Validation of Analytical Method for Determination of Adenine and Hypoxanthine Purine Bases in Melinjo Chips by HPLC-UV

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Abstract: Melinjo chips, which is commonly consumed by Indonesian people, are considered as one of the causes which triggers gout disease due to its purine content. The method to analyze purine in food is limitedly known by food laboratories in Indonesia. The objective of this research was to validate the analytical method for purine bases determination in melinjo chips by HPLC-UV. Adenine and hypoxanthine were of the known purine bases and chosen to be analyzed due to their characteristics which cause more uric acid accumulation in the body rather than other purine bases, guanine and xanthine. Guanine and xanthine were insoluble in the mobile phase used in this study, so that they might not be able simultaneously analyzed with adenine and hypoxanthine. Adenine and hypoxanthine standards were used in the instrumental performance experiment, method linearity and recovery test. The results showed that the HPLC-UV instrument with RP-C18 column and UV 257 nm detection had a good linearity in the concentration range of 7.81–125.00 µg/mL. The coefficients of determination (R2) were more than 0.999 for both adenine and hypoxanthine. Adenine and hypoxanthine were detected by HPLC-UV at retention time of 5.9–6.8 and 4.8–5.5 min respectively, and both retention times had an acceptable precision, less than 2.0 %. Detection limit (LOD) and quantification limit (LOQ) of the instrument were found at 0.72 and 2.39 µg/mL for analysis of adenine, while for analysis of hypoxanthine were at 0.69 and 2.30 µg/mL, respectively. The analytical method showed a good linearity at a concentration range of 50–800 µg/g sample with R2 more than 0.990 for both adenine and hypoxanthine analysis. Method detection limit (MDL) of adenine and hypoxanthine analysis was 19.44 and 14.42 µg/g respectively. Accuracy of the method was determined by a recovery test at spiking concentrations of 100, 500, and 1000 µg/g. In the analysis of adenine, the respective recovery results were 79.33%, 89.39%, and 90.37% with respective precisions were 5.19%, 4.50%, and 3.46%. While in the analysis of hypoxanthine, the recovery results were 66.75%, 92.29%, and 100.15%, and the precisions were 2.98%, 3.15%, and 2.22%, respectively. Based on these results, the analytical method for determination of purine bases in melinjo chips has been validated and was found to be accurate at concentration more than 100 µg/g wet weight of sample.

1 INTRODUCTION

Purines consist of adenine and guanine found in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), whereas hypoxanthine and xanthine are purine-derived natural compounds that are rarely found as bases in DNA and RNA, but often act as important intermediate compounds in the process of formation and breakdown of nucleotides (Garret 2005). Since purines can be synthesized and reused by human body, the need for purines from food is very small. Purines from food that are absorbed by the body but are not needed will be catabolized to produce the final product of uric acid (Zöllner 1982). In the research of Kaneko et al. (2014), total purines in food were described as follows: in cereals 157−759 µg/g, beans 188−776 µg/g, soybean products 200−2931 µg/g, dried seaweeds 154−5917 µg/g, eggs not detected (nd = <2 µg/g), dairy products nd−129 µg/g, mushrooms 69−3795 µg/g, fruits 24−35 µg/g, beef meat 774−1064 µg/g, chicken meat 700−1539 µg/g, pork meat 814−1197 µg/g, and fish meat 669−2114 µg/g.

Gout or also known as gouty is a condition of the accumulation of uric acid crystals in the joints. The accumulation occurs due to the excess of uric acid production or suboptimal excretion of uric acid as a product of purine catabolism. According to the

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research results of Clifford et al. (1976a) and Clifford and Story (1976b), adenine and hypoxanthine show a greater impact on gouty or uric acid levels, compared to guanine and xanthine.

The analytical method for purine base analysis in food has rarely developed by food laboratories in Indonesia. Melinjo (*Gnetum gnemon* L.) is stigmatized to cause uric acid due to its purine content, however a study on serum uric acid, after consuming the product of melinjo (fried melinjo chips), mentioned it didn't raise the uric acid level (Saifudin et al 2018). Considering that melinjo chips are generally consumed by Indonesian people, an analysis of purine content in melinjo chips is important to give an information for consumers of the product. Thus, there is a need for a validated analytical method to analyze the purine content in melinjo chips.

