A Method for the Quantitation of 5′-Mononucleotides in Foods and Food Ingredients

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A method is presented for the quantitation of the flavor-potentiating 5′-mononucleotides in foods and food ingredients. Extracted nucleotides, nucleosides, and purines are separated by ion-pairing reversed-phase high-performance liquid chromatography. The method is not affected by high levels of salts in the sample, and recovery of 5′-mononucleotides after sample treatment and analysis is 102 ± 3%. In addition to measurements of 5′-mononucleotides in foods and food ingredients, data are presented to illustrate the method's utility in monitoring nucleotide levels during food or ingredient processing or preparation.

INTRODUCTION

Like monosodium glutamate (MSG), guanosine 5′-monophosphate (5′-GMP) and inosine 5′-monophosphate (5′-IMP) are umami substances that have become important flavor potentiators in the food industry. Their widespread utilization thus necessitates that they be quantitated in a broad spectrum of sources from flavor additives such as yeast extracts to food products such as bouillons and sauces. Although anion-exchange chromatography has been the prevailing method for the separation and quantitation of nucleotides (Harwick and Brown, 1975; Floridi et al., 1977; McKeag and Brown, 1978; Nissinen, 1980; Riss et al., 1980; Freese et al., 1984), its application to flavor mononucleotides in foods is limited because of the frequent presence of interfering levels of salts. The advent of high-performance liquid chromatography (HPLC) has allowed the separation of cellular nucleosides and nucleotides by other methodologies such as reversed-phase HPLC (Wakizaka et al., 1979; Taylor et al., 1981) and ion-pairing reversed-phase HPLC (Hoffman and Liao, 1977; Qureshi et al., 1979; Walseth et al., 1980; Knox and Jurand, 1981; Payne and Ames, 1982). Certainly, recent publications in the flavor industry [e.g., Kenney (1990)] point out the rapidly growing importance of HPLC in flavor research and flavor quality control. However, I found that none of the published HPLC methods for nucleotide separation and quantitation could be directly applied to the extraction and determination of 5′-mononucleotides in foods and food ingredients. Toward this end, an extraction procedure and one of the above HPLC methodologies were modified for application to food products; the results are reported herein.

MATERIALS AND METHODS

Materials. All nucleotides, nucleosides, and purine bases were purchased from Sigma (St. Louis, MO). PIC A, regular (tetrabutylammonium phosphate), was purchased from Waters Associates (Milford, MA). Methanol for HPLC was of Optima grade from Fisher Scientific (Fair Lawn, NJ). Water for HPLC was produced by a Milli-Q system (Millipore/Waters). Yeast products were obtained as samples from the suppliers; the foods were purchased at a local supermarket.

Standard Samples. Standard nucleotides were dissolved in 0.1 M potassium phosphate buffer, pH 7.5. The true concentration of each nucleotide in its standard solution was determined by its absorbance measured in an AVIV 14DS UV–vis scanning spectrophotometer with the use of the following molar extinction coefficients at pH 7 (Beaven et al., 1955; Bock et al., 1956): 5′-CMP, 9.0×10^3 at 271 nm; 5′-AMP, 15.4×10^3 at 259 nm; 5′-UMP, 10.0×10^3 at 262 nm; 5′-GMP, 13.7×10^3 at 252 nm; 5′-IMP, 12.2×10^3 at 248.5 nm; and 5′-TMP, 9.6×10^3 at 267 nm. After the elution position of each individual nucleotide was determined, aliquots of each of the nucleotides were combined into a mixture of the six standards. The nominal concentration of individual nucleotides in the mixture of standards was 50 μM.

