

## DEVELOPMENT OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR ANALYZING DISODIUM 5'-GUANYLATE AND DISODIUM 5'-INOSINATE LEVELS IN FLAVOR ENHANCERS

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### **ABSTRACT**

**Objective:** This study aimed to develop a selective analytical method for assessing disodium 5'-guanylate and disodium 5'-inosinate levels in flavor enhancers.

**Methods:** The levels were assessed using high-performance liquid chromatography (HPLC) with a photodiode array detector (PDA) (wavelength =255 nm) and a SunFire® C18 column (250 mm × 4.6 mm × 5 µm). The mobile phase comprised a mixture of potassium phosphate buffer and an ion pair reagent-hexane-1-sulfonic acid sodium salt - with a flow rate of 1.2 mL/min. The ion pair was used to generate a neutral equilibrium, which resulted in increased retention of the analytes. Optimized analysis conditions were then validated regarding accuracy, precision, linearity, selectivity, and the limits of detection and quantification.

**Results:** The average levels of disodium 5'-inosinate in the six analyzed samples were 0.24±1.46, 0.21±2.69, 0.58±3.26, 0.21±0.84, 0.22±3.59, and 0.47±2.21%, respectively. Regarding disodium 5'-guanylate, the average levels were 0.15±2.85, 0.15±0.12, 0.41±3.80, 0.16±1.72, 0.27±1.18, and 0.34±1.83, respectively.

**Conclusion:** The optimal conditions for analyzing disodium 5'-guanylate and disodium 5'-inosinate using HPLC with a PDA and SunFire C18 column were λ=255 nm, a mobile phase of potassium phosphate buffer and sodium hexane sulfonate, and a flow rate of 1.2 mL/min. For disodium 5'-inosinate, its average levels in samples A-F were 0.24±1.46, 0.21±2.69, 0.58±3.26, 0.21±0.84, 0.22±3.59, and 0.47±2.21%, respectively. Meanwhile, the average levels of disodium 5'-guanylate in the samples were 0.15±2.85, 0.15±0.12, 0.41±3.80, 0.16±1.72, 0.27±1.18, and 0.34±1.83%, respectively.

**Keywords:** Disodium 5'-guanylate, Disodium 5'-inosinate, High-performance liquid chromatography; ion pair, Photodiode array.

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### **INTRODUCTION**

Flavor enhancers are used to maintain and improve the quality and stability of food. They are also used to maintain the nutritional value of food, which can be reduced during processing or packaging, to fulfill community needs [1].

Flavor enhancers are widely used in several countries, including Indonesia, because they can create a savory taste, also known as "umami." Monosodium glutamate (MSG) is a white crystalline compound that is widely used as a flavor enhancer. In addition to MSG, the commonly used flavor enhancers in Indonesia include disodium 5'-inosinate and disodium 5'-guanylate. These compounds are categorized as nucleotide compounds that are generally used in conjunction with MSG to strengthen the flavor [2].

Disodium 5'-guanylate and disodium 5'-inosinate are metabolized in the body and absorbed in the duodenum. The nucleosides are actively absorbed, whereas purines are generally absorbed through passive diffusion mechanisms across the intestinal wall. Disodium 5'-guanylate undergoes dephosphorylation to guanosine, followed by hydrolysis to guanine, deamination to xanthine, and oxidation to form uric acid. Meanwhile, disodium 5'-inosinate is derived from the dephosphorylation of ATP to AMP followed by deamination [3,4].

Although flavor enhancers are safe for consumption, their excessive use can endanger community health, and thus, further examination of these nucleotides needed. A quantitative analysis method that can determine the levels of flavor enhancers in small sample quantities is desired.

Several methods have been previously used by researchers to analyze disodium 5'-guanylate and disodium 5'-inosinate levels.

Previous research conducted by Qiu *et al.*, Vinas *et al.*, and Yang *et al.* used high-performance liquid chromatography (HPLC) with a photodiode array detector (PDA) [2,5-9]. The mobile phase used by Qiu *et al.* and Vinas *et al.* was phosphate buffer and methanol. Yang *et al.* used a Pentadecafluoroctanoic acid ion pair and methanol as the mobile phase. However, the researchers were unable to separate disodium 5'-guanylate and disodium 5'-inosinate through HPLC because the analyses were performed only by differentiating the pH and composition of the mobile phase.