Purines are known able to be analyzed by high performance liquid chromatography (HPLC) instrument with UV detection in ppm levels (mg/kg or µg/g). The aromatic ring functional group in purine molecules can absorb strongly light at ultraviolet (UV) wavelengths. This can be used for both quantitative and qualitative analyses of purines (Garret 2005). Reversed phase HPLC (RP-HPLC) has been proven to be very efficient for nucleic acid analysis (Titkova et al. 1983), and has been commonly used to separate and quantify purine bases (Kaneko et al. 2014).

RP-HPLC-UV method requires the hydrolysis of nucleic acids to become nucleotides and free purine bases using strong acids. Brulé et al. (1989) developed a sample preparation method for purine base analysis with RP-HPLC using acid hydrolysis. In the research of Brulé et al. (1989), samples were hydrolyzed using 11.6 N perchloric acid for 1 hour at 100 °C, pH was adjusted using NH4OH to obtain pH 4.0, and to mark with distilled water in a 50 mL volumetric flask, filtered, and finally analyzed by RP-HPLC. The purine bases were isocratically separated with a RP-HPLC column C18 and a mobile phase of 0.1 M potassium phosphate buffer mixture and phosphoric acid at pH 4.0.

In a research conducted by Sotelo et al. (2002) regarding the determination of purine bases in sea urchin gonads, samples were hydrolyzed with a mixture of trifluoroacetic acid/formic acid (1/1, v / v) at 90 °C for 15 min, and to mark in 250 mL volumetric flask, and dried with a rotary vacuum evaporator at 75 °C. The purine base was dissolved with 10 mL buffer KH2PO4 0.3 M (pH 4.0) and filtered using a filter membrane before being analyzed with RP-HPLC equipped with a UV-VIS detector at a wavelength of 255 nm. Gradient analysis was carried out using a mobile phase buffer solution KH2PO4 0.3 M with a pH of 4.0.

The main objective of this research was to validate the purine base analysis method with a HPLC instrument. In this study a method validation of the purine base analysis was conducted on melinjo chip sample with a HPLC instrument equipped with RP-HPLC column and a UV-Vis detector which was set for UV detection, called as RP-HPLC-UV method. The purine bases chosen were adenine and hypoxanthine which are known to have a greater impact on the increase in uric acid in the body than guanine and xanthine (Clifford et al 1976a). Adenine and hypoxanthine were analyzed by RP-HPLC with operating conditions referring to the adenosine analysis method in royal jelly in the study of Xue et al. (2009). The sample preparation method was adapted from the qualitative analysis procedure for xanthine purine base (AOAC 2012a). The melinjo chips were hydrolyzed with 6 N HCl at 100 °C for 1 hour, neutralized with 25% NH4OH, and then treated with aquabidest in a 10 mL volumetric flask. Finally, the sample was passed through a SPE (Solid Phase Extraction) column containing silica, and is injected into a HPLC equipped with a UV-Vis detector.

2 MATERIAL AND METHOD

2.1 Materials

The materials used in this research were melinjo chips (Sriti, Sriti Food Co., Jakarta, Indonesia). The chemicals used for analysis include adenine and hypoxanthine standards (98-99%, Sigma, Sigma-Aldrich, USA), phosphoric acid 0.4% (Merck, Germany), methanol (pa, Merck, Germany), ethanol 80% (pa, Merck, Germany), aquabidest, NH4OH 25% (Merck, Germany), HCl 37% (Merck, Germany), and silica 60 (Merck, Germany).

Analytical balance, oven, food processor, hot plate, magnetic stirrer, vacuum filter, spatula, vortex, Millipore nylon filter membrane 0.45 µm, column SPE (Solid Phase Extraction), and glasswares were used. The analytical instruments used were pH-meter and high performance liquid chromatography (HPLC) LC 6A model (Shimadzu, Shimadzu Corp., Kyoto, Japan), equipped with a SPD-10AV model UV-Vis detector (Shimadzu, Shimadzu Corp., Kyoto, Japan), Chromatopac semi-automatic data recorder (Shimadzu, Shimadzu Corp., Kyoto, Japan), and C18 column (Zorbax, Agilent Technologies, USA).

2.2 Method Validation

In general, this study consisted of four parts, namely a preliminary test for the determination of retention time precision and resolution of separation, HPLC-UV instrument performance testing, development of purine base analysis procedure, and validation of purine base analysis method on melinjo chip. The validation of the analytical method included the specificity of the method, the linearity of the method, the accuracy and precision of the method by recovery test, the limit of the method detection, and the intralab reproducibility. Adenine and hypoxanthine were the purine bases chosen in this study.

