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A Chromatographic-Spectrophotometric Method for Determining Nucleotide Composition of RNA in Plants [1, 2]

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Introduction

Most methods for estimating the four principal ribonucleic acid (RNA) constituents have been applied to animal tissue, but less work has been done on plant sources, usually yeast and wheat germ (8, 17). This study is an attempt to simplify and improve the quantitative extraction of RNA from plant tissue for analysis by means of a rapid and simple column fractionation, followed by spectrophotometric analysis for individual nucleotides. Such a method was needed in a program for study of biochemical relationships between plants susceptible and resistant to disease in relation to environment.

The use of trichloracetic acid for extraction of RNA (14) from plant material for direct ultraviolet absorption analysis is undesirable because of the absorption of the solvent below 280 mμ (11). Ultraviolet-absorbing impurities result from treatment with 1 M KOH and incubation at 37 C for 15 hours (13). Similar difficulties arise in perchloric acid extraction. Cold perchloric acid alters the absorption curve of RNA (11) and may dissolve some deoxyribonucleic acid (DNA) (10, 11).

DeDeken-Grenson and DeDeken (3), after comparison of the Schmidt-Thannhauser, Ogur-Rosen, and Schneider methods, modified the Schmidt-Thannhauser procedure by treating the alkaline hydrolysate containing the ribonucleotides with Dowex-2 in the chloride form to remove impurities left in the supernatant. Smillie and Kratkov (15), with a similar modification using Dowex-1, concluded that the technique is suitable for a wide range of plants. These workers did not fractionate the nucleotides.

The methods of Cohn (1) and Hurlbert, et al. (7) employed the use of resin columns and fraction collectors to separate the nucleotides into four fractions. These methods provided the desirable separations but employed considerable volumes of solution and were time consuming. In the method outlined below, only two fractions are necessary, the entire analysis is less tedious, and smaller samples can be used.

Methods

Extraction of Plant Tissue: Fresh plant material was steamed in the autoclave for 3 minutes at 100 C, dried at 100 C for 2 to 3 hours, and ground in a Wiley mill, using a 60-mesh screen. Fibrous material was removed with a fine screen and discarded. Samples were stored in a freezer at −20 C.

RNA was removed quantitatively from the dried material by four extractions, two of 50 ml each of 0.55 M sodium chloride solution, and two of 25 ml each, by steaming each extraction mixture in the autoclave at 100 C for 20 to 30 minutes. The resultant supernatants were pooled, and an equal volume of 95% ethyl alcohol was added. The mixture was cooled at 2 C overnight. The resultant suspension was centrifuged batch-wise (ca 1,200 g for 10 min for each batch) in a 50-ml tube. The supernatant (containing any free nucleotides) was discarded. This procedure resulted in a white gelatinous preparation of RNA.

Hydrolysis of RNA: One milliliter of 0.5 M KOH was added to the RNA in the centrifuge tube and the mixture was incubated 40 hours at 30 C. The hydrolysate was transferred quantitatively to a 15 ml calibrated centrifuge tube with distilled water, adjusted to pH 8 to 9 with 1 M HCl, and made to 10 ml. Centrifugation removed the small amount of protein present. The supernatant (can be stored at 2 C for several days) was ready for column fractionation.

Time is saved by a preliminary estimation of total RNA following hydrolysis but before column frac-

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tionation. This may prevent overloading the column and is best done by measuring the absorbance of a small aliquot at 268 mμ. For barley (*Hordeum vulgare* L.) leaf extract, a 100-fold dilution was satisfactory. The quantity of plant meal may be varied and suitable dilutions made to obtain proper absorbance values. In our experience, the content of barley leaves varies considerably with age and environment. For field-grown barley leaf meal, 300 mg has given good results.

