system relates to either the retention volume or the peak area. The reproducibility of the peak area and the retention volume was studied in a series of six chromatographic runs, conducted under the same experimental conditions. The accuracy of the six analyses was $\pm 3.0\%$. The reproducibility of V_R values was $\pm 3.0\%$ for bases, nucleosides and nucleoside mono- and polyphosphates.

Acid-soluble nucleotide pools

The chromatographic system described was developed for testing simultaneously bases, nucleosides and nucleoside mono- and polyphosphates from the cell acid-soluble pools. The performance can be seen from the chromatographic profiles in Figs. 2 and 3, which give the results of the chromatographic analysis of the nu-

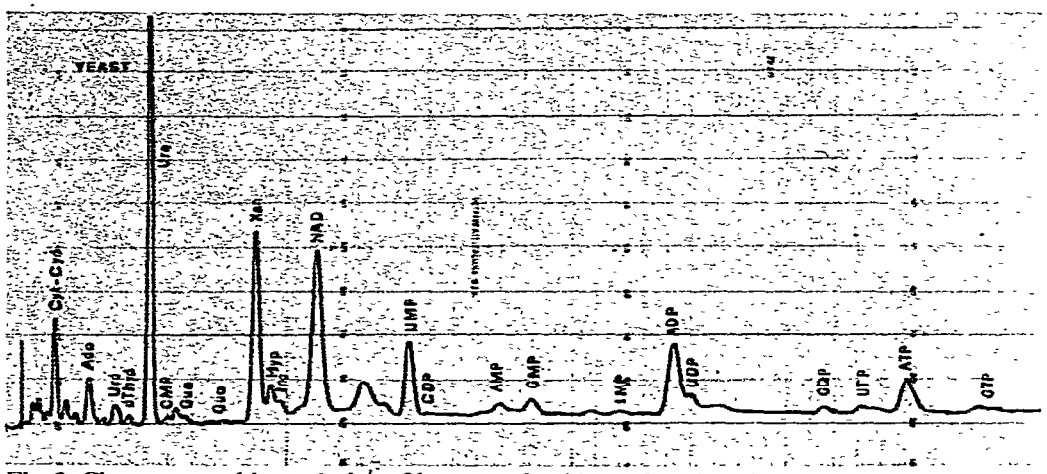

Fig. 2. Chromatographic resolution of bases, nucleosides and nucleoside mono- and polyphosphates from PCA extract of Baker's yeast. Sample size, 250 μl of the extract prepared as described (see Experimental). Column temperature, flow-rate and effluent monitoring conditions as in Fig. 1.

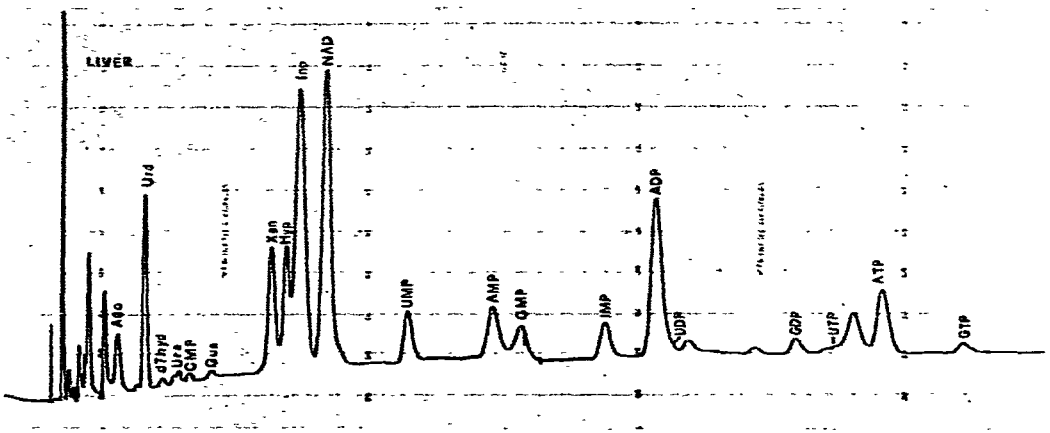

Fig. 3. Separation of acid-soluble nucleotide pool from rat-liver extract. Sample size, column temperature, flow-rate and effluent monitoring conditions as in Fig. 2.

cleotide pools from yeast and rat liver, respectively. The quantitative results are not reported here because it is beyond the scope of this work. The two chromatographic profiles clearly show that a greater number of components are present than in the calibration mixture. Of the unknown compounds, only the peak eluted after Ino has been tentatively identified as NAD^+; the nature of the others was not investigated.

In conclusion, almost all of the acid-soluble cell nucleotide compounds can be identified and determined by single-column chromatography.

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