Determination of Biogenic Amines Using Image Analysis of Ninhydrin-Visualized Biogenic Amine Spots in Thin Layer Chromatography

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Image analysis was performed on ninhydrin-visualized spots of putrescine dihydrochloride, cadaverine dihydrochloride, spermidine trihydrochloride and tyramine hydrochloride in thin layer chromatography using the ImageJ software. The original image was processed and analyzed to determine Rf values, pixel area, mean gray values and circularity. The TLC methodology produced adequate differential separation of tyramine hydrochloride and cadaverine dihydrochloride from putrescine dihydrochloride and spermidine trihydrochloride in biogenic amine mixtures and fish paste extract. Regression equations showed adequate linearity when the pixel areas ($R^2 > 0.946$, $\rho<0.01$) and mean gray values ($R^2 > 0.866$, $\rho<0.01$) were utilized. TLC-image analysis using ImageJ software shows good potential in quantifying biogenic amines in aqueous solutions and food samples due to its low cost and simplicity.

KEYWORDS: biogenic amines, image analysis, mean gray values, pixel areas, Rf values, thin layer chromatography

INTRODUCTION

Biogenic amines or polyamines are non-volatile, nitrogenous, organic compounds produced from the microbial degradation of protein-rich food such as fish and fish products, meat and fermented foods (den Brinker, Kerr, & Rayner, 2002), following the decarboxylation of free amino acids by gastrointestinal bacteria (Kalac & Krausova, 2005). Basically, all food items which are rich in proteins or free amino acids may promote bacterial production of biogenic amines by decarboxylases produced by several bacterial genera such as *Bacillus, Citrobacter, Clostridium, Klebsiella, Escherichia, Proteus, Pseudomonas, Shigella, Photobacterium, Lactobacillus, Pediococcus,* and *Streptococcus* (Karovicova & Kohajdova, 2005). Histamine, tyramine, putrescine and cadaverine are the most commonly found biogenic amines in food (Ladero, Calles, Fernandez, & Alvarez, 2010).

The names of biogenic amines are based from the amino acids that give rise to them. For instance, histamine is named after histidine, tryptamine is named after tryptophan and phenylethylamine is named after phenylalanine. Cadaverine, however, is derived from lysine while putrescine is derived from ornithine or arginine (European Food Safety Association, 2010). Kantaria and Gokani (2011) classified biogenic amines as aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine), or heterocyclic (histamine, tryptamine).

In very high concentrations, biogenic amines have various toxicological effects which are associated with allergy-like symptoms, neurological, and blood pressure problems (Ladero et al., 2010; Shukla, Kim, & Kim, 2011). The roles of biogenic amines in cell growth and proliferation have also been studied extensively because of their involvement in tumor development (Kalac & Krausova, 2005). Fermented food products such as red wines, cheese, fish pastes, sausages, and lactic acid fermented vegetables are rich in putrescine, cadaverine, spermidine, spermine, histamine and tyramine (Karovicova & Kohajdova, 2005) and daily consumption of these food items may also increase the dietary intake of biogenic amines. Because of these health risks, there is a need to determine the concentration of biogenic amines in fermented food items.

Detection of biogenic amines in various samples can be performed using enzymatic methods, immunoenzymatic methods, flow injection analysis, fluorometric methods, gas chromatography, high performance thin layer chromatography, high performance liquid chromatography, and capillary electrophoresis (Etienne, Ifremer, &

Nantes, 2006), although these methods are expensive and require longer preparation time and analysis. Thin layer chromatography has been used as a simpler alternative to detect the presence of biogenic amines in biological samples since quantification is performed qualitatively. Sherma (2000) emphasized that thin layer chromatography (TLC) has multiple applications in the area of food composition, intentional additives, adulterants, contaminants, and decomposition involving determination of amino acids, lipids and fatty acids, sugars, biogenic amines, vitamins, and organic acids.

In the local setting, thin layer chromatography was used with image analysis and densitometry to quantify biogenic amines in fermented sausages (Bandolin, Pham, & Barraquio, 2010) and cheese samples (Vallejos, Pham, & Barraquio, 2011) using Biosoft™ Quantiscan for Windows (Biosoft 2004). Most biogenic amines are non-chromophoric or non-fluorophoric and derivatization is often required in most TLC methods through the use of dansyl chloride or o-phathaladehyde (Kantaria & Gokani, 2011; Ayesh, Ibraheim, El-Hakim, & Mostafa, 2012) to visualize them in TLC plates. Visualization of biogenic amines can also be performed using a cheaper alternative such as nihydrin (Morincova, Dicakova, & Bystricky, 2009; Bandolin et al., 2010) since the amino group of biogenic amines can form complexes with ninhydrin, forming a purple complex.

In addition, other image analysis software such as Sorbfil TLC Video densitometer software, and Scion Image were already utilized by other studies to identify and quantify the compounds in thin layer chromatography (Zakrzewska, Parczewski, Kazmierczak, Ciesielski, & Kochana; 2007; Tie-xin & Hong, 2008; Phattanawasin, Sotanaphun, Sriphong, Kanchanphibool, & Piyapolrungroj, 2011), suggesting the wide application of image analysis in analytical procedures. Thin layer chromatography is a cheaper and faster method to separated compounds in a given sample, but is limited in providing an accurate quantification of the separated compound. ImageJ, an image processing program created by Wayne Rasband of the Research Services Branch, National Institute of Mental Health in Bethesda, Maryland, has several features which could be used to process and analyze images in most commonly used image formats (Abramoff, Magalhaes, & Ram, 2004). ImageJ can be used to process images in TIFF, GIF, JPEG, PNG, DICOM, BMP, PGM and FITS formats (Ferreira & Rasband, 2012). To our knowledge, very few studies utilized ImageJ as a tool to quantify and describe visualized biogenic amine spots and other compounds in thin layer chromatography.