Therefore, this study aimed to develop a more selective analytical method for assessing disodium 5'-guanylate and disodium 5'-inosinate levels using HPLC with a PDA.

### **METHODS**

An LC-20AD HPLC system (Shimadzu) was equipped with a pump, SunFire® C18 column, PDA (SPDM-20A), manual injector, data processor, HPLC syringe (SGE), 0.45-µm-diameter Whatman membrane, oven, desiccator, analytical scales, volume pipette, latex balloons, and glass tools.

Six flavor enhancer samples (A-F) were generated using a standard mixture of disodium 5'-guanylate and disodium 5'-inosinate, bidistilled water, distilled water, potassium dihydrogen phosphate, sodium hexane sulfonate, phosphoric acid, and methanol.

The standard mixture was generated by dissolving 100 mg each of disodium 5'-guanylate and disodium 5'-inosinate in 50 mL of distilled water in a 100-mL flask. The mixture was stirred until homogeneous. Then, 1-, 1.5-, 2-, 2.5-, 3-, and 3.5-mL aliquots of the 1000 mg/mL solution were added into a 10-mL flask, diluted with a solvent to obtain final concentrations of 100, 150, 200, 250, 300, and 350 mg/mL, respectively, and stirred until homogeneous.

The mobile phase was prepared by dissolving 17 g of potassium dihydrogen phosphate in 300 mL of bidistilled water in a 500-mL flask and stirring until homogenous (FI V, 2015). The pH was adjusted to  $3.50 \pm 0.02$  using phosphoric acid. To this mixture, 850 mg of sodium hexane sulfonate was added followed by dissolving and stirring until homogenous. The mixture was sonicated for 15 min.

The mobile phase composition, flow rate, and stability were assessed to optimize the analytical conditions. The mobile phase composition was examined by injecting 20 mL of a 200 mg/mL standard solution into the HPLC-PDA system at phosphate buffer/methanol ratios of 100:0, 95:5, and 90:10. The mobile phase composition was assessed on the basis of separation between the two compounds. The flow rate and column temperature were 1.2 mL/min and 30°C, respectively, and the analysis was performed twice for each composition.

The flow rate was optimized by first injecting 20 mL of the 200 mg/mL standard solution into the HPLC-PDA system using flow rates of 0.8, 1, and 1.2 mL/min. The injection was performed twice for each flow rate. Stability testing was performed by injecting the same volume of the standard solution into the system on three consecutive days. Optimization was confirmed using the peak retention times, sharpness of the peak, and width of the analyte peak area.

The analytical methods were validated by creating a calibration curve through injecting 20-mL aliquots of the standard solution at concentrations of 100, 150, 200, 250, 300, and 350 mg/mL into the HPLC-PDA system under the selected analytical conditions. Regression of the peak area (y) against the analyte concentration (x) was analyzed, and the calibration curve was created. The correlation coefficient (*r*) of the linear regression equation was used to view the linearity of the curve by calculating the linearity factors of the line, that is,  $r_p$ ,  $r_i^2$ ,  $V_{x_0}$ , and  $\Delta y/\Delta x$ .

The limits of detection (LOD) and quantitation (LOQ) were obtained by statistically calculating the calibration curve linear regression line equation. LOD was obtained using the formula  $LOD = \frac{3Sy/x}{b}$ , whereas LOQ was obtained using the formula  $LOQ = \frac{10Sy/x}{b}$ . In both formulas, *b* was the slope, and the calibration curve equation was  $y=bx+a$ . Furthermore, a selectivity test was conducted to compare the standard chromatogram with the sample chromatograms to identify impurity between the two compounds.

Four samples were created by added 600 mg of powder to a 20-mL volumetric flask together with one of three concentrations (100, 250, and 350 mg/mL) of the standard solution for the accuracy and precision tests. Six replicates were used for each concentration.