2.3 Preliminary Retention Time Precision and Peak Resolution Test

The preliminary test was carried out by separately injecting the standards adenine and hypoxanthine which had been dissolved in 0.4% (90%) phosphoric acid and methanol (10%) at several concentrations to determine the chromatogram profile and the retention time of each compound. Once adenine and hypoxanthine appeared at different retention times, both were then re-injected in the form of a mixture to determine the peak resolution of the two compounds. The resolution shows the ability of the column to separate the two peaks and is declared good if it has a value greater than 1.50 (Zhang 2007). This test was done in duplicate. Resolution was determined using the equation (1).

$$Rs = 2\ (tR\text{-}B - tR\text{-}A) / wb\text{-}A + wb\text{-}B \quad (1)$$

tR-A and tR-B is the retention times of the two peaks (compound A is the compound that was eluted first), while wb-A and wb-B are the width of the baseline (the bottom) of the two peaks.

The operating conditions of HPLC for analysis of purine bases in melinjo chips refer to the adenosine analysis method in royal jelly in Xue et al. (2009) with modifications of the isocratic elution method and the flow rate of mobile phase. The analysis of purine adenine and hypoxanthine bases using HPLC with UV-Vis detector was performed with the condition as follows: Zorbax C18 (octadecyl silane or ODS) column, particle size 5 µm, L 250 mm, inner diammeter 4.6 mm, isocratic mobile phase of phosphoric acid 0.4% in water mixed with methanol (pro analysis) at ratio 90:10 and the pH adjusted to 4.0 by NH4OH 1 M, flow rate at 0.5 mL/min, ambient temperature, 20 µL injection volume, and detection at UV 257 nm.

2.4 HPLC-UV Instrument Performance Test for the Analysis of Adenine and Hypoxanthine Purine Bases

Standard stock solution. Standard stock solutions of adenine and hypoxanthine were made with a concentration of 500 µg/mL by dissolving 0.025 g of each standard into 50 mL of the HPLC mobile phase. The mobile phase consists of a mixture of 0.4% (90%) phosphoric acid and methanol pro analysis (10%). The standards adenine and hypoxanthine were mixed to obtain a standard mixed concentration with a concentration of 250 µg/mL.

Instrument linearity. Linearity was tested by injecting a serial solution of a mixture of adenine and hypoxanthine at concentrations of 1.95, 3.91, 7.81, 15.62, 31.25, 62.50, 125.00, and 250.00 µg/mL into HPLC with the condition above, so that the peak area of the serial concentrations were known. Testing on standard mixed solutions with eight different concentrations was carried out in triplicate from three different serial standard solutions. The HPLC results were then used for making a calibration curve which plotted between the concentrations (µg/mL) and the averaged peak area, then the coefficient of determination (R2) was calculated. Linearity is considered good if it has R2 greater than 0.990 (AOAC 2012b).

Precision of peak area and retention time. The precision determination of the area and retention time was done by injecting seven times a standard mix solution of adenine and hypoxanthine at the same concentration into the HPLC, in this case a concentration of 7.81 µg/mL was used. This concentration was chosen because it gave an acceptable recovery result (greater than 80%) at relatively low concentration. The peak area and retention times from the seven repeatations of injection were calculated for their average, standard deviation (SD), and relative standard deviation (RSD). RSD acceptance in this test was less than 2.0% (JECFA 2006).

Instrument detection limit (limit of detection or LOD) and quantification limit (limit of quantification or LOQ). The LOD of instrument was determined from the above seven injections of a standard mix solution. Each concentration of each standard from each injection was calculated using a calibration curve obtained from the above test. The SD of the concentrations obtained from the seven repeatations of injection was calculated, then the LOD was determined as three times of the SD, meanwhile the LOQ was determined as ten times of the SD.

2.5 Analytical Procedure Orientation

The sample preparation procedure for purine base analysis in melinjo chip was using the acid hydrolysis method adapted from AOAC (2012a), AOAC Official Method 960.56 Microchemical Tests for Xanthine Alkaloids, with some modifications. The modifications were the HCl volume used to hydrolyze the sample as well as the length of the hydrolysis process. The operating conditions of HPLC-UV for purine base analysis in emping melinjo samples followed the adenosine analysis method in royal jelly (Xue et al. 2009) with some modifications. The modifications were made in terms of the elution method with the isocratic mobile phase and its flow rate. In the preparation stage, the melinjo chips that have been mashed with a food processor were hydrolyzed with 6 N HCl at different HCl volumes, 0.5, 1.0, and 2.0 mL at 100 °C for 1 hour, neutralized with 25% NH4OH, and to mark with aquabidest in a 10 mL volumetric flask. Then the sample solution was passed through a solid phase extraction (SPE) column containing silica (approximately 1 gram, weighed after activated in an oven 105 °C for at least 2 hours) and then it was injected into the HPLC equipped with a UV-Vis detector with detection at UV 257 nm as above condition.

