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By Santyas Kuniaty

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Validation of rhodamin b analysis method in beef sausage with visible spectrophotometry instruments

Santyas Kurniaty[1], Nina Salamah[2]
[1,2]Faculty of Pharmacy, Universitas Ahmad Dahlan, Jl. Prof. Dr. Soepomo, S.H., Janturan, Umbulharjo, Yogyakarta, Indonesia
[2]Ahmad Dahlan Halal Center, Universitas Ahmad Dahlan, Jl. Prof. Dr. Soepomo, S.H., Janturan, Umbulharjo, Yogyakarta, Indonesia
Corresponding author: nina.salamah@pharm.uad.ac.id

ABSTRACT

Sausage is made from meat that has been chopped and then mashed, seasoned, and put in a symmetrical elliptical sleeve, made either from animal intestines or artificial wrapping (casing). To attract consumer interest, synthetic food coloring such as Rhodamin B is added. Rhodamin B is a dye prohibited from being used in products, according to the Director General of POM No. 00386/C/SK/II/1990, because the use of Rhodamin B for a long time can cause liver damage and cause cancer. The purpose of this study was to validate the analytical method of rhodamine B in beef sausages by visible spectrophotometry. The sample used in this study was a beef sausage with a reddish-pink characteristic and without a brand. The validation parameters of the method used in this study are linearity, precision, LOD, LOQ, and accuracy. The data obtained were then analyzed using the SPSS Kolmogorov-Smirnov method, then ANOVA was used. The validation results of linearity test methods, LOD LOQ, precision, and accuracy met the requirements of PPOP GMP in 2013.
Keywords: Sausage, Rhodamine B, UV-Vis, Validation

INTRODUCTION

Sausages are food products from a mixture of finely ground meat (containing not less than 75% meat) with flour or starch with or without the addition of permitted spices and other food additives and put into sausage casings. A very important component of meat in the manufacture of sausages is protein. Meat protein increases the breakdown of meat during cooking to form a compact product structure (Badan Standardisasi Nasional, 1995).

A coloring agent is a food additive that can improve or give color to food. The addition of food coloring is intended to improve the color of food that changes or turns pale during processing or to color colorless food to make it look more attractive (Pertiwi et al., 2013). Food coloring is a food additive in the form of natural and synthetic dyes, which can give or improve color when added or applied to food (Kemenkes RI, 2012).

In Indonesia, the use of coloring agents for food (both permitted and prohibited) is regulated in the Regulation of the Minister of Health of the Republic of Indonesia Number: 239/Men.Kes/Per/V/85 concerning certain dyes declared as hazardous substances and the Regulation of the Minister of Health of the Republic of Indonesia Number 033 of 2012 concerning Food Additives. Rhodamine B is a synthetic dye which, when in the form of a greenish crystalline powder in the form of a high-concentration solution, is purplish red, and in low concentration, it is bright red. Rhodamine B belongs to the xanthenes dye group, a base made from meta-diethylamino phenol and phthalic anhydride, a material that is inedible and highly fluorescent (Purnamasari & Saebani, 2013). Rhodamine B can produce attractive colors with deep colors and is very fluorescent when dissolved in water and ethanol.

Rhodamine B has a considerable carcinogenic potential; hence its usage is forbidden. Long-term rhodamine B consumption can cause the body to store more of it. It can result in signs of enlarged liver and kidneys, diminished liver function, liver damage, bodily physiological disturbances, or even liver cancer (Hidayah et al., 2017).

Permatasari et al. (2014) researched street food suspected of containing Rhodamin B in traditional markets in Bandar Lampung City by random sampling and conducted qualitative tests using Paper Chromatography. Quantitative tests were measured using UV-Vis Spectrophotometry and showed positive results. As many as 50% of the samples contained Rhodamin B, 15 of the 30 samples.

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RESEARCH METHOD

Materials

The materials used in this study were 2% Ammonia, 10% Ammonia, 10% Acetic Acid (Merck), Aquadestilata, standard rhodamine B (Merck), wool yarn, ether, distilled water, 10% ammonia, 70% ethanol (Bratachem), silica gel GF 254 nm, eluent, n-Butanol (Merck): Ethyl Acetate (Merck): Ammonia (Merck) (10:4:5), Sausage samples were taken at the market in Yogyakarta City. The tools used are analytical balance, hot plate, filter paper Whatmann No. 1, spectrophotometry UV 1800 (Shimadzu), balance, water bath, UV lamp 366 nm, and glassware.