The measurement results in the recovery test can also be used to determine the thoroughness of the analytical method. Precision was measured as the standard deviation or relative standard deviation (*r*)  $\leq 2\%$ .

Samples A-F were prepared using the same treatment. In total, approximately 1.4 g of A, B, and D, 600 mg of samples C and F, and 2 mg of sample C were added into 20-mL volumetric flasks. Distilled water was added to the flasks to the specified limit, and the mixtures were stirred until homogenous. All samples were sonicated for 15 min and filtered into a 10-mL vial using 0.45-μm Whatman syringe filters.

The sample level was determined by injecting 20 mL of each sample into the HPLC system and measuring the width of the peak area. The experiment was repeated 3 times. The obtained uptake was incorporated into the calibration curve equation to determine the levels of the nucleotide in each sample.

## RESULTS AND DISCUSSION

The results for mobile phase optimization are presented in Table 1.

Regarding the buffer mixture of potassium phosphate and sodium hexane sulfonate with methanol (100:0), the produced chromatograms could be separated well. Separation occurred between disodium 5'-inosinate and disodium 5'-O-guanylate because the cluster on sodium hexane sulfonate in the mobile phase interacts with the amine group of disodium 5'-guanylate. This interaction led to the formation of neutral ion pairs, making disodium 5'-guanylate less polar than disodium 5'-inosinate compound [10,11].

These findings resulted in short retention time for disodium 5'-inosinate, which eluted earlier than disodium 5'-guanylate. Concerning the 95:5 and 90:10 mixtures of potassium phosphate and sodium hexane sulfonate with methanol, the generated chromatograms were less well separated than those for the 100:0 mixture.

At a flow rate of 0.8 mL/min, the retention times of disodium 5'-inosinate were 4.616 and 4.580 min, whereas those of disodium 5'-guanylate were 5.801 and 5.789 min. At 1.0 mL/min, the retention times of disodium 5'-inosinate were 3.276 and 3.251 min, compared to 4.160 and 4.155 min for disodium 5'-guanylate. At 1.2 mL/min, the retention times of disodium 5'-inosinate were 2.766 and 2.730 min, whereas those of disodium 5'-guanylate were 3.305 and 3.495 min. Thus, 1.2 mL/min was chosen as the optimal flow rate because it provided the fastest retention times.

In the stability test, the stabilities of both compounds decreased from the 1<sup>st</sup> day to 3<sup>rd</sup> day. The decline of stability was caused by the

**Table 1: Mobile phase optimization analysis of disodium 5'-guanylate and disodium 5'-inosinate**

Mobile phase	Compound	Area (μV/s)	Retention time (min)	Following factor (Tf)	HETP	Theoretical plate value (N)	Resolution
100:0	Inosinate	1,358,507	2.787	1.978	143.232	6981.698	3.786
	Guanylate	2,967,251	3.865	1.338	95.821	10436.128	
	Inosinate	1,178,734	2.829	1.376	127.754	7827.517	2.648
	Guanylate	2,916,445	3.872	1.344	92.119	10855.495	
95:5	Inosinate	584,182	2.461	0.995	28.932	34563.568	1.123
	Guanylate	2,468,073	2.627	1.652	82.398	12136.274	
	Inosinate	635,854	2.286	0.934	114.521	8732.022	0.965
	Guanylate	2,253,558	2.505	1.724	166.123	6019.629	
90:10	Inosinate	335,180	2.439	1.014	41.952	23836.689	1.192
	Guanylate	2,548,024	2.635	1.598	91.697	10905.484	
	Inosinate	355,297	2.486	1.024	28.596	34970.289	0.907
	Guanylate	2,592,340	2.674	1.606	89.485	11175.082	

instability of the two compounds during storage when dissolved in water. In this study, we determined that the disodium 5'-guanylate and disodium 5'-inosinate standard solution could be used for 2 days based on the wide peak areas on day 3.

The results obtained from the system suitability test met the system suitability test requirement because the r or repeatability values were  $\leq 2\%$ . The system suitability test result illustrated that the current method might provide more optimal analysis conditions than those used in previous studies (Tables 2 and 3). The addition of sodium hexane sulfonate permitted the separation of disodium 5'-inosinate and disodium 5'-guanylate. In addition, short retention times were achieved using the current method, which should permit a relatively rapid analysis.