Chromatographic Procedure. A Dionex BIOLC HPLC equipped with an autosampler and a variable-wavelength detector was used throughout. A Synchropak 250 mm × 4.6 mm RPP-100 C-18 column (Synchrom, Lafayette, IN) was employed. Three different columns of this type from the same manufacturer gave similar resolution among components and identical sequence of elution of nucleotides and nucleosides; however, the retention times of the compounds varied slightly from column to column. Quantitative results from the three columns were identical. A flow rate of solvent through the column was maintained at 1.0 mL/min. Nucleotides were detected by their absorbance at 254 nm. In samples of unknown composition, peaks were tentatively identified by their retention time. The identity of each of the tentatively identified peaks was further supported by the coelution of each with the authentic compound. Instrument control and data handling were accomplished through the use of Dionex AI 400 software. The solvent systems of Qureshi et al. (1979) were used in this study; the aqueous solvent, solvent A, contained water/glacial acetic acid/PIC A in a 97.5:1.5:1.0 (v/v/v) ratio, while the organic solvent, solvent B, consisted of methanol/glacial acetic acid/PIC A in a 97.5:1.5:1.0 (v/v/v) ratio. The volume of PIC A used in each solvent corresponds to a concentration of 3.5 mM. Columns were washed with H_2O and stored in methanol when not in use.

Sample Preparation. Samples were either yeast in some phase of processing or a commercial food or flavor product. Thus, the principal steps of sample preparation were (i) extraction of the nucleotides followed by (ii) acid precipitation of any macromolecules from the extract with recovery of all of the free 5′-mononucleotides. During various stages of process development, yeast suspensions were sampled directly and treated with perchloric acid as described below. Food and flavor products were pretreated in one of two ways. Water-soluble samples were suspended in water at 1 g dry weight/10 mL final volume. They were then stirred at 10 °C for 1 h before an aliquot was removed for perchloric acid treatment.

Water-insoluble samples (the rice dish and the stuffing mix) were treated in two ways. First, 15 mL of H_2O/g of dry sample was added to each sample so that the mixture was "soupy" enough that it could be stirred on a magnetic stirrer. The mixture was stirred at 10 °C for 1 h, a sample was centrifuged to remove debris, and an aliquot was removed for perchloric acid treatment. Second, each food dish was prepared according to its manufacturer's directions. It was then added to a volume of water 2–5 times its prepared weight, blended for 2 min, stirred at 10 °C for 15 min, centrifuged to remove debris, and aliquoted for perchloric acid treatment.

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Table I. Recovery of 5'-Mononucleotides from Various Sample Treatment Protocols

treatment[a]	% recovery[b] 5'-GMP	5'-IMP	5'-AMP
nucleotides in H_2O; injected directly	100 ± 4	100 ± 2	100 ± 3
nucleotides added to H_2O; treatment 1[c]	97 ± 4	100 ± 5	95 ± 4
nucleotides added to H_2O; treatment 2[d]	98 ± 3 (12)	99 ± 4 (12)	99 ± 4 (12)
nucleotides added to heated broken yeast cells; treatment 1	46 ± 10	102 ± 5	110 ± 2[e]
nucleotides added to heated broken yeast cells; treatment 2	97 ± 4 (9)	96 ± 4 (9)	110 ± 3[e] (9)
nucleotides added to heated broken yeast cells, 8.66 mg/mL each; treatment 2	102 ± 5 (11)	102 ± 3 (11)	
nucleotides added to heated broken yeast cells, 0.05 mg/mL each; treatment 2	105 ± 7 (6)	99 ± 2 (6)	

[a] The concentration of each nucleotide in the sample before treatment was 0.15 mg/mL unless another concentration is indicated. [b] Average and standard deviation of three samples unless a larger number of samples is indicated. [c] Treatment 1: Precipitation with 0.6% $HClO_4$ and neutralization with 1 M $KHCO_3$. [d] Treatment 2: Precipitation with 0.6% $HClO_4$ and neutralization with 1 M $KHCO_3$–50 mM Na_4EDTA. [e] Yeast cells contained endogenous 5'-AMP; recovery of exogenously added 5'-AMP was 99 ± 3% when data were corrected for the amount of endogenous nucleotide.

The perchloric acid treatment step was performed as follows. A 0.7 mL volume of sample was added to 0.7 mL of ice-cold 1.2 M $HClO_4$ and mixed. The mixture was allowed to stand on ice for 5 min and then centrifuged on a bench-top centrifuge at 12000*g* for 2 min to clarify the supernatant. An 0.8-mL aliquot of the supernatant was then added to 0.85 mL of ice-cold 1.0 M $KHCO_3$/50 mM Na_4EDTA in a 15-mL screw-top conical centrifuge tube, and the contents were thoroughly mixed. After setting on ice about 15 min, the sample was centrifuged to pellet the $KClO_4$, and an aliquot of the supernatant was further diluted with water (if necessary) or directly injected onto the column for analysis. Samples were either centrifuged or filtered prior to injection. It is important that the quantity of $KHCO_3$ be sufficient to completely neutralize the $HClO_4$ so that the pH of the neutralized sample is around 6.5. The EDTA is necessary to ensure complete recovery of 5'-GMP (see Results and Discussion).