Samples to be analyzed by HPLC-UV consist of unspiked samples (without the addition of standards) and spiked samples (with the addition of standards). The standard mixture of adenine and hypoxanthine with a concentration of 400 µg/g sample was applied. Thus, the recoveries obtained by using procedures with different acid hydrolysis time lengths and volumes were determined and the procedure with the best recovery was chosen for the method validation below. This orientation test was done in duplicate. Analysis of the adenine and hypoxanthine purine bases content in the sample was compared to the results of the standard injection only from the instrumental performance test above. If the peak of each purine base in the sample could be detected proportional to its concentration, then the developed procedure was used for the method validation stage.

2.6 Method Validation

The validation of the purine adenine and hypoxanthine analytical method consisted of: method specificity, linearity, accuracy and precision, detection limit, and intralab reproducibility. Method validation was carried out following EURACHEM (1998).

Analytical Procedure. Sample preparation. In the sample preparation stage, a total of 0.5 grams of melinjo chips that have been mashed with a food processor were weighed using an analytical balance. Then, the sample was hydrolyzed with 6 N HCl at 100 °C for 1 hour. The 6 N HCl volume used for acid hydrolysis was 0.5 mL (the selected volume resulting from the above development). The hydrolyzed sample was then neutralized with NH4OH 25%. The solution was transferred into a 10 mL volumetric flask and was fixed to the mark with aquabidest. The analytical solution was passed through an SPE column containing about 1 gram of silica before being injected into HPLC.

Determination by HPLC. The analysis of purine adenine and hypoxanthine bases using HPLC with UV-Vis detector was performed isocratically following the conditions described above. The purine base in sample was calculated by multiplying the concentration from calibration curve (from instrumental performance test) with the final sample volume (10 mL), then divided by sample weight.

Method specificity. Specificity test was done by injecting a standard mixture of adenine and hypoxanthine, samples without standard addition, and samples that have been added with the standard mixture of adenine and hypoxanthine. In this study a standard mixed concentration of 400 µg/g of sample was used. Thus, at least three chromatograms were obtained. If the chromatogram shows well-separated peaks and these peaks were not having interference by other peaks of sample components, then the specificity of the analytical method was considered good.

Method linearity. This test was carried out using samples spiked with adenine and hypoxanthine standards at concentrations of 50, 100, 200, 400, and 800 µg/g, then sample treatment according to the analytical procedure for the sample above was applied to these mixtures, and then injected into the HPLC. The linearity test of the method was carried out in triplicate by making three series of samples which were spiked at the specified concentrations. After that, the method callibration curve was made, that is a plot between the peak area and the purine concentrations (µg/g). The linearity requirement for the method is R2 greater than 0.990 or r greater than 0.995 (AOAC 2012b).

Accuracy by recovery test. This test was carried out using samples spiked with standards at three different concentrations, low (100 µg/g), medium (500 µg/g), and high concentrations (1000 µg/g). Each analysis was carried out at seven replications. The percentage of recovery was using formula (2).

Acceptance of the recovery percentages is according to AOAC (2012b).

$$\text{Recovery (\%)} = \frac{\text{Conc found in spiked sample} - \text{Conc found in unspiked sample}}{\text{Spiked Concentration}} \text{ x } 100\ \% \quad (2)$$

Method precision. The data obtained in the recovery test for accuracy at three different concentrations were used for the determination of method precision by calculating the RSD in each purine analysis at low, medium, and high concentrations. The value of RSD analysis (RSDa) was compared to RSD Horwitz (RSDh). Good precision was for a RSDa value smaller than RSDh. RSDh was calculated using formula (3).

$$\text{RSDh} = [2 \exp (1\text{-}0.5 \log C)] \quad (3)$$

C = Analyte concentration (in fraction of sample)

Method detection limit. The method detection limit was determined from a plot between the standard deviations and the concentrations of adenine and hypoxanthine from the recovery test results at concentrations of 100, 500, and 1000 µg/g. Through a linear equation of the curve, the standard deviation SD0 was determined when the concentration was equal to zero. MDL value is three times the SD0 value obtained. Determination of the MDL was referred to EURACHEM (1998).

Intralab reproducibility. Intralab reproducibility test was carried out using the same melinjo chip sample and the same operator and laboratory, but carried out on different weeks. The results of the analysis were then calculated for the mean and the RSD values. The RSD value obtained was then compared with its RSDh. Good intralab reproducibility was that with a smaller RSDa value than its RSDh. In addition, the results obtained were processed by one-way ANOVA followed by Duncan posthoc test if there was a significant difference at 5% level, using the IBM Statistic SPSS 20 program between the results of analysis from different weeks.

3 RESULTS AND DISCUSSION

3.1 Retention Time and Resolution of Adenine and Hypoxanthine

The chromatogram obtained showed that adenine appeared at 5.9-6.8 min, meanwhile hypoxanthine appeared at 4.8-5.5 min. The quite different retention times of adenine and hypoxanthine gave the fact that the two compounds could be analyzed simultaneously. Figure 1 shows the chromatograms of standard adenine, standard hypoxanthine, and the mixture of adenine and hypoxanthine at 62.50 µg/mL. The analysis of the standard mixture have a resolution of 4.51. The resolution shows the ability of the column to separate the two peaks and is declared good if it has a value greater than 1.50 (Zhang 2007). Thus, the resolution of the peak adenine and hypoxanthine was acceptable, so that the two compounds can be analyzed simultaneously further.

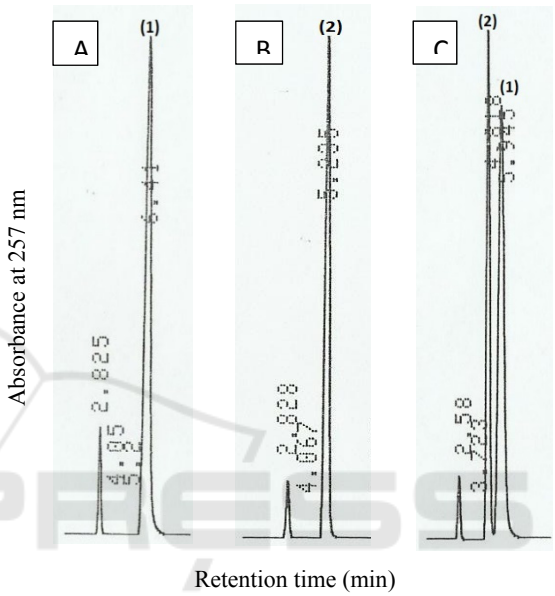

Figure 1: Chromatograms of standards adenine (A), hypoxanthine (B), a mixture of adenine and hypoxanthine (C) at a concentration of 62.50 µg/mL in mobile phase solution. HPLC column was C18, and the isocratic mobile phase was phosphoric acid 0.4% in water mixed with methanol (pro analysis) at ratio 90:10 and the pH adjusted to 4.0 by NH4OH 1 M. Peaks: (1) adenine, (2) Hypoxantine.

3.2 Instrument Performance Test

Instrument linearity test results are presented in Table 1. Instrument linearity test results in the table shows good slope precision, with RSD values less than 5%. Meanwhile, the value of the intercept obtained showed a lack of precision both in the analysis of adenine and hypoxanthine with RSD values greater than 20%. However, the linearity of the HPLC-UV instrument is considered as good due to the R2 more than 0.990, which means the instrument is able to produce a linear response to the concentration of the analyte at various levels of concentration.

Table 1: The results of HPLC-UV instrument linearity test for the analysis of adenine and hypoxanthine standards at serial concentrations of 1.95, 3.91, 7.81, 15.62, 31.25, 62.50, 125.00, and 250.00 µg/mL.