Methods

Validation of Analysis Method

Linearity

From 20 ppm of Rhodamin B mains solution, concentration of 0.8 ppm, 1.0 ppm, 1.2 ppm, 1.4 ppm, 1.6 ppm, 1.8 ppm, and 2.0 ppm were made by pipetting each 0. 4 ml, 0.5 ml, 0.6 ml, 0.7 ml, 0.8 ml, 0.9 ml, and 1.0 ml into a 10 ml volumetric flask then added a basic solution (10% ammonia dissolved in 70% ethanol) %) to the boundary mark. The solution was homogenized, and the absorbance was measured at the wavelength obtained at the maximum wavelength measurement with Visible spectrophotometry.

Determination of linearity seen from the regression data, namely the correlation coefficient, the y-intercept, the direction of the regression line, and the sum of the squares of the residuals of the acceptable regression line. Linearity is calculated using the linear regression equation y = a + bx. The linearity results are said to be good if the linearity results in a correlation (R) close to 1 (one).

Precision

Weigh 50 mg of Rhodamin B, put it into a 100 ml measuring flask, and add it with a basic solution (10% ammonia in 70% ethanol) up to the mark. Then six solutions with the same concentration were put in a 10 ml measuring flask, and then added a basic solution (10% ammonia in 70% ethanol) was up to the mark. The absorbance was measured at the maximum wavelength obtained with a Uv-Vis spectrophotometer.

The precision determination is calculated from the standard deviation or relative standard deviation. The precision calculation aims to determine the coefficient of variation (CV), which the formula can calculate:

$$CV = \frac{SD}{X} \times 100\%$$

Good precision results occur when the CV value or relative standard deviation is less than 2% (Rohyami et al., 2018).

LODs and LOQs

From the main solution of Rhodamine B 20 ppm, concentrations of 0.8 ppm, 0.6 ppm, 0.4 ppm, 0.2 ppm, and 0.1 ppm were made using a 0.4 ml pipette; 0.3ml, 0.2ml, 0.1 ml, and 0.05 ml then added a basic solution (10% ammonia in 70% ethanol) to the mark of the 10 ml volumetric flask. Then the absorbance was measured at the maximum wavelength obtained with a Uv-Vis spectrophotometer.

LOD is a parameter to determine the smallest content of the sample but cannot be quantified precisely. The formula calculates the LOD value:

$$\text{LOD} = \frac{(3\,X\,sy/x)}{Slope}$$

LOQ is the smallest sample level that can be analyzed and calculated. The formula calculates the LOQ value:

$$\text{LOQ} = \frac{(10\,X\,sy/x)}{Slope}$$

Accuracy

Ten grams of sample was added by taking the diluted Rhodamine B standard (9x replication). The addition of standard Rhodamine in the sample was carried out by weighing 10 mg of standard

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Rhodamine then, putting it in a 100.0 ml volumetric flask, adding an alkaline solution (10% ammonia in 70% ethanol) to the mark, then taking 0.075 ml; 0.094 ml and 0.113 ml were added with a basic solution (10% ammonia in 70% ethanol) in a 10.0 ml volumetric flask up to the mark. The addition of the standard Rhodamine B solution to the sample was based on the results of the determination of Rhodamine B levels with the highest average concentration, namely in the G code sample of 0.94 ppm. During the preparation of the sample, this solution was added. The extraction process was then completed by adding 20 mL of a 2% ammonia solution that had been dissolved in 70% ethanol, and it was allowed to sit overnight. Next, Whatmann No. 1 filter paper was used to filter the solution by filtrate. Wool thread free of fat with a length of 40 cm is placed into the acid solution and boiled for 10 minutes. The dye will color the wool thread. One and the colored solution is placed into the Erlenmeyer. The solution is heated on the hotplate, and the residue from evaporation is dissolved in 10 mL of distilled water containing acid. After being removed, the wool thread is cleaned with distilled water. When the wool thread is cooked in an alkaline solution (10 mL of 10% ammonia in 70% ethanol), the dye is released from the woolen thread and dissolved in the alkaline solution. The extracted solution was placed in a cuvette, and its absorbance was assessed using a Uv-Vis spectrophotometer at its maximum wavelength.

The determination of accuracy is calculated from the percentage of the recovery. The accuracy value expressed as a percentage of the recovery or good recovery must meet 98-102% (Badan Pengawas Obat dan Makanan Republik Indonesia, 2013).

Data Analysis

The research data were analyzed concerning the validation parameter of the analysis method in GMP 2018 and the Indonesian Pharmacopoeia VI 2020.

RESULT AND DISCUSSION

The sample used in this study was red-pink and unbranded beef sausage taken from a market in Yogyakarta city. Rhodamine B is a dye suspected of being used as a coloring agent for sausages. Rhodamin B is prohibited from being used in food, drinks, cosmetics, etc. However, in small doses, according to the Director General of POM No. 00386/C/SK/II/1990, regarding changes to the attachment to Regulation of the Minister of Health (Permenkes) No.239/Menkes/Per/85 because the use of Rhodamine B for a long time can cause liver damage and cause cancer. Rhodamine B is a synthetic dye in the form of a greenish crystalline powder, odorless, in the form of a high concentration solution, purplish red in color, and has a strong fluorescence (Direktur Jenderal Pengawas Obat dan Makanan Republik Indonesia, 2013).