Linear regression calculation of the calibration curve obtained using six concentrations produced equations of  $y=6158.36x+216.232.90$  for disodium 5'-inosinate (Fig. 1) and  $y=12,950.90x+1,327,349.42$  for disodium 5'-guanylate (Fig. 2). The linearity test results obtained from the calibration curve equations indicated that the compounds met the linearity test criteria because r for disodium 5'-guanylate was 0.99915 and that for disodium 5'-inosinate was 0.99920.

The LOD for disodium 5'-inosinate was 5.34 mg/mL, and the LOQ was 17.78 mg/mL. Meanwhile, the values for disodium 5'-guanylate were 5.53 and 18.45 mg/mL, respectively. These results were much lower than those obtained in prior studies. Thus, we concluded that the current methods are superior to previously reported strategies.

The results of the selectivity test illustrated the absence of any interference or nuisance chromatogram at the retention times of disodium 5'-guanylate and disodium 5'-inosinate (Figs. 3 and 4). In the chromatogram, there was a small peak in the second minute that did not correspond to either compound (Fig. 4). However, no other aberrant peaks were observed. Thus, the developed method is selective for disodium 5'-inosinate and disodium 5'-guanylate.

The accuracy and precision test results for disodium 5'-guanylate and disodium 5'-inosinate at three different concentrations met the criteria with values of 98–102%. In addition, the precision test data met the requirement of a relative standard deviation of  $\leq 2\%$  (Tables 4 and 5).

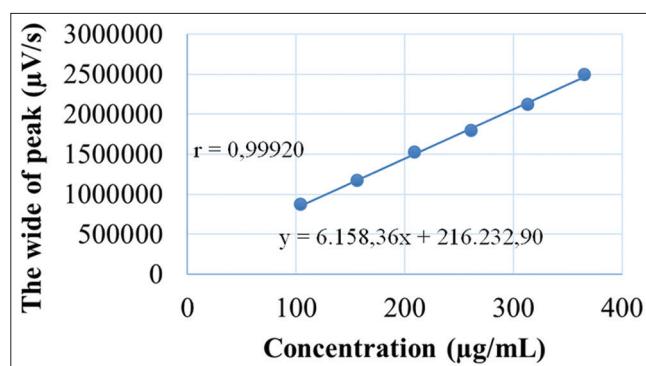
The assay results for the six samples of results assay of six samples of disodium 5'-guanylate and disodium 5'-inosinate are shown in Tables 6 and 7.

**Table 2: Results of the disodium 5'-inosinate system suitability test**

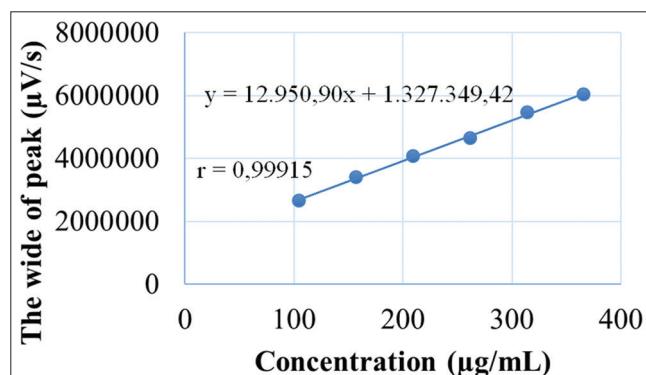
Area ( $\mu\text{V/s}$ )	Retention time (min)	Following factor (Tf)	HETP	Theoretical plate value (N)	Resolution	Standard deviation	Coefficient of variation
1,321,399	2.754	1.800	65.860	12428.445	4.095	17753.02	1.13
1,290,754	2.758						
1,328,312	2.752						
1,305,528	2.756						
1,290,561	2.774						
1,286,000	2.750						