RESULTS AND DISCUSSION

Development of the Method for 5'-Mononucleotide Quantitation. The ion-pairing reversed-phase HPLC solvent system of Qureshi et al. (1979) was chosen for our applications after it was observed that the levels of ions in many sources for analysis thwarted attempts to quantitate 5'-mononucleotides by ion-exchange HPLC. Furthermore, reversed-phase HPLC in the absence of an ion-pairing agent did not provide adequate resolution of the nucleotides from food sources. It was also necessary to modify the published gradient program of Qureshi et al. (1979) to achieve complete separation of the 5'-mononucleotides. With this new gradient program, a different elution sequence for the nucleosides and nucleotides was observed from that reported earlier (Qureshi et al., 1979).

As illustrated in Figure 1, a complete separation of the 5'-mononucleotides was achieved with the new gradient program. The elution program included a 5-min wash with solvent A after sample injection, a 15-min linear gradient to 10% solvent B, a 3-min isocratic wash at 10% solvent B, a 6-min linear gradient to 40% solvent B, a 1-min isocratic wash at 40% solvent B, a 4-min linear gradient back to 100% solvent A (initial conditions), and finally, a 10-min re-equilibration wash with 100% solvent A before the next sample injection. Unlike the program

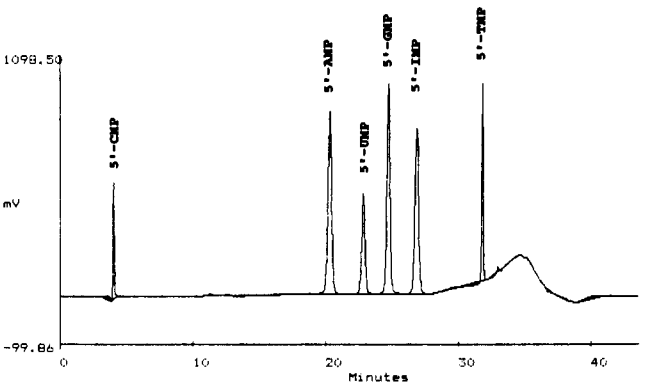

Figure 1. Elution profile of 5'-mononucleotide standards. The quantity of each nucleotide injected onto the column was 0.278 μg of 5'-CMP, 0.484 μg of 5'-AMP, 0.383 μg of 5'-UMP, 0.569 μg of 5'-GMP, 0.585 μg of 5'-IMP, and 0.415 μg of 5'-TMP. Full-scale absorbance was 0.05 at 254 nm.

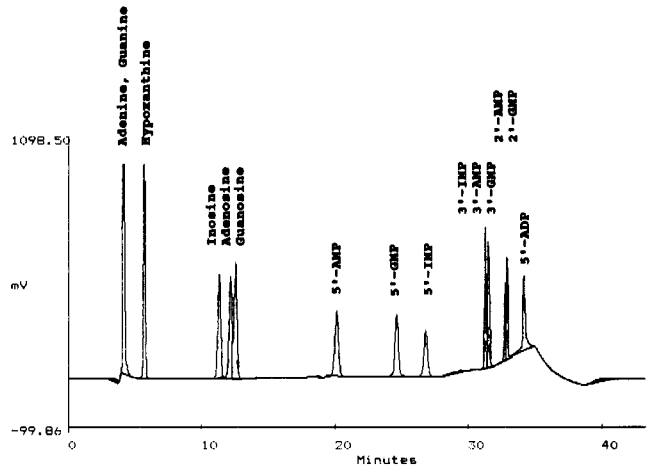

Figure 2. Separation of purines, nucleosides, and nucleotides. The nominal amount of each compound injected onto the column was about 0.3 μg. Full-scale absorbance was 0.05 at 254 nm.

of Qureshi et al. (1979), this gradient program did not require washing with methanol every four samples. As many as 48 h of continuous runs were made without a noticeable effect on the column's resolving properties; more extended schedules of runs were not tried.

On a routine, day-to-day basis, the peak areas for each of five of the six standards agreed to within ±2.5% for multiple injections. 5'-CMP, because of its location near the breakthrough peak was less precise (±6%). The area of each peak was a linear function of the quantity of 5'-mononucleotide injected, normally between 0.04 and 0.9 μg. These quantities were of the appropriate range for the detector sensitivity routinely employed, which was 0.05 absorbance unit full scale. Furthermore, very few UV-absorbing compounds were observed to elute in the region at which 5'-GMP and 5'-IMP elute; this greatly simplifies recognition and quantitation of these two flavor mononucleotides.

As observed by others (Hoffman and Liao, 1977; Qureshi et al., 1979; Walseth et al., 1980; Knox and Jurand, 1981; Payne and Ames, 1982), the number or location of phosphate ester bonds on a given nucleoside has a profound impact on its retention time. This is illustrated in Figure 2 by the elution times for the purines, their nucleosides, and their various nucleoside phosphate esters. To provide maximal resolution among the 5'-mononucleotides, some resolution among the other compounds was compromised by our elution program. Nevertheless, it was still possible to identify and quantitate most of these various nucleo-

Table II. 5'-Mononucleotide Contents of Selected Foods and Food Ingredients

product	% of dry product (w/w) ± SD (n)		
	5'-AMP	5'-GMP	5'-IMP
A (yeast extract)	1.64 ± 0.15 (2)	1.79 ± 0.03 (3)	ND[a]
B (yeast extract)	0.39 ± 0.01 (3)	0.41 ± 0.05 (3)	ND
C (yeast autolysate)	0.10 ± 0.01 (4)	<0.01	ND
D (yeast extract)	0.18 ± 0.01 (3)	1.48 ± 0.02 (3)	1.50 ± 0.10 (3)
E (instant beef bouillon)	ND	0.024 ± 0.002 (2)	0.032 ± 0.002 (2)
F (beef-flavored broth)	ND	0.016 ± 0.003 (2)	0.013 ± 0.003 (2)
G (gravy mix)	ND	ND	ND
H (rice and sauce before prep)	0.005 ± 0.001 (2)	0.065 ± 0.004 (2)	0.078 ± 0.006 (2)
I (rice and sauce after prep)	ND	0.031 ± 0.002 (2)	0.033 ± 0.003 (2)
J (stuffing mix before prep)	ND	0.007 ± 0.003 (2)	0.007 ± 0.003 (2)
K (stuffing mix after prep)	ND	0.012 ± 0.003 (2)	0.010 ± 0.002 (2)

[a] ND, none detected at the level assayed.

sides and nucleotides. This can prove to be very useful in developing and monitoring production processes as will be discussed later.

The method of treatment of the sample before HPLC analysis was found to be a critical aspect of the methodology. As illustrated in Table I, it was discovered that recoveries of 5'-GMP were reduced by as much as 50–70% in some samples when the acid-treated extract was neutralized with only $KHCO_3$. This loss of 5'-GMP was a time-dependent phenomenon that occurred after neutralization of the acid-treated material. The inclusion of EDTA with the $KHCO_3$ in the neutralizing solution eliminated this loss of 5'-GMP. No further investigations were initiated to elucidate the component(s) responsible for the diminution of 5'-GMP. As a final test of 5'-mononucleotide recovery using the procedure, the recoveries of 5'-GMP and 5'-IMP were determined in various yeast samples to which known amounts of each of the nucleotide standards had been added and which were then subjected to our analysis procedure. The average recovery was 102 ± 3% for 26 samples to which the 5'-mononucleotides had been added over a 170-fold concentration range (Table I).

Applications of the Method. As stated earlier, the major advantage of ion-pairing reversed-phase chromatography over ion-exchange chromatography is that the functionality of the former is unaffected by the ionic composition of the sample. Thus, analysis may be applied directly to autolysates, extracts, fermentor creams, or processed foods. This, of course, suggests that, in addition to its utility for the quantitation of flavor mononucleotides in finished products, it may be used as a quantitative monitor during process development or production. Such an application is illustrated in Figure 3, which examines the nucleoside and nucleotide levels at one step during process development.