The sample was prepared by mixing all the samples, then pulverized with a blender, after being smooth, then weighed as much as 10.00 grams. Replication 5 times. The sample that had been weighed and replicated was soaked with 20.0 ml of 2% Ammonia solution (which was dissolved in 70% ethanol), then left overnight. The addition of ammonia is to attract the dyes contained in the beef sausage sample. After being left overnight, the solution was filtered using Whatman No.1 paper.

Then the filtrate is left in the fume hood to evaporate the remaining solvent before, after being left in the fume cupboard, the solution is heated on a hotplate. Then the residue from the evaporation is dissolved with 10.0 ml of distilled water containing acid. Next, the dye is withdrawn with woolen thread. The woolen thread used is 20 cm long. The wool yarn is put into an acid solution (10 ml of distilled water and 5 ml of 10% acetic acid) and then heated for 10 minutes. The purpose of heating is to get the extract from the sample. The woolen threads that have changed color are removed and then washed with distilled water. After that, the wool thread is put into an Erlenmeyer containing sufficient alkaline solution (10% ammonia dissolves in 70% ethanol), then the solution obtained is put into a 10.0 ml measuring flask, and then an alkaline solution (10% ammonia dissolved in 70% ethanol) there is a limit sign. Soaking in an alkaline solution will fade the dye absorbed into the wool yarn during the previous process. Then the solution is read as the maximum lambda of the sample by visible spectrophotometry.

In the quantitative test of Rhodamine B, namely the determination of Rhodamine levels using the Visible Spectrophotometry method. The choice of this method was based on the fact that Rhodamine B is a colored compound or molecule because it has a chromophore group. The quantity of Rhodamine B color is very sharp due to the presence of 2 ausochrome groups, dimethyl amine. Then because this

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method is also easy, cheap, and simple, and based on the reading results, the maximum wavelength of the standard Rhodamine B is 556.0 nm, the reading will be more precise if you use Visible Spectrophotometry.

Based on the 2013 GMP Guidelines Operational Guidelines, parameters that can be used to show that the analytical method is suitable for its intended use and properly validated include linearity, the limit of detection or called LOD (Limit of Detection), quantitation limit or called LOQ (Limit of Quantitation), precision and accuracy (Badan Pengawas Obat dan Makanan Republik Indonesia, 2013).

Linearity is a validation method that shows the ability of the tool to obtain test results that are proportional to the concentration of the analyte in the sample. Linearity was carried out with a single measurement of samples with different concentrations, namely by making seven solutions with different concentrations and then reading the absorbance with the maximum wavelength obtained earlier. The results of determining the linearity test can be seen in Table I; a linear regression equation is obtained: Y = 0.2902 x + 0.0219 with a coefficient value (r) of 0.998. Based on these data, the linearity test meets the linearity requirements for PPOB, namely $r \leq 0.99$. These results indicate that the instrument's response at analyte concentrations is comparable.

Table I. Linearity test.

Concentration (ppm)	Intake volume (μl)	Absorbance
0.8	400	0.257
1.0	500	0.303
1.2	600	0.378
1.4	700	0.430
1.6	800	0.487
1.8	900	0.533
2.0	1000	0.609

LOD (Detectable Limit) is the lowest concentration of analyte that can be detected but cannot be quantified. LOQ (Quantified Limit) is the lowest concentration that can be determined with an acceptable level of precision and accuracy.

The LOD LOQ test was carried out by preparing five solutions with concentrations below the standard curve, namely 0.8 ppm; 0.6 ppm; 0.4 ppm; 0.2 ppm, and 0.1 ppm, then read the absorbance using visible spectrophotometry with a maximum wavelength of 556.0 nm. How to determine the LOD LOQ by looking for linear regression of the standard curve of the adsorbent y with sample concentration x. Y = bx + a expresses this. In the results of this study, the value of the linearity curve was Y = 0.1072 x + 0.1721. Then calculate the absorbance value of the measurement results and compare it with the corrected absorbance value.

Table II. LOD LOQ test.

