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RAPID COLUMN CHROMATOGRAPHIC DETERMINATION OF RIBONUCLEOTIDES IN SOUP PREPARATIONS

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SUMMARY

The authors describe a rapid and simple column chromatographic method for the determination of 5′-ribonucleotides in soup preparations by the separation and determination of the two purine bases hypoxanthine and guanine, obtained from the ribonucleotides by hydrolysis with HCl. The purines were separated on a column of a cation-exchange resin, and determined by the continuous U.V.-spectrometric monitoring of the effluent. The recovery of 5′-ribonucleotides introduced as additives into a soup preparation whose purine base content was accurately known was 100.1 ± 1.8% and 102.0 ± 1.2% on the basis of hypoxanthine and guanine, respectively.

INTRODUCTION

The current use of 5′-ribonucleotides as flavour enhancers calls for a method for their rapid analysis in foods, which can be used for production control. The literature contains various methods for the determination of ribonucleotides and the purine bases they give on acid hydrolysis. These methods are based on paper chromatography[1–9], thin-layer chromatography[10–18], and ion-exchange or gel-filtration chromatography[20–37].

Since COHN's first work[19], ion-exchange chromatography has been widely used for the separation and determination of mixtures of purines and pyrimidine bases, nucleosides, and nucleotides. The work of ANDERSON *et al.*[33] and GREEN *et al.*[36] is of particular interest in this connection because of their techniques and the resolutions obtained.

Owing to the complex nature of the mixtures to be separated, these methods involve the use of long resin columns with a low flow rate, at the expense of sensitivity. Furthermore, they are complicated and time-consuming, since the resin is generally an anion-exchanger and cannot be regenerated in the column after each analysis. To speed up the work, very high column pressures of up to 4000 p.s.i. are

sometimes used[36], in which case the usual glass columns must be replaced by stainless steel ones.

The present task was simpler, namely to separate and determine only the purine bases, and we could therefore choose a rapid, simple, and sensitive method not requiring sophisticated equipment, and which would be suitable for the routine analysis of 5′-ribonucleotides introduced into foodstuffs.

EXPERIMENTAL

Apparatus and reagents

The chromatographic column (30 × 0.9 cm) was packed to a height of 13 cm with the cation-exchange resin Aminex A5 (Bio-Rad Lab., Richmond, Calif.), the spherical particles of which had a diameter of 8–12 μ. The column was used in conjunction with a Mini-Pump (Milton Roy Co., Fla., U.S.A.) and a Beckman DB-G double-beam spectrophotometer (catalogue no. 97290) fitted with a Beckman 10 in. recorder and a microcell with a quartz window, a light path of 10 mm, and a total volume of 0.3 ml.

The reagents were 3 *N* HCl, 0.1 *N* HCl, 0.3 *M* acetic acid, adjusted to pH 3.70 ± 0.02 with 40% NaOH, and 0.3 *M* acetic acid, adjusted to pH 5.20 ± 0.02 with 40% of NaOH.

Procedure

A sample of 5–10 g was weighed out, the amount depending on the meat extract content and the yeast autolysate content. It was dissolved in 30–40 ml of water, and cooled with running water until the fats had solidified. The solids were filtered off by passing the solution through glass wool, and the filtrate was collected in a 100 ml beaker. The filtrate was then acidified to pH 1 with 3 *N* HCl and passed through the same glass wool. The filtrate was collected in a 100 ml volumetric flask, the beaker and the glass wool were washed with 0.1 *N* HCl, and the washings were also collected in the volumetric flask; the latter was then filled up to the mark with 0.1 *N* HCl.

TABLE I

RECOVERY OF 5′-RIBONUCLEOTIDES ADDED TO A SOUP PREPARATION

Sample	*Hypoxanthine added* (mg)*	*Hypoxanthine found (mg)*	*Recovery (%)*	*Guanine added* (mg)*	*Guanine found (mg)*	*Recovery (%)*
1	0.584	0.573	98.2	0.804	0.822	102.3
2	0.584	0.574	98.3	0.804	0.818	101.7
3	1.166	1.169	100.1	1.618	1.624	100.4
4	1.168	1.172	100.3	1.618	1.681	103.9
5	2.920	3.005	102.9	4.020	4.116	102.4
6	2.920	2.949	101.0	4.020	4.076	101.4
		Average recovery	100.1 ± 1.8		Average recovery	102.0 ± 1.2

* 5, 10 and 25 mg of 5′-ribonucleotides (mixture of sodium guanilate and sodium inosinate 50:50) were added to 5 g of the soup preparation corresponding respectively to 0.1, 0.2 and 0.5%.

Of this solution 20 ml were transferred quantitatively into a 30 ml ampoule, which was then sealed in a flame and kept at 121° for 2 h. The contents were transferred quantitatively into a 50 ml flask and filled up to the mark with the pH 3.70 buffer. A few millilitres of the solution were passed through a Whatman No. 42 filter paper, and 1 ml was placed on the chromatographic column. The first eluent was the pH 3.70 buffer; 27 min later, this eluent was replaced by the pH 5.20 buffer. The column temperature was kept at 55° by a thermostat.

Twelve minutes after the emergence of guanine, the wavelength of the spectrophotometer was changed from 248 mμ, while the slit was always kept at 0.5 mm.

After each analysis, the column was regenerated by washing with 1 *N* NaOH until the NaOH front had descended to three-quarters of the column, and the latter was then conditioned for 30 min with the pH 3.70 buffer.

Calculations

The following formulae were used to find the amount of 5'-ribonucleotides added to the sample from the total amount of hypoxanthine, guanine, adenine and creatinine with the aid of the data given in Table II.

$$A_m = C_r \cdot F \quad (1)$$
$$A_y = A_T - A_m \quad (2)$$
$$H_m = R_1 \cdot A_m \quad (3)$$
$$H_y = R_3 \cdot A_y \quad (4)$$
$$G_y = R_2 \cdot A_y \quad (5)$$

where:

A_T = total amount of adenine from the chromatogram
A_m = amount of adenine in the meat extract
A_y = amount of adenine in the yeast extract
H_m = amount of hypoxanthine in the meat extract
H_y = amount of hypoxanthine in the yeast extract
G_y = amount of guanine in the yeast extract
C_r = amount of creatinine determined by HADORN's method[38]
F = ratio between adenine and creatinine of the meat extract determined experimentally (see Table II)
R_1 = ratio between hypoxanthine and adenine of the meat extract determined experimentally (see Table II)
R_2 = ratio between guanine and adenine of the yeast extract determined experimentally in two different types of extract (see Table II)
R_3 = ratio between hypoxanthine and adenine of the yeast extract determined experimentally in two different types of extract (see Table II).

When the values of H_m, H_y, and G_y were calculated by eqns. (3), (4) and (5), from the total hypoxanthine and guanine given by the chromatogram one can obtain, by difference, the hypoxanthine and the guanine from the ribonucleotides added.

RESULTS AND DISCUSSION

The proposed method is rapid, accurate, and easy to carry out. The cation

TABLE II

PURINE-BASE CONTENT OF SOME MEAT EXTRACTS AND YEASTS EXTRACTS

Sample	*Quality*	*Hypoxanthine (%)*	*Guanine (%)*	*Adenine (%)*	*Creatinine (%)*	$F = \frac{A_m^*}{C_r}$	$R_1 = \frac{H_m}{A_m}$	$R_2 = \frac{G_y}{A_y}$	$R_3 = \frac{H_y}{A_y}$
1	Meat extract	1.30	trace	0.18	6.93	0.0261	7.18	—	—
2	Meat extract	1.32	trace	0.20	8.01	0.0252	6.53	—	—
3	Meat extract	1.52	trace	0.22	7.60	0.0287	6.96	—	—
4	Meat extract	1.45	trace	0.21	7.65	0.0271	7.02	—	—
5	Meat extract	1.36	trace	0.21	7.45	0.0286	6.38	—	—
6	Meat extract	1.51	trace	0.23	7.61	0.0302	6.55	—	—
7	Meat extract	1.34	trace	0.19	7.09	0.0269	7.03	—	—
8	Meat extract	1.74	trace	0.26	7.73	0.0338	6.67	—	—
9	Meat extract	1.29	trace	0.17	7.16	0.0230	7.79	—	—
10	Meat extract	1.41	trace	0.25	7.90	0.0322	5.53	—	—
	Average values	1.42	trace	0.21	7.51	0.0281	6.76	—	—
11	Yeast extract I°	0.034	0.339	0.464	—	—	—	0.731	0.0733
12	Yeast extract	0.024	0.220	0.381	—	—	—	0.577	0.0630
13	Yeast extract	0.041	0.326	0.439	—	—	—	0.743	0.0934
14	Yeast extract	0.052	0.372	0.482	—	—	—	0.772	0.1080
15	Yeast extract	0.028	0.188	0.366	—	—	—	0.514	0.0765
16	Yeast extract	0.018	0.323	0.465	—	—	—	0.695	0.0387
17	Yeast extract	0.050	0.406	0.480	—	—	—	0.846	0.1040
18	Yeast extract	0.050	0.396	0.485	—	—	—	0.817	0.1030
19	Yeast extract	0.034	0.246	0.380	—	—	—	0.648	0.0900
20	Yeast extract	0.073	0.396	0.399	—	—	—	0.993	0.1830
21	Yeast extract	0.082	0.395	0.404	—	—	—	0.978	0.203
	Average values	0.044	0.328	0.431	—	—	—	0.756	0.103
22	Yeast extract II°	0.142	0.876	0.677	—	—	—	1.294	0.210
23	Yeast extract	0.134	0.846	0.698	—	—	—	1.212	0.192
24	Yeast extract	0.099	0.767	0.638	—	—	—	1.202	0.155
25	Yeast extract	0.098	0.833	0.688	—	—	—	1.211	0.142
26	Yeast extract	0.108	0.791	0.697	—	—	—	1.145	0.155
27	Yeast extract	0.132	0.860	0.656	—	—	—	1.311	0.201
28	Yeast extract	0.129	0.842	0.658	—	—	—	1.280	0.196
29	Yeast extract	0.141	0.877	0.664	—	—	—	1.321	0.212
30	Yeast extract	0.103	0.880	0.693	—	—	—	1.270	0.149
31	Yeast extract	0.112	0.890	0.694	—	—	—	1.282	0.161
	Average values	0.120	0.846	0.676	—	—	—	1.252	0.177

* For explanation of symbols see *Calculations*.

exchanger in a 13 × 0.9 cm column separates the purine bases very well from one another and from interfering substances (*cf.* Fig. 1). The pressure need not be more than 1 atm for the eluent to migrate down the column. The bases can be determined accurately, because they are well resolved from other substances that absorb in the U.V. region. Furthermore, the speed with which the individual bases leave the column enables one to reach a high sensitivity: even 0.02 μmole of a base can be determined very well.

To check the validity of the method, a mixture of sodium guanylate and sodium inosinate was added to the soup preparation, and the amounts of total hypoxanthine

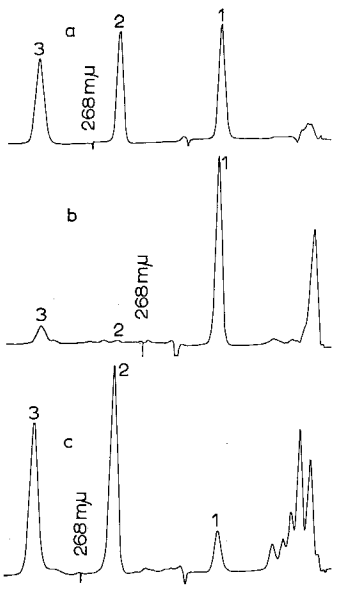

Fig. 1. (a) Chromatogram of a purine standard solution; (b) chromatogram of a meat extract; (c) chromatogram of a yeast extract. 1 = Hypoxanthine; 2 = guanine; 3 = adenine.

and guanine were determined after hydrolysis with HCl. The mixture of 5′-ribonucleotides and the soup preparation were analysed separately, so as to determine the purine base content. The results thus obtained are shown in Table I, where it can be seen that the recovery is practically quantitative, and that the method has a high accuracy.

To find the mean values of the hypoxanthine, guanine, and adenine content, many meat extract and yeast extract samples were analysed, the results being shown in Table II. This Table also gives the values of some of the ratios F, R_1, R_2, and R_3, which are used to find the amounts of 5′-ribonucleotides added to the samples from the total amount of hypoxanthine, guanine, adenine and creatinine by means of the formulae specified above.

Since the quantities of purine bases in the various meat extracts examined do not vary much, the values of the ratios F and R_1 are also fairly constant.

This is less so in the case of the yeast extracts, of which two types were analysed: the one called extract I was obtained by traditional means, while extract II was prepared by a special technique whereby a product richer in crude ribonucleotides could be obtained. These crude ribonucleotides are a mixture of guanylic and adenylic acid, in which the latter had not yet been subjected to enzymatic oxidative deamination, converting it into inosinic acid.

Inspection of the data in Table II reveals a considerable difference between the two types of autolysates as regards the values of hypoxanthine, guanine, and adenine and the values of R_2 and R_3. We think it is better to use the values for extract II (richer in 5′-ribonucleotides) for the calculations since, by using the

values for extract I, one would often run the risk of regarding some ribonucleotides naturally present in a product as additives.

These variations in the amounts of bases in the meat extracts and the yeast extracts unfortunately decrease the accuracy in the determination of the 5'-ribonucleotides introduced as additives. However if the flavour enhancer is a mixture composed of equal amounts of sodium guanylate and sodium inosinate, more reliable values can be obtained for the amounts present by carrying out checks on hypoxanthine and guanine.

On the other hand, the method is particularly useful for routine production control and for checking the guanylate and the inosinate content of a commercial mixture of 5'-ribonucleotides. In the first case, the original hypoxanthine and guanine content of the food is accurately known, and so the amount of 5'-ribonucleotides added can be determined quickly and accurately.

Determinations carried out with some commercial formulations of (50:50) 5'-ribonucleotides showed that these have a fairly constant content of hypoxanthine and guanine. The mean values used in the calculations were 11.89 and 16.08% for hypoxanthine and guanine, respectively.

CONCLUSIONS

The chromatographic method described above is rapid, accurate, and easy to perform. It is therefore useful for production control and for checking commercial mixtures of 5'-ribonucleotides. The method is also suitable for the determination of 5'-ribonucleotides added to foodstuffs, provided that a probable value is acceptable. The variation in the natural ribonucleotide content does not permit very accurate determination in such cases.

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