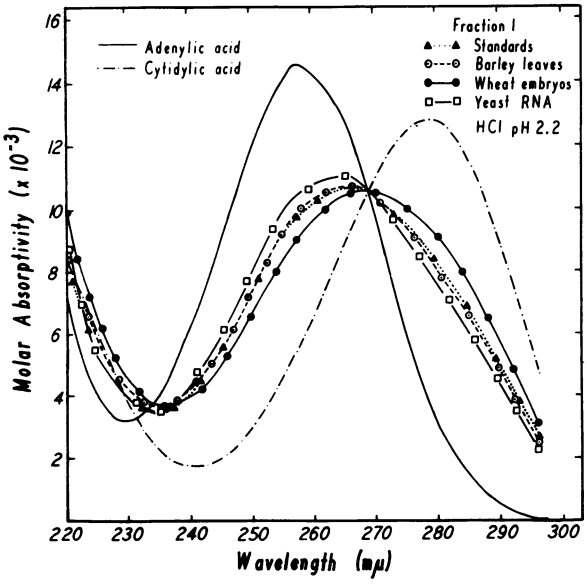
Adsorption Column Fractionation of Mononucleotides: Columns (1 cm in diameter & 6 cm in length) were prepared from Dowex 1-× 8 (200–400 mesh) by washing with 1 M HCl, followed by distilled water until the effluent was neutral. The hydrolysate was poured onto the column, followed by distilled water. The column was then washed with 0.01 M ammonium chloride (*2*) until the effluent was neutral to indicator paper, followed by distilled water. These washes prepared the column for uniform extraction with acid. Fraction 1 (F_1, adenylic & cytidylic acids) was eluted with 100 ml of HCl (pH 2.2) and Fraction 2 (F_2, guanylic & uridylic acids) with the same volume at pH 1.1, both at a flow rate of 0.75 ml per minute. Columns could be reused for successive determinations by washing again with 1 M HCl, followed by distilled water.

Columns, to which no nucleotides had been applied, gave blank absorption values up to 0.007 absorbance units, depending upon the wavelength. These corrections were always applied to absorbance observations.

Spectroscopic Analysis of Fractions: *Standards.* The values used here were chosen after a careful spectrophotometric study of standard nucleotides, [California Corp. for Biochemical Research, Grade CFP or A, 3′ (2′) isomers] with a Beckman Model DU spectrophotometer at pH 2.2 in HCl, pH 7.2 in 0.1 M phosphate buffer, and pH 12.0 in NaOH. For analytical purposes, pH 1.1 (figs 1 & 2) was chosen as most satisfactory, because the curves for the pairs of compounds in F_1 and F_2 are least related or most dissimilar in acid solution. Zscheile, Murray, Baker, and Peddicord (*19*) showed the impossibility of direct spectroscopic analysis of this four-component system without fractionation. The spectra of adenylic, cytidylic, and uridylic acids are constant in the pH range 1.1 to 2.2. The spectrum of guanylic acid changes slightly in the regions 232 to 248 and 280 to 296 mμ.

Solutions of standard nucleotides were first prepared in HCl (pH 1.1) and stored at room temperature. On checking their absorption curves after a period of several months, uridylic acid appeared to be the only very stable acid; curves for both adenylic and cytidylic acids had changed appreciably and that for guanylic acid more markedly, indicating hydrolysis. Standards (ca 7×10^{-4} M) were then prepared in HCl at pH 2.2 and stored at 2 to 4 C to increase the period of stability to 3 or 4 weeks.

To calculate concentrations in F_1 of an unknown, the observed absorbance at 268.7 mμ was divided by 10.6×10^3 liters/mole cm, the molar absorptivity common to both adenylic and cytidylic acids at this crossing point of their absorption curves (fig 1). Likewise, for F_2 the absorbance at 268 mμ was divided by 8.52×10^3 liters/mole cm, the average molar absorptivity for guanylic and uridylic acids at this wavelength, where their absorption curves approach coincidence (table I & fig 2).



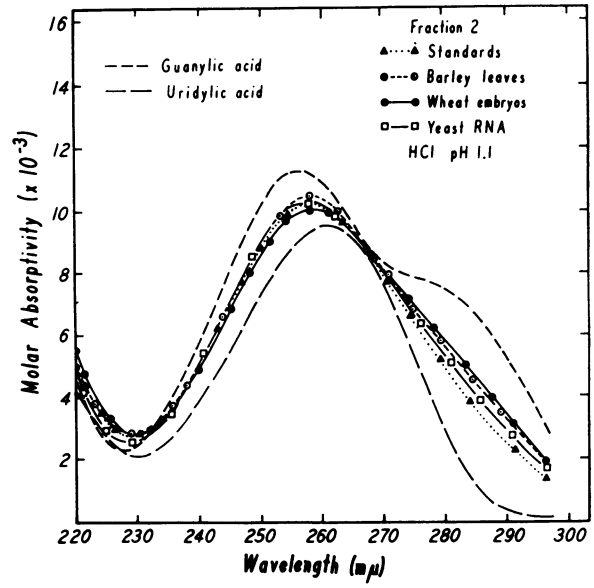


Fig. 1. Absorption spectra of RNA constituents of Fraction 1 on the molar basis, with absorption curves of Fraction 1 from several sources, superposed to agree at 268.7 mμ, a coincident point of the standard curves.