Two characteristics of the process are evident from the analysis. First, 5'-mononucleotides have been produced, and their respective levels can be quantitated. Second, it can also be deduced that a substantial quantity of 5'-AMP and 5'-GMP has been dephosphorylated during a previous step. If the causative agent (heat, pH, enzyme) of the phosphate ester hydrolysis can be identified and its detrimental action overcome, then the yield of 5'-mononucleotides would be increased by 70%.

Table II presents the quantitative analysis of flavor-potentiating 5'-mononucleotides from a selection of commercial yeast-based flavor enhancers and some selected food products. 5'-AMP levels are included as it can serve as a direct precursor of 5'-IMP. The data from the samples selected for this table illustrate applications of the methodology. As discussed above, yeast products as flavor potentiators can be evaluated quantitatively for their

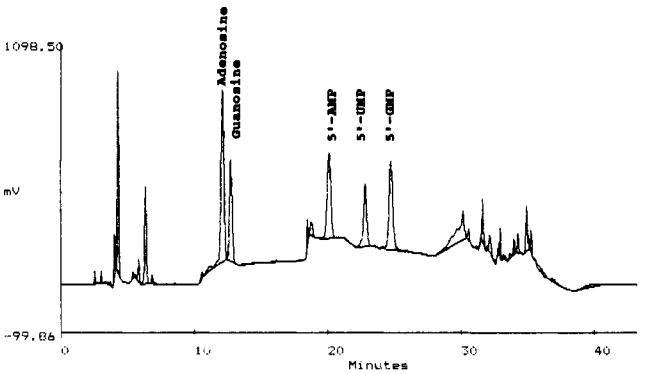

Figure 3. Measurement of the flavor-potentiating nucleotides produced during an experimental process development. Labeled peaks were verified by coelution with standards. Full-scale absorbance was 0.05 at 254 nm. The peak for 5'-AMP represents 0.207 μg of nucleotide for a sample content of 0.39% 5'-AMP on a dry weight basis. The peak for 5'-UMP represents 0.216 μg of nucleotide for a sample content of 0.41% 5'-UMP on a dry weight basis. The peak for 5'-GMP represents 0.260 μg of nucleotide for a sample content of 0.49% 5'-GMP on a dry weight basis.

content of 5'-GMP and 5'-IMP. The methodology allows evaluation of individual process steps as well, since separation and quantitation of purines, nucleosides, and other nucleotides are also possible. For example, high levels of 3'-mononucleotides apparent in chromatograms from the analysis of product C suggest that nucleases specific for the 5'-phosphodiester bond predominated during autolysis. High levels of nucleosides in product B suggest that further hydrolysis of the 5'-mononucleotides occurred during production/processing. One of the foods, product G, yielded no measurable 5'-GMP or 5'-IMP, although they were listed as ingredients; however, significant levels of guanosine and inosine (equivalent to 0.02% of each by weight) were present. These results suggest that the flavor mononucleotides were hydrolyzed during a heating/drying processing step subsequent to their addition. It is also interesting to note that about 50% of the GMP and IMP was lost during preparation of the rice in sauce; the lost nucleotides could be accounted for by an equivalent production of guanosine and inosine. Perhaps flavor nucleotide loss occurs during preparation of the rice in sauce and apparently not during stuffing preparation because of the shorter heating times necessary for preparation of the stuffing (Shaoul and Sporns, 1987; Matoba et al., 1988).

In conclusion, a method is presented for the quantitation of 5'-mononucleotides in foods, in food ingredients, and during process development. The method is simple, quantitative, and virtually free of interference from other components found in food samples normally tested.

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Registry No. 5'-AMP, 61-19-8; 5'-GMP, 85-32-5; 5'-IMP, 131-99-7; 3'-IMP, 572-47-4; 3'-AMP, 84-21-9; 3'-GMP, 117-68-0; 2'-AMP, 130-49-4; 2'-GMP, 130-50-7; 5'-ADP, 58-64-0; adenine, 73-24-5; guanine, 73-40-5; hypoxanthine, 68-94-0; inosine, 58-63-9; adenosine, 58-61-7; guanosine, 118-00-3.