**Table 3: Results of the disodium 5'-guanylate system suitability test**

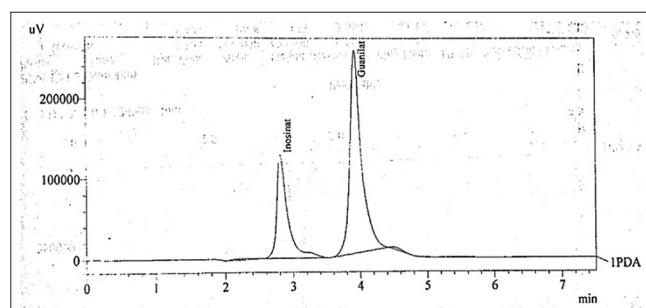
Area ( $\mu\text{V/s}$ )	Retention time (min)	Following factor (Tf)	HETP	Theoretical plate value (N)	Resolution	Standard deviation	Coefficient of variation
3,638,956	3.429	2.090	98.175	10693.812	4.095	44527.68	1.04
3,557,692	3.453						
3,581,573	3.502						
3,614,875	3.442						
3,530,988	3.454						
3,530,759	3.438						



**Fig. 1: Calibration curve results for disodium 5'-inosinate**



**Fig. 2: Calibration curve results for disodium 5'-guanylate**



**Fig. 3: Selectivity test result for the standard solution of disodium 5'-guanylate and disodium 5'-inosinate**

**Table 4: Accuracy and precision data for disodium 5'-inosinate**

Concentration (mg/mL)	Standard area (MV/s)	Blank area (MV/s)	Addition area (MV/s)	Addition standard area (MV/s)	Measured concentration (mg/mL)	Standard deviation	Coefficient of variation	UPK
100.6	818,783	1,033,363	1,859,837	826 474	101.54	1.36	1.36	100.94
		1,056,300	1,862,096	805 796	99.00			98.41
		1,063,906	1,867,763	803 857	98.77			98.18
		1,063,909	1,868,026	804 117	98.80			98.21
		1,036,241	1,864,106	827 865	101.72			101.11
		1,053,307	1,866,617	813 310	99.93			99.33
		1,033,363	2,767,031	1733668	249.73	2.28	0.90	99.30
		1,056,300	2,818,767	1762467	253.88			100.95
		1,063,906	2,826,532	1762626	253.91			100.96
		1,063,909	2,833,286	1769377	254.88			101.34
251.5	1,745,930	1,036,241	2,816,139	1779898	256.39			101.95
		1,053,307	2,805,196	1751889	252.36			100.34
		1,033,363	3,339,051	2305688	355.57	4.33	1.23	100.99
		1,056,300	3,363,075	2306775	355.74			101.03
		1,063,906	3,304,672	2240766	345.56			98.14
		1,063,909	3,328,098	2264189	349.17			99.17
		1,036,241	3,340,452	2304211	355.35			100.92
		1,053,307	3,319,369	2266062	349.46			99.25

**Table 5: Accuracy and precision data for disodium 5'-guanylate**

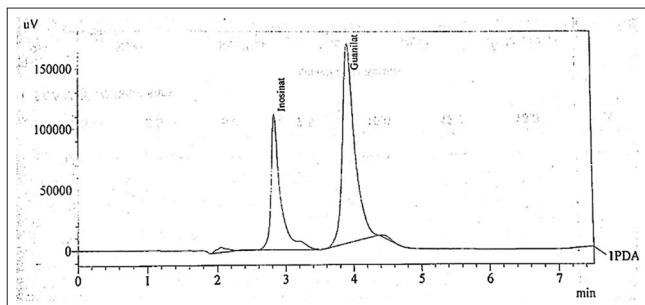
Concentration (pg/mL)	Standard area (μV/s)	Blank area (μV/s)	Addition area (μV/s)	Addition standard area (μV/s)	Measured concentration (pg/mL)	Standard deviation	Coefficient of variation	UPK
101.4	2,190,785	2,574,667	4,771,224	2,196,557	101.67	0.76	0.75	100.26
		2,599,811	4,798,040	2,198,229	101.74			100.34
		2,649,174	4,830,181	2,181,007	100.95			99.55
		2,659,993	4,886,124	2,226,131	103.04			101.61
		2,607,785	4,789,689	2,181,904	100.99			99.59
		2,618,648	4,819,113	2,200,465	101.85			100.44
		2,574,667	7,386,952	4,812,285	258.39	1.95	0.76	101.93
		2,599,811	7,367,856	4,768,045	256.01			100.99
		2,649,174	7,397,280	4,748,106	254.94			100.57
		2,659,993	7,370,878	4,710,885	252.94			99.78
253.3	4,721,283	2,607,785	7,407,074	4,799,289	257.69			101.65
		2,618,648	7,385,116	4,766,468	255.92			100.96
		2,574,667	8,780,940	6,206,273	359.85	1.50	0.42	101.39
		2,599,811	8,772,823	6,173,012	357.92			100.85
		2,649,174	8,785,150	6,135,976	355.77			100.25
		2,659,993	8,836,908	6,176,915	358.15			100.91
		2,607,785	8,805,541	6,197,756	359.36			101.26
		2,618,648	8,817,616	6,198,968	359.43			101.28