Fig. 2. Absorption spectra of RNA constituents of Fraction 2 on the molar basis, with absorption curves of Fraction 2 from several sources, superposed to agree at 268 mμ, where the standard curves approach coincidence.

Table I

Molar Absorptivities at Analytical Wavelengths (liters/mole cm)

Wavelength	268.7 mμ	268 mμ	284 mμ	290 mμ
Adenylic acid	10.6×10^3	...	1.75×10^3	...
Cytidylic acid	10.6×10^3	...	11.85×10^3	...
Guanylic acid	...	8.68×10^3	...	5.21×10^3
Uridylic acid	...	8.36×10^3	...	0.28×10^3

Compositions of F_1 and F_2 in terms of individual nucleic acids were readily calculated from absorbance readings at 268.7 and 284 mμ for F_1 and at 268 and 290 mμ for F_2 (table I). The following equations employ these molar absorptivities. Relationships of the curves are illustrated in figures 1 and 2. C_1 and C_2 represent concentrations of adenylic and cytidylic acids, and C_3 and C_4 those of guanylic and uridylic acids, respectively. A = absorbance.

Equations for Fraction 1:

$$A_{268.7} = (10.6 \times 10^3\ C_1) + (10.6 \times 10^3\ C_2)$$
$$A_{284} = (1.75 \times 10^3\ C_1) + (11.85 \times 10^3\ C_2)$$

Equations for Fraction 2:

$$A_{268} = (8.68 \times 10^3\ C_3) + (8.36 \times 10^3\ C_4)$$
$$A_{290} = (5.21 \times 10^3\ C_3) + (0.28 \times 10^3\ C_4)$$

Solution of the above equations:

Fraction 1

Adenylic acid (Molarity) =
$(11.07\ A_{268.7} - 9.90\ A_{284}) \times 10^{-5}$

Cytidylic acid (Molarity) =
$(9.901\ A_{284} - 1.631\ A_{268.7}) \times 10^{-5}$

Fraction 2

Guanylic acid (Molarity) =
$(20.329\ A_{290} - 0.681\ A_{268}) \times 10^{-5}$

Uridylic acid (Molarity) =
$(12.674\ A_{268} - 21.111\ A_{290}) \times 10^{-5}$

Comparison With Adsorption Analysis by Fraction Collector: Analyses of yeast (*Saccharomyces cerevisiae* Meyen) RNA (C grade, California Corp. for Biochemical Research, Los Angeles, Cal.) and barley leaves were checked by use of a fraction collector according to an abbreviated version of the method described by Hurlbert, et al. (7), using their formic acid system. A gradient elution system of 300 ml of water and 450 ml of 4 M formic acid was used. With yeast RNA and barley leaf nucleotides 5 and 10 ml syphons were used, respectively. Syphons were calibrated for delivery of the graduated solvent system during the fractionation and corrections applied. All four nucleotides were eluted by a 500 ml volume of eluate. Absorbance values at 260 mμ were compared to that of water.

Fractions containing each nucleotide were pooled, evaporated to dryness at room temperature and made to 25 ml in 0.1 M HCl. Absorption curves were determined for identification and concentrations were calculated from maximum absorbance values for each nucleotide.

Results

To determine the recovery of the standard nucleotides in F_1 and F_2, each pair of nucleotides (about 3×10^{-6} moles) was added individually to a column and eluted as indicated in the method. For the mixture of standards reported in table II, errors in individual results were ± 0 to 4 %. Errors in totals in F_1, F_2, or $F_1 + F_2$ were 1 to 2 %. To supplement these analyses, other mixtures (with similar total amounts) were analyzed, with results presented in table III. These mixtures represent extreme variations in composition, and errors are acceptably low. None of our measurements indicated any deviation from Beer's Law.

Curves for Fractions: In figure 1 are the standard curves for adenylic and cytidylic acids at pH 2.2. Curves for F_1 isolated chromatographically from a mixture of the four standards (mixture 1 of table III) and from barley leaves, young wheat (*Triticum aestivum* L.) embryos (4–5 mm in length) and yeast RNA are calculated to agree with the standard curves at their crossing point, 268.7 mμ. In figure 2 are similar curves for F_2 at pH 1.1, placed to agree at 268 mμ between the standard curves where they approach coincidence most closely.

Comparison With Fraction Collector Method: Values from fraction collector adsorption analysis are recorded in table II under composite analysis. As a check on this procedure, absorbance values for each individual fraction were corrected for increasing solvent absorption due to increasing concentrations of formic acid during the fractionation. Absorbance values for individual fractions containing each nucleotide were added and amounts calculated as presented in table II, under addition of absorbances. This table summarizes comparative results on mixtures of standards, and on yeast and barley leaf RNA by column—spectrophotometry and fraction collector methods and presents corresponding base ratios.

Discussion

Various extraction procedures for RNA were tried, using fresh barley leaves. Leaves were boiled in 95 % ethanol, disintegrated in a waring blendor, and extracted with acetone for removal of chlorophyll and plant pigments. The remaining tissue was first extracted by boiling with 0.55 M NaCl solution. Incomplete extraction resulted. Extraction by salt

Table II

Comparative Analytical Results* on RNA from Several Sources by 2 Methods

Nucleotide	Column-spectrophotometry					Fraction collector			
	(moles/l) Standards		(moles/g dry wt)			(moles/g dry wt) Addition of absorbances		Composite analysis	
	Known	By analysis	Yeast RNA	Barley leaves (Atlas)	Barley leaves (Atlas 46)	Yeast RNA	Barley leaves (Atlas)	Yeast RNA	Barley leaves (Atlas)
	× 10^5		× 10^4	× 10^6	× 10^6	× 10^4	× 10^6	× 10^4	× 10^6
Adenylic acid	1.42	1.37	6.03	5.32	5.28	7.05	5.53	6.35	5.50
Cytidylic acid	1.55	1.54	5.13	5.39	5.40	5.84	5.88	5.30	5.41
Sum (F_1)	2.97	2.91	11.16	10.71	10.68	12.89	11.41	11.65	10.91
Guanylic acid	1.36	1.34	7.99	8.02	7.58	9.54	7.22	8.18	6.65
Uridylic acid	1.54	1.53	7.19	5.34	5.55	7.84	5.12	7.74	5.30
Sum (F_2)	2.90	2.87	15.18	13.36	13.13	17.38	12.34	15.92	11.95
Total (F_1 + F_2)	5.87	5.78	26.34	24.07	23.81	30.27	23.75	27.57	22.86
Ratios									
A/U	0.922	0.895	0.840	0.990	0.953	0.902	1.080	0.820	1.040
G/C	0.877	0.870	1.560	1.490	1.402	1.630	1.230	1.540	1.230
Pu/Py	0.900	0.883	1.138	1.242	1.175	1.213	1.159	1.114	1.133

* Averages of two completely separate determinations.

solution was recently discussed by Smillie and Kratkov (15). Further attempts to extract RNA by autoclaving at 10 pounds pressure for 10 minutes resulted in considerable decomposition, as shown by variable base ratios and poor precision. A further disadvantage in the use of fresh tissue was the extraction of large amounts of contaminating protein.

No additional RNA was removed from dried leaf material by more than four extractions. Steaming periods under 20 minutes gave incomplete extraction and no additional extraction resulted from a 60-minute period.

Published work (4, 5) indicates that alkaline hydrolysis of RNA with 1 M KOH for 24 hours at room temperature failed to reduce all of the compounds to mononucleotides, a fraction amounting to about 5 % of oligonucleotides remaining in the case of RNA from yeast and mammalian tissue. Further hydrolysis with 1 M KOH for 24 hours converted the oligonucleotides to mononucleotides. These oligonucleotides are high in purine bases.

Comparisons were made on RNA preparations hydrolyzed with 0.5 M KOH at 30 C for 18, 40, and 60 hours. The results for the 40- and 60-hour periods were similar, both being higher than for the 18-hour period. For the 40- and 60-hour periods, higher amounts of both adenylic and guanylic acids were found.

The absorbance value at 268 mμ before fractionation indicated about 20 % extraneous absorption for barley leaves and 28 % for young wheat embryos. Materials causing this extraneous ultraviolet absorbance were removed by the column and were due to non-nucleotide impurities, which can be shown by corresponding absorption curves. Such measurements are reliable guides for determining the load suitable for the column.

The column with dimensions described provides good separation of F_1 and F_2 when their absorbance values at 268 mμ are in the range 0.21 to 0.32. Work with standard components showed that when larger quantities were applied to the column, poor separation and inaccurate recovery from the column resulted.

The supernatant resulting from hydrolysis and centrifugation was tested for protein (12). Trace

Table III

Comparative Analytical Results on Standard Mixtures of Nucleotides

Acid mixture	Known composition %				Error relative to amount present %				Error relative to total RNA %			
	1	2	3	4	1	2	3	4	1	2	3	4
Adenylic acid	25	12	50	0	2.5–4.0	< 7	6.7	...	< 1.0	0.8	2.9–3.4	< 0.15
Cytidylic acid	25	38	25	25	0–2.0	< 1	1.5	< 2	< 0.5	0.1–0.2	< 0.5	< 0.5
Guanylic acid	25	12	0	50	0–3.0	1–5	...	2.4–7.0	< 0.7	0.3–0.6	0.8–1.7	1.1–3.4
Uridylic acid	25	38	25	25	0–2.0	1–2	2.5–7.0	4–6.5	< 0.5	0.6–0.7	0.8–2.0	1.1–1.7

amounts were noted from yeast RNA, two barley varieties, and young wheat embryos. F_1 and F_2 from wheat embryos gave negative tests for protein.

Spectroscopic measurements on the more recently purified standards used here are probably more accurate than values reported by Smith and Markham (16) because of improved purification methods. Ratios of absorption at $\frac{280}{260}$ and $\frac{250}{260}$ mμ are in good agreement with those of Volkin and Cohn (18). The specific wavelengths used here for analysis have not been used elsewhere on the same fractions. Other workers have used 340, 290, 280, 278, 265, 262, 250, 245, and 230 mμ in various combinations for estimation of nucleic acids and ratios of absorption at such wavelengths were used as indications of purity.

The customary use of λ 260 mμ to estimate RNA in acid solutions is not the best choice, since different base ratios will obviously give different apparent results with possibly large errors. If a single wavelength is needed to estimate total RNA, 268.7 mμ is the best available, where differences in molar absorptivities are minimum (average value 9.52×10^{-3}). A more favorable wavelength might be near 270 mμ at pH 7.2, in which case three of the component curves approach coincidence.

Magasanik, et al. (8) employed wavelengths 245 and 265 mμ compared to 290 mμ and developed equations for guanylic and uridylic acid concentrations, based on absorption differences at these wavelengths. They separated this pair of constituents from adenylic and cytidylic acids by paper chromatography at pH 3.6. Their work is the most closely related study to the method reported here. Their method handled quantities of 0.008 to 0.070 mg of nucleotides, whereas the procedure described here requires quantities of 1 to 2 mg for best results.

Smith and Markham (16) hydrolyzed RNA with 1 N HCl at 100 C for 1 hour, separated the resultant purines and pyrimidine nucleotides by paper chromatography, and determined absorbance values of material from the eluted spots. The apparent standard absorption values for cytidylic and uridylic acids in 0.1 N HCl were close to those from the curves of figures 1 and 2. They reported some loss (5 %) of pyrimidine nucleotide during hydrolysis and studied yeast RNA samples as small as 0.1 mg. In comparison of molar ratios of bases referred to the average, their results agree with ours for cytidine and guanine, but are higher than ours for adenine and lower for uridine. This may be due in part to differences in standard absorption values. The same method for determining nucleotide composition was recently employed by Miura and Egami (9) in their studies on yeast RNA.

Loring, et al. (6) presented a method for the spectrophotometric analysis of purine and pyrimidine components after separation of purine bases by silver precipitation from an acid hydrolysate, followed by conversion of the pyrimidine nucleotides to cytidine and uridine by acid phosphatase. They purified the nucleoside fraction by filtration through Dowex-1 and employed wavelengths 262 and 280 mμ for analysis of adenine and guanine mixtures at pH 1.0 and 260 and 278 mμ for cytidine and uridine mixtures at pH 2.0. On artificial mixtures of purified components they reported recovery of 99 ± 2 % of each constituent, after a correction of 3 to 4 % was made for deamination of cytidilic acid. This method is considerably longer than that reported here and involves more steps, possibly leading to more decomposition.