Hence, this study was conducted to attempt to develop a simple thin layer chromatography-image analysis method to detect and quantify biogenic amines in biogenic amine mixtures and sample fish pastes using the "particle analysis" pathway of the ImageJ software. Specifically, this study aimed to compare the visualized spots of putrescine dihydrochloride, cadaverine dihydrochloride, spermidine trihydrochloride and tyramine hydrochloride in terms of their Rf values, pixel area, mean gray value and circularity, determine the regression equations for quantifying biogenic amines using their pixel areas and mean gray values and quantify the biogenic amines present in biogenic amine mixtures and fish paste solutions.

MATERIALS AND METHODS

Reagents

Authentic standards of putrescine dihydrochloride, cadaverine dihydrochloride, spermidine trihydrochloride, and tyramine hydrochloride (Sigma Aldrich, Germany) were purchased from Chemline Scientific Corporation. Acetone, acetic acid, methanol, ninhydrin and ammonium hydroxide were obtained from Fisher Scientific and purchased from Saint Louis University Natural Science Research Unit (SLU-NSRU). All reagents utilized in the study were analytical grade.

Preparation of Standard Solutions and Mixtures

In a separate sterilized Erlenmeyer flask, 500 mg of each of the biogenic amines were dissolved in 50 mL methanol according to a modified methodology of Bandolin et al. (2010). The standard solutions were diluted into 2 mg/mL, 4 mg/mL, 6mg/mL, 8mg/mL using methanol as the diluent. For biogenic amine mixtures, 100 mg of each of the biogenic amine standards were mixed together in 400 mL of methanol. The solutions and mixtures were stored inside the refrigerator (≈4^{o}C) until used.

Thin Layer Chromatography Conditions

The methodology of Valls, Bello, and Kodaira (2002) was used with few modifications. The mobile phase was 5% ammonium hydroxide in acetone while the developing agent was 0.2% ninhydrin with 2% acetic acid in methanol. The developing chamber was rinsed with the

mobile phase twice before placing the TLC plates. A volume of 10μL of the biogenic amine standard solutions ranging from 2mg/mL to 10mg/mL (interval of 2mg/mL) was applied on five silica coated TLC plates (Merck) using a micropipette at a distance of 1.0 cm from each spot. After running the procedure of twenty-five minutes, the TLC plates were oven-dried at 95°C for five minutes until dry. The spots were visualized by dipping the TLC plates in acidified ninhydrin solution and oven-dried again at 95°C for five minutes until the spots were visible. The image of the chromatogram was captured using a Canon PowerShot A490. The image was not edited before the image processing using ImageJ software. The Rf value was computed using the distance of the center of the spots and the distance of the mobile phase. The distance of the spots and solvent front were determined using the pixel numbers set on ImageJ software.

The Rf value was calculated using the formula:

$$R_f = \frac{\text{Distance migrated by substance (pixels)}}{\text{Distance travelled by solvent (pixels)}}$$

The circularity of the biogenic amine spots using their pixel areas was determined using the formula:

$$\text{Circularity} = 4\,\pi\,(\text{area}/\text{perimeter}^2)$$

Preparation of Linear Regression Equations

The ImageJ program was used to determine the mean gray value and pixel area of the image of the chromatogram after image processing through background subtraction, conversion to 8-bit format and inversion of color patterns. The computed mean gray values and pixel areas of the biogenic amine samples (n=5) in different concentrations were used to formulate the regression equations. The concentrations of biogenic amines were determined using a simple linear regression equation $y = mx + b$ where x refers to the concentration of biogenic amine (mg/mL) and y refers to the pixel area or mean gray value of the visualized spots.

Extraction of Biogenic Amines from Fish Paste Samples

Fish paste samples were obtained from Baguio City Market. The

method of Bandolin et al. (2010) was used with few modifications. In a clean test tube, 10 grams of homogenized fish paste sample was mixed with 10 mL of methanol and vortexed for one minute. The extract was transferred to an Erlenmeyer flask and immersed in a water bath at 60°C for 15 minutes, cooled, and transferred to a 50 mL volumetric flask. Extraction of the same sample was done until subsequent extracts appeared clear and free of suspended particles. Additional methanol was added to the volumetric flask to yield 50 mL of the extract. The extract was transferred to tubes and centrifuged at 5000 rpm for ten minutes to remove the suspended particles. The test tubes were sterilized before analysis to avoid bacterial contamination of the extract. Samples were freshly prepared daily before analysis.

Treatment of Data

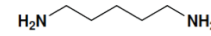
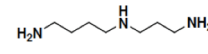
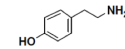
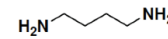
The results of image analysis of TLC chromatograms were presented using tables. Data collected from the analysis of images were presented as mean ± SD. Least Squares Method was utilized to obtain the linear regression formulae of the standard biogenic amines using their mