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Separation of ribonucleotides, ribonucleosides, deoxyribonucleotides, deoxyribonucleosides and bases by reversed-phase high-performance liquid chromatography

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Abstract

A procedure is described for the separation of 21 purine and pyrimidine bases, ribonucleotides, ribonucleosides, deoxyribonucleotides and deoxyribonucleosides in a single chromatographic run by reversed-phase high-performance liquid chromatography using a Waters Resolve C_{18} 5-μm Radial-Pak cartridge and a gradient elution system. The method is fast, taking 35 min for one run. It has proved useful in analysing flavour-enhancing compounds of nucleic acid origin in yeast autolysates.

1. Introduction

We are interested in studying the products of DNA and RNA degradation that are formed during the autolysis of yeasts. Yeast autolysates containing these products have commercial applications in the food industry as flavour ingredients [1], while yeast autolysis and the degradation of nucleic acids during wine, champagne and beer fermentations can impact on the final quality of these beverages [2,3].

Many high-performance liquid chromatography (HPLC) procedures for separating individual classes of purine and pyrimidine compounds have been developed and these are well documented in the literature [4–6]. Generally, anion-exchange chromatography is used for separating the highly charged nucleotides, while reversed-phase chromatography is used for separating the less charged nucleosides and bases. Recently, methods based on reversed-phase HPLC to separate mixtures of these compounds have been reported [7–12]. Although the published methods are successful in their respective applications, none of them can separate a mixture of ribonucleotides, ribonucleosides, deoxyribonucleotides, deoxyribonucleosides and bases in one chromatographic run. Some of the procedures have the additional disadvantage of requiring long analysis times.

We report, here, a reversed-phase HPLC procedure which is able to separate a mixture of 21 nucleotides, nucleosides and bases. The analysis is rapid, taking only 35 min for one run, and was used successfully for the analysis of RNA

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and DNA degradation products in yeast autolysates.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Bio-Rad HPLC gradient processor system which included two Bio-Rad Model 1330 HPLC pumps and a Bio-Rad automation interface with an Apple Model IIe computer; a Waters U6K injector and a Waters Model 440 absorbance detector with two wavelengths of 245 and 280 nm. Retention times and peak areas or peak heights were measured with a Waters 745 data module.

2.2. Column

The column used was a Waters Resolve C_{18} 5-μm Radial-Pak cartridge (100 mm × 8 mm I.D) equipped with a Waters RCM-100 cartridge holder. Packing material of the cartridge was a silica-based reversed-phase C_{18} HPLC matrix of 5-μm spherical beads.

2.3. Chemicals and chromatographic standards

Potassium dihydrogenphosphate (analytical-reagent grade) was obtained from BDH (Sydney, Australia). Methanol (HPLC grade) was obtained from Waters–Millipore (Sydney, Australia). Standards of individual nucleotides, nucleosides and nucleic acid purine and pyrimidine bases (as listed in Table 1) were purchased from United States Biochemical Corporation (Cleveland, OH, USA).

Stock solutions of standards (1%, w/v) were made up by dissolving individual compounds in de-ionised water and were stored at −20°C. Working solutions of standards were made by diluting the stock solutions to 0.01% (w/v).

2.4. Preparation of solvents for HPLC elution

Solvent A was a solution of 0.02 *M* K_2HPO_4 with pH adjusted to 6.30 with concentrated potassium hydroxide solution. Solvent B was a solution of methanol in distilled, de-ionised water (60%, v/v). Prior to use, the eluents were filtered through a membrane filter, pore size 0.45 μm (Millipore, Sydney, Australia), and degassed by vacuum and sonification for 5 min.

2.5. Chromatographic conditions

Before injection of samples, the column was equilibrated with solvent A for 5 min at a flow-rate of 3 ml/min. This step served two purposes. Firstly, it washed out residuals from previous injections and secondly, it stabilised the baseline of the system for the next injection.

After injection of the sample (20 μl), elution was started with 100% solvent A and 0% solvent B. The ratio of solvent B in the elution system was increased linearly from 0 to 40% over 18 min. Solvent B was then increased to 100% over 5 min and the column was then flushed with 100% solvent B for a further 2 min in order to remove strongly absorbed compounds. The system was automatically returned to its starting condition (*i.e.* 100% solvent A and 0% solvent B) over 5 min by computer control. The elution of one sample took 35 min with the solvent flow-rate being maintained at 3 ml/min throughout the program. The elution was performed at ambient temperature (25°C).

2.6. Autolysis of yeasts

Cells of *Saccharomyces cerevisiae* x2180 were grown in 0.5% yeast extract–5% glucose medium at 25°C for 48 h. Cells were harvested by centrifugation, washed, and suspended in 0.2 *M* sodium phosphate–citric acid buffer, pH 7.0. Autolysis was initiated by incubating the suspension at 40°C with orbital shaking at 200 rpm [13]. Samples were withdrawn daily and separated by centrifugation (5000 *g* for 5 min) into cell pellets and cell-free autolysate. The autolysate fraction was filtered through a 0.45-μm pore-sized membrane filter (Millipore) and used in the HPLC without further treatment.

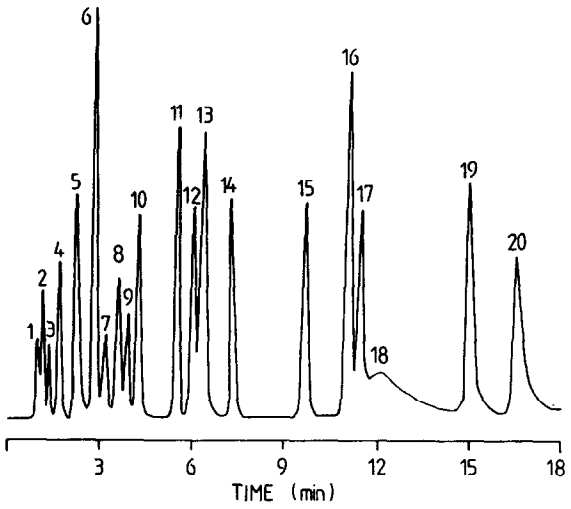

Fig. 1. Chromatogram of a standard mixture of nucleotides, nucleosides and purine and pyrimidine bases (0.01%, w/v, each). Injection volume, 20 μl; detection wavelength, 254 nm. Peaks: 1 = 5′-CMP; 2 = 5′-UMP; 3 = 5′-dCMP; 4 = 5′-GMP; 5 = cytosine; 6 = uracil; 7 = 5′-dTMP; 8 = 5′-AMP; 9 = 5′-dGMP; 10 = cytidine; 11 = uridine; 12 = deoxycytidine; 13 = 5′-dAMP and thymine; 14 = guanine; 15 = guanosine; 16 = deoxyguanosine; 17 = thymidine; 18 = adenine; 19 = adenosine; 20 = deoxyadenosine. For conditions see Experimental section.

3. Results and discussion

Fig. 1 shows the separation of a mixture of standards comprising 21 individual ribonucleotides, ribonucleosides, deoxyribonucleotides, deoxyribonucleosides and bases. Peaks of the chromatogram were identified by matching their respective retention times with those of standard compounds injected individually. Out of the 21 components, 19 were separated, but 5′-dAMP and thymine did not separate and eluted together as one peak (peak 13). All the compounds were eluted within 18 min. A further 17 min were needed to wash the column and to return it to its initial conditions for the next injection. Nucleotides eluted in the first 7 min, followed by bases and nucleosides, as expected in a reversed-phase HPLC system. If a sample contains nucleotides, or one is interested in the analysis of nucleotides only, the analysis time can be shortened to about 25 min.

The retention times and capacity factors of the 21 compounds are presented in Table 1 and showed high degrees of reproducibility. For compounds eluted in the first few minutes, deviations of retention times were less than 0.03 min. Compounds eluting at later times showed slightly greater deviations, but these were not more than 0.05 min, except for deoxyadenosine, which came out last.

An important factor influencing the separation was control of pH in solvent A, which must be kept at pH 6.30 ± 0.05. Deviation from this value resulted in overlapping of some peaks. Increasing the flow-rate to 4 ml/min or decreas-

Table 1
The retention times of 21 nucleotides, nucleosides and purine and pyrimidine bases as separated by HPLC on a Waters Resolve C_{18} 5-μm Radial-Pak cartridge

Component	Retention time (min)[a]	Capacity factor[b]
Purine and pyrimidine bases		
Ade	11.85 ± 0.05	17.52
Cytosine	2.14 ± 0.02	2.34
Guanine	7.07 ± 0.03	10.05
Uracil	2.64 ± 0.02	3.13
Thymine	6.45 ± 0.05	9.08
Nucleosides		
Adenosine	14.68 ± 0.05	21.94
Cytidine	3.94 ± 0.03	5.16
Guanosine	9.55 ± 0.04	13.92
Uridine	5.25 ± 0.03	7.20
Deoxyadenosine	16.18 ± 0.08	24.28
Deoxycytidine	5.73 ± 0.03	7.95
Deoxyguanosine	10.81 ± 0.05	15.89
Thymidine	11.26 ± 0.05	16.59
Nucleotides		
5′-AMP	3.41 ± 0.02	4.33
5′-CMP	1.00 ± 0.02	0.56
5′-GMP	1.67 ± 0.02	1.61
5′-UMP	1.15 ± 0.02	0.80
5′-dAMP	6.10 ± 0.03	8.53
5′-dCMP	1.34 ± 0.02	1.10
5′-dGMP	3.65 ± 0.02	4.70
5′-dTMP	3.04 ± 0.02	3.75

[a] The data indicate average retention times ± variation taken from 10 analyses.

[b] Capacity factor was calculated as $(t_R - t_0)/t_0$, where t_R is the retention time of solute and t_0 is the retention time of a non-retaining compound (calculated as system void volume/flow-rate); $t_0 = 0.64$ min.

ing it to 0.5 ml/min did not improve peak resolution; the latter had the adverse effect of causing peak broadening. It is unfortunate that the system did not resolve 5′-dAMP and thymine. To resolve these two compounds, it was necessary to elute with solvent A adjusted to pH 6.5. However, under this condition resolution of several other components was lost due to peak overlapping (*e.g.* 5′-AMP, 5′-dGMP).

Fig. 2 shows the resolution of nucleic acid degradation products in an autolysate of the yeast, *Saccharomyces cerevisiae*. The majority of peaks in the chromatogram were readily identified by comparing their retention times with those of the standards. One of the major peaks (denoted as ×) did not correspond to any component in the mixture of standards and could be 3′- or 2′-isomers of ribonucleotides.

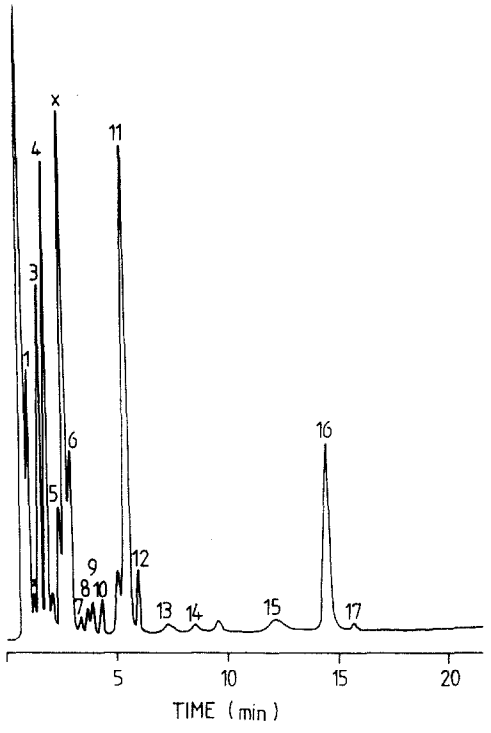

Fig. 2. Chromatogram of an autolysate sample; the autolysate was taken from a sample after cells of *Saccharomyces cerevisiae* had been autolysed at 40°C for 10 days at pH 7.0. Injection volume, 20 μl; detection wavelength, 254 nm; Peaks: 1 = 5′-CMP; 2 = 5′-UMP; 3 = 5′-dCMP; 4 = 5′-GMP; 5 = cytosine; 6 = uracil; 7 = 5′-dTMP; 8 = 5′-AMP; 9 = 5′-dGMP; 10 = cytidine; 11 = uridine; 12 = deoxycytidine; 13 = guanine; 14 = guanosine; 15 = adenine; 16 = adenosine; 17 = deoxyadenosine. For conditions see Experimental section.

There are several published methods which can separate ribonucleotides from ribonucleosides and bases [8,14,15]. Also, methods have been described for separation of mixtures of ribonucleotides and deoxyribonucleotides [16,17]. However, these methods cannot separate mixtures of ribonucleotides and ribonucleosides from deoxyribonucleotides and deoxyribonucleosides in one step. To deal with samples that contain all of these components, such as yeast autolysates, group separation has to be carried out first, which can be time-consuming. Methods are known which can separate ribonucleosides from deoxyribonucleosides [18–20], but they cannot separate nucleotides from nucleosides and bases and the above-mentioned disadvantage of group separation also applies to these methods.

The HPLC procedure presented in this paper is a new method that has the capacity to separate a mixture of 21 purine and pyrimidine bases, ribonucleotides, ribonucleosides, deoxyribonucleotides and deoxyribonucleosides. It is fast and retention times have good reproducibility. An additional advantage of the method is that sample preparation (membrane filtration) is simple and quantitative. There is no need for solvent extraction of the sample, as required by many other methods, which is tedious and contributes to poor reproducibility. It has been successfully used for analysing the degradation products of nucleic acid origin in yeast autolysates, and may be used for analysis of similar products in beer, wine and champagne. However, the present method cannot separate different isomers of nucleotides. Methods for such separation are described elsewhere [21]. For the present study, the primary interest is 5′-isomers of nucleotides, which have desirable flavour-enhancing properties [22]. Our study shows that 5′-isomer nucleotides are the major degradation products of nucleic acids during yeast autolysis.

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