A solution of each standard (twice the quantity used in mixture 1 of table III) was passed individually through the adsorption column under the same conditions used in analysis. The absorption curves of the eluates were higher at the shorter wavelengths than for the standards before adsorption; below 240 mμ for guanylic and uridylic acids, below 248 mμ for adenylic acid, and below 256 mμ for cytidylic acid. Spectroscopic study of the acid washes that should theoretically be free of absorption because of absence of certain constituents showed that contamination of F_2 with constituents expected only in F_1 were negligible (< 1 % of amount present), as was also the amount of uridylic acid that appeared in F_1; guanylic acid appeared in F_1 to the extent of 6 % of the amount present. This indicates that the column may be more easily overloaded with guanylic acid, to cause total F_1 and apparent cytidylic acid to be high (by a calculated amount less than 6 % of the guanylic acid in the above case, because of the relationship of molar absorptivities).

The fractions collected from the column for composite analysis by the fraction collector were studied spectroscopically in comparison with standard curves. Measurements of the curves of fractions from yeast RNA and barley leaves showed that absorbance was slightly higher than for the standard at wavelengths below 245 and above ca 278 mμ for adenylic acid, above 278 mμ for cytidylic acid, below 250 mμ for guanylic acid, and below ca 254 to 258 mμ for uridylic acid.

Careful study of the absorption curves for F_1 and F_2 (figs 1 & 2) revealed close agreement with expected positions for wavelengths above ca 240 to 250 mμ. At shorter wavelengths all the curves deviated toward higher values than expected. Since even the standards shared this change, it may be inferred that the column procedure contributed to this source of error at the shorter wavelengths. Biological extracts frequently contain impurities absorbing the short-wave ultraviolet, making necessary the use of longer wavelengths.

It is apparent from table II that satisfactory results can be obtained on unknowns. Agreement with fraction collector methods was satisfactory, considering the many sources of error known to apply to quantitative addition of the fraction data. Composite data were in general lower than those from addition of absorbances. The relative instability of guanylic acid is contrasted with greater stability of uridylic acid. Addition of absorbances may add many small

errors, whereas, the composite method may lead to more decomposition, especially of guanylic acid.

The method reported here is simple and rapid but requires rigid adherence to certain limitations of column capacity. It does not consider the possible occurrence of nucleotides other than the four most common ones. For their detection, paper chromatography is the best method. To our knowledge, those of less frequent occurrence are seldom present to the extent of more than several percent of the total. As such, they would not interfere seriously with the main system of analysis, particularly if their curves are similar to their counterpart considered here. We consider the dependable limits of error of this method as less than approximately ± 4 % for each nucleotide on the basis of amount present and ± 3 % on the basis of total RNA. Precision is usually subject to less than ± 1 % error. If amounts of any nucleotide are relatively small, the analysis for it may have considerable error as a part of the amount present, but the percentage of the total nucleotide sample accounted for will still be in error no more than ca ± 2 %. Unfamiliar materials should always be checked by paper chromatography to determine the presence of new or different nucleotides or to check for the presence of very small amounts of the four components considered here.

Most of our analyses were applied to barley leaf meal and yeast RNA. In a brief study of RNA from young wheat embryos, we found that absorption curves for F_1 and F_2 deviated from analytical expectancy, especially that for F_2 in the region below 260 mμ. Paper chromatography did not positively reveal any other constituent in addition to the four components considered here, but the uridylic acid spot's location was not exactly as expected. Possibly an isomer occurred in this source.

Summary

RNA from barley (*Hordeum vulgare* L.) leaves and yeast (*Saccharomyces cerevisiae* Meyen) was analyzed for each of its four principal components, adenylic, cytidylic, guanylic, and uridylic acids, with an error of ± 4 % or less. Total recoveries were good. Following alkaline hydrolysis, fractionation into two groups of two components each by an adsorption column provided mixtures suitable for ultraviolet spectrophotometric analysis. The procedure was simple and short. Details of extraction, purification, hydrolysis, fractionation, and spectrophotometry were critically reviewed. Limitations were specified. Checks with a fraction collector agreed well. The method was applied to mixtures of standards, yeast RNA, and barley leaves.

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