	Slope	*Intercept*	R	R^2
Adenine				
1	65840	92344	0.9998	0.9997
2	66281	139408	0.9999	0.9998
3	66773	44532	0.9999	0.9999
Average	66298	92095	0.9999	0.9998
SD	467	47438	$5.77.\ 10^{-5}$	$1.00.10^{-4}$
RSD (%)	0.70	51.51	0.01	0.01
Hypoxanthine				
1	45434	103801	0.9995	0.9991
2	43748	60649	0.9998	0.9997
3	43076	92983	0.9995	0.9990
Average	44086	85811	0.9996	0.9993
SD	1215	22452	$1.73.10^{-4}$	$3.79.10^{-4}$
RSD (%)	2.76	26.16	0.02	0.04

The chromatogram precision determined in this study covers the precision of peak area and the precision of retention time. Each precision was shown by its RSD value obtained from seven injections. The precision of retention time in analysis of adenine by the HPLC-UV was 0.67%, meanwhile the precision of peak area was 3.18%. The similar result was obtained for hypoxanthine, the precision of retention time was 0.96% and the precision of peak area was 2.97%. The acceptable precision is 2.0% or less according to JECFA (2006). The precisions of all retention times were acceptable, however the precisions of peak area were not. The poor peak area precision was caused by the use of semi-automatic data recorders and printers, so that the peak area could be affected by the feed speed. Besides this, according to Barwick (1999), the precision of peak area was probably influenced by the flow rate of the mobile phase. A constant mobile phase flow rate can only be produced by HPLC pumps that in good condition. However, the HPLC flow rate has been callibrated by an external calibration service.

LOD and LOQ values obtained in the analysis of adenine and hypoxanthine were 0.72 and 2.39 µg/mL, respectively. Meanwhile, the LOD and LOQ values in the hypoxanthine analysis were 0.69 and 2.30 µg/mL, respectively. As a comparison, in the research of Sotelo et al. (2002) regarding the determination of purine base levels in gonads of sea urchins with HPLC instruments, the detection limits of adenine and hypoxanthine were 0.076 and 0.060 µg/mL, respectively, which are ten times lower than those obtained in this current study.

The precision of the analysis results in determining the detection limit and the quantification limit of the instrument was determined by calculating RSD of analysis (RSDa) and RSD Horwitz (RSDh). The RSDa of adenine analysis (3.90%) was smaller than 2/3 RSDh (8.12%). Similar result obtained for hypoxanthine analysis, the RSDa value (3.97%) was smaller than 2/3 RSDh (8.19).

3.3 Analytical Procedure Orientation

Filtering the final sample solution with silica using a SPE column was aimed to remove impurity components contained in the sample. In developing this analytical procedure, samples prepared with the addition of 6 N HCl at various volumes were injected into the HPLC, where the samples consisted of unspiked samples (without the addition of standards) and spiked samples (with the addition of standards). Each standard was added at a concentration of 400 µg/g sample which was ten times of the instrument LOQ.

The results of sample analysis, without the addition of spikes, using HCl volumes of 0.5, 1.0, and 2.0 mL showed that the average melinjo chips contained adenine in the concentration range of 70.37-171.88 µg/g sample and hypoxanthine in the concentration range of 48.37-155.58 µg/g sample. The total of purine bases in emping melinjo was predicted between 500-1500 µg/g. The recoveries obtained at the use of HCl volumes of 0.5, 1.0, and 2.0 mL were presented in Tables 2. The results show that HCl volume of 0.5 mL provides the best recovery result of 92.99% and 113.84% for the analysis of adenine and hypoxanthine, respectively. This was acceptable according to AOAC (2002b), which mentioned the acceptable recovery between 85-110%.

Table 2: The results of the analytical procedure orientation of adenine and hypoxanthine analysis in melinjo chip using different volumes of HCl for acid hydrolysis in the sample preparation stage*.

Volume of HCl 6 N (mL)	Concentration of unspiked sample (µg/g)	Concentration of spiking (µg/g)	Concentration of spiked sample (µg/g)	Recovery (%)
Adenine				
0.5	79.43 ± 2.61	400	451.37 ± 12.82	92.99
1.0	171.88 ± 8.85	400	495.54 ± 11.35	80.91
2.0	70.37 ± 3.48	400	559.03 ± 17.25	122.16
Hypoxanthine				
0.5	103.29 ± 12.12	400	558.64 ± 39.18	113.84
1.0	48.37 ± 4.42	400	650.55 ± 18.93	150.54
2.0	155.58 ± 0.76	400	703.58 ± 5.60	137.00

*in duplicate

3.4 Method Specificity

The chromatogram in Figure 2 shows that the peaks of adenine and hypoxanthine could be separated to each other, either analyzed in standard mix solution or in melinjo chips. Both adenine and hypoxanthine peaks were not interferred by other peaks of sample component. Guanin and xanthine which could be extracted during the sample preparation process, were not be able analyzed by HPLC because they cannot be eluted with the mobile phase used in this study. This mentioned that the method had a good specificity. Adenine and hypoxanthine in the melinjo sample were detected at 5.9–6.8 min and 4.8–5.5 min, respectively. In Sotelo et al. (2002) study regarding the determination of purine bases in sea urchin gonads by HPLC, adenine and hypoxanthine respectively detected at about 18 and 11 min. A considerable difference between the retention times obtained in this study and Sotelo et al. (2002) due to the different mobile phase used. The mobile phase used in the Sotelo et al. (2002) study was a 0.3 M KH_2PO_4 buffer solution, whereas in this study a mixture of 0.4% phosphoric acid (90%) mixed with methanol p.a. (10%) was as the mobile phase. In general, hypoxanthine was detected earlier than adenine.