Concentration (ppm)	Absorbance	Equation	Y from Equation y^	(y – y^)	$(y – y^\wedge)^2$
0.1	0.183	A = 0.1721	0,1828	0,0002	0,00000004
0.2	0.195	B = 0.1072	0,1935	0,0065	0,00004225
0.4	0.213	R = 0.9990	0,2149	-0,0019	0,00000361
0.6	0.236	Y = 0.1072x + 0.1721	0,2364	0,0004	0,00000016
0.8	0.259		0,2578	0,0012	0,00000144
				Total	0.0000475

$(Sy/x)2 = \frac{\Sigma(y-y^\wedge)^2}{n-2} = \frac{0.0000475}{5-2} = 0.00001583$
Sy/x = 3.9786 x 10-3 = 0.00397
LOD = (3sy/x)/b = (3 x 0.0039786) / 0.1072 = 0.112 ppm
LOQ = (10sy/b) = (10 x 0.0039786) / 0.1072 = 0.371 ppm

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Based on the data in Table II, then calculated using this formula, the results of calculating the limit of Rhodamine B concentration in detectable samples (LOD) of 0.112 ppm, these results indicate that Rhodamine B in the sample can still be detected significantly but cannot be quantified by Visible Spectrophotometry. While the LOQ results obtained were 0.371 ppm, the results indicated the concentration of Rhodamine B, which could be used in quantitative analysis precisely.

The precision test aims to show the closeness of the results of studies carried out repeatedly with the same sample (accuracy). The requirement for the RSD value is ≥ 2%. The precision test was carried out by making six solutions with the same concentration and then reading the absorbance value with Visible Spectrophotometry using the maximum wavelength obtained, 556.0 nm. The absorbance value obtained is then calculated as the RSD value.

Table III. Precision test.

Replication	Absorbance
1	0.278
2	0.277
3	0.272
4	0.281
5	0.280
6	0.279
x	0.2778
Standard Deviation	3.1885×10^{-3}
RSD	1.1477%

Based on Table III, the RSD value was 1.17%. Based on these results, the research results are valid because they meet the parameter requirements of the precision test results. The accuracy test is a validation test that aims to show how close the measurement results are to the true value. Accuracy is expressed as the percent recovery of the added analyte. The accuracy test was carried out by adding the Rhodamine B standard to a sample whose concentration was known. The addition of standard Rhodamine B was divided into three different concentration levels, namely at a concentration of 80%, 100%, and 120%. Then read the Visible Spectrophotometry and repeat it three times. Accuracy requirements in method validation, according to Badan Pengawas Obat dan Makanan Republik Indonesia (2013), are 98.00% - 102.00% with an RSD value of ≥ 2%

Accuracy is determined by calculating the percent recovery value using the following formula:

$$Recovery = \frac{CF - CA}{C^*A} \times 100\%$$

Description:
CF : total concentration value of the sample obtained from the measurement
CA : actual sample concentration value
C*A : concentration value of the added analyte

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Table IV. Accurate test.

Absorbance	Standard Rhodamin B + concentration (μg)	Rhodamin B concentration in the sample (μg)	Standard Concentration added (μg)	recovery (%)	Average %Recovery	RSD (%)
0.508	1.675		0.75	98.00		
0.511	1,685	0.94	0.75	99.33	99.20	1.15
0.513	1,692		0.75	100.27		
0.563	1,865		0.94	98.40		
0.565	1,871	0.94	0.94	99.04	99.22	0.76
0.568	1,882		0.94	100.21		
0.612	2,033		1,13	98.21		
0.618	2.055	0.94	1,13	100.09	99.85	1.53
0.622	2,068		1,13	101.25		

Based on Table IV, in this study, the percentage of recovery was obtained at a concentration of 80%, namely 98.00%; 99.33% and 100.27%; at a concentration of 100%, namely 98.40; 98.40% and 100.21% and at a concentration of 120% namely 98.21%; 100.09% and 101.25% with an RSD value at a concentration of 80% of 1.15% < 2.00%, at a concentration of 100% RSD value of 0.76% < 2.00% and at a concentration of 120% of 1.53% < 2.00%. Based on these data, the results were obtained following the percent recovery requirements stipulated in PPOP CPOB in 2013, and there is a closeness of the degree of analysis results to the actual analyte.

Based on the five parameters used in the validation method on Rhodamin B, the beef sausage sample obtained the results; namely, in the linearity test, the value of r was 0.998, so it was said to meet the requirements. In the LOD test, the results obtained were 0.112 ppm and LOQ 0.371 ppm; in the precision test, the RSD results were obtained at 1.17%, which can be said to be eligible because the RSD value was ≥ 2.00%. In the accuracy test, the percent recovery was by the requirements. PPOP CPOB in 2013, namely in the range of 98.00% to 101.25%, and the percent recovery requirement based on PPOP CPOB in 2013 was 98.00% to 102.00%.

CONCLUSION

Conclusion In this study, the results of the five parameters validation of the analytical method, namely linearity, precision, LOD, LOQ, and accuracy, all parameters met the requirements according to the provisions of PPOB CPOB 2013.

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