**Table 6: Assay results for disodium 5'-inosinate**

Sample	Area (MV/s)	Level (mg/mL)	Average level (mg/mL)	Content (%)	Total per wrap (mg)
A	1,269,324	171.00	169.34	0.24±1.46	26.4
	1,252,504	168.27			
	1,255,511	168.76			
B	1,122,092	147.09	144.00	0.21±2.69	42.0
	1,091,776	142.17			
	1,095,206	142.73			
C	1,271,251	171.31	173.56	0.58±3.26	464.0
	1,308,043	177.29			
	1,275,910	172.07			
D	1,145,990	150.97	151.91	0.21±0.84	18.9
	1,155,803	152.57			
	1,153,539	152.20			
E	1,637,261	230.75	233.41	0.22±3.59	55.0
	1,678,785	237.49			
	1,644,985	232.00			
F	1,068,839	138.45	140.60	0.47±2.21	42.3
	1,081,440	140.49			
	1,095,993	142.86			

**Table 7: Assay results for disodium 5'-guanylate**

Sample	Area (MV/s)	Level (mg/mL)	Average level (mg/mL)	Content (%)	Total per wrap (mg)
A	2,720,841	107.60	108.45	0.15±2.85	16.5
	2,701,761	106.12			
	2,772,979	111.62			
B	2,667,899	103.51	103.60	0.15±0.12	30.0
	2,670,820	103.74			
	2,668,451	103.55			
C	2,889,710	120.64	123.97	0.41±3.80	328.0
	2,986,487	128.11			
	2,922,371	123.16			
D	2,752,583	110.05	109.62	0.16±1.72	14.4
	2,765,902	111.08			
	2,722,527	107.73			
E	4,924,726	277.77	277.23	0.27±1.18	67.5
	4,928,342	278.05			
	4,900,235	275.88			
F	2,698,907	105.90	103.97	0.34±1.83	30.6
	2,651,779	102.27			
	2,670,833	103.74			

**Fig. 4: Selectivity test result of the sample solution of disodium 5'-guanylate and disodium 5'-inosinate**

## CONCLUSION

The optimal conditions for analyzing disodium 5'-guanylate and disodium 5'-inosinate using HPLC with a PDA and SunFire C18 column were  $\lambda=255$  nm, a mobile phase of potassium phosphate buffer and sodium hexane sulfonate, and a flow rate of 1.2 mL/min.

For disodium 5'-inosinate, its average levels in samples A-F were  $0.24\pm1.46$ ,  $0.21\pm2.69$ ,  $0.58\pm3.26$ ,  $0.21\pm0.84$ ,  $0.22\pm3.59$ , and  $0.47\pm2.21\%$ , respectively. Meanwhile, the average levels of disodium 5'-guanylate in the samples were  $0.15\pm2.85$ ,  $0.15\pm0.12$ ,  $0.41\pm3.80$ ,  $0.16\pm1.72$ ,  $0.27\pm1.18$ , and  $0.34\pm1.83\%$ , respectively.

## CONFLICTS OF INTEREST

Authors declare no conflicts of interest in this research.

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