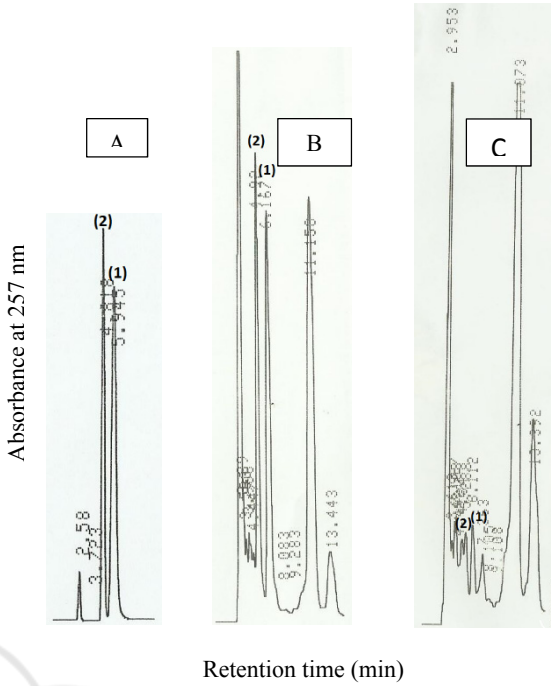

Figure 2: Chromatograms of a standard mixture of adenine and hypoxanthine (a), unspiked sample (b), and spiked sample at spiking concentration of 400 µg/g sample (c). Peaks: (1) adenine, (2) Hypoxanthine.

3.5 Method Linearity

The callibration curve of the method in the analysis of adenine and hypoxanthine had a linear equation obtained in the linearity test method for adenine analysis is y = 3637.7x + 305030 with R and R2 values of 0.9998 and 0.9996, respectively. Meanwhile testing conducted on hypoxanthine yielded a curve with the equation of y = 2535.8x + 157650, while R and R2 values were 0.9978 and 0.9956, respectively. R and R2 values obtained in the adenine and hypoxanthine tests met the requirements set by AOAC (2012b), that is, R was greater than 0.995 or R2 was greater than 0.990. Thus, the method used had a good linearity, which could provide a linear response to the concentration of analytes in the sample.

3.6 Method Accuracy and Precision

According to AOAC (2002b), the acceptable percentages of recovery for concentrations of 100 and 500 µg/g is 85-110%, while for concentration of 1000 µg/g is 90-108%. The recovery test results were presented in Table 3. The results at the spike of 500 and 1000 µg/g in adenine analysis met the AOAC (2002) requirement, namely by recovery of 89.39%

and 90.37%, respectively. While at 100 µg/g spiking, the recovery did not meet the standard, which was 79.33% (less than 85%). The recovery results in hypoxanthine analysis, were only acceptable at the spiking concentration of 500 and 1000 µg/g, with recoveries of 92.29% and 100.15%, respectively. While at the spiking concentration of 100 µg/g, its recovery was far below the AOAC standard, which was 66.75%. This might be due to the relatively low concentration of spikes and the analytes content naturally in the sample (without the standards addition). The loss of analytes during sample preparation seems more significant in the results of the analysis.

The precision of the analytical method was determined through the recovery test. The precision of the method is considered good if the RSDa value is smaller than the RSDh. If the RSDa value is smaller than 2/3 RSDh, then the precision of the method is even better. Determination of the 2/3 RSDh value for a more stringent standard is to ensure the results of the analysis. The method precision is presented in Table 4. In the adenine analysis with the addition of standard at 100, 500, and 1000 µg/g, RSDa value was smaller than RSDh, but only at concentrations of 1000 µg/g RSDa value was smaller than 2/3 RSDh. This shows that in the analysis of adenine, the spiking at 100 and 500 µg/g was less precise than the spiking at 1000 µg/g. On the other hand, in the hypoxanthine analysis, the RSDa values were all smaller than 2/3 RSDh. Thus, hypoxanthine analysis had a good precision at all spiking studied.

Table 3: Recovery of analytical method for the determination of adenine and hypoxanthne in melinjo chip by HPLC-UV.

Concentration of spiking (µg/g)	Concentration found in spiked sample (µg/g)	Concentration found in unspiked sample (µg/g)	Averaged recovery (%)
Adenine			
100	222.04 ± 11.53*	142.71 ± 1.35	79.33
500	591.97 ± 26.65*	145.00 ± 4.70	89.39
1000	1044.26 ± 36.19**	140.53 ± 4.51	90.37
Hypoxanthine			
100	285.30 ± 8.49*	218.55 ± 7.44	66.75
500	592.29 ± 18.67*	130.84 ± 2.74	92.29
1000	1261.64 ± 28.07**	260.12 ± 11.83	100.15

*Obtained from 7 replications
**Obtained from 5 replications

Table 4: Precision of analytical method for the determination of adenine and hypoxanthne in melinjo chip by HPLC-UV.

Concentration of spiking (µg/g)	SD (µg/g)	RSDa* (%)	RSDh** (%)	2/3 RSDh (%)
Adenine				
100	11.53	5.19	7.09	4.73
500	26.65	4.50	6.12	4.08
1000	36.19	3.46	5.62	3.75
Hypoxanthine				
100	8.49	2.98	6.83	4.55
500	18.67	3.15	6.12	4.08
1000	28.07	2.22	5.46	3.64

*RSDa is relative standard deviation of the analysis
**RSDh is RSD Horwitz 2(1-0.5 log c) with c is a fraction in sample

3.7 Method Detection Limit

The method detection limit was determined by plotting the standard deviations obtained from recovery test and the concentrations of adenine and hypoxanthine found. Through the linear equation, the standard deviation at zero concentration was determined (SD0). The method detection limit is three times the value of SD0. The linear curve is presented in Figure 3.

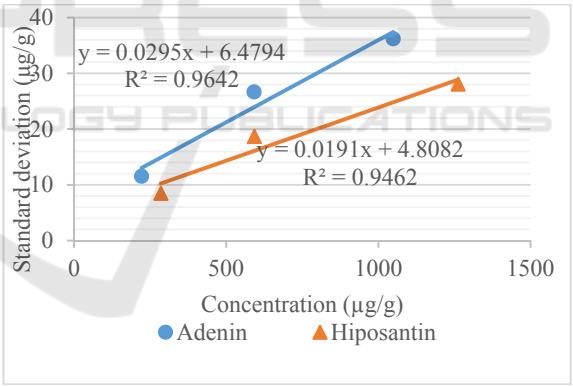

Figure 3: A plot between analyte concentration (µg/g) and its standard deviation from recovery test of adenine and hypoxanthine analyses by HPLC-UV to determine SD0 for calculating method detection limit (3SD0).

Based on the curve in Figure 3, the SD0 value for adenine was 6.4794. Thus, the method detection limit value for adenine analysis was 19.44 µg/g. While the SD0 value for hypoxanthine was 4.8082, therefore the method detection limit for hypoxanthine analysis was 14.42 µg/g. The curve had R2 values greater than 0.900 which was acceptable.

3.8 Intralab Reproducibility

The values of intralab reproducibility in the analysis of adenine and hypoxanthine were greater than the RSDh value. Intralab reproducibility values for adenine and hypoxanthine analyses were 34.53% and 39.89%, respectively. The results of one-way ANOVA followed with Duncan test gave that they were significantly different, the result from week 1 was different from the results from weeks 2 and 3. Meanwhile, the results of hypoxanthine analysis at weeks 1, 2, and 3 were significantly different to each other. This might be caused by a poor area precision observed in the instrument performance test results.

4 CONCLUSIONS

The analysis procedure for alkaloids in AOAC Official Method 960.56 can be applied to the analysis of the purine adenine and hypoxanthine bases in melinjo chips with HPLC-UV. Development of the analytical procedures carried out showed that the analytical method using acid hydrolysis provided the best recovery in the use of 6 N HCl volumes of 0.5 mL. The developed procedure can then be used at the method validation stage.

In general, the method had a good performance on all validation parameters except the intralab reproducibility. The analytical method for the determination of adenine and hypoxanthine in melinjo chip by HPLC-UV was validated and gave an accurate result at concentrations more than 100 µg/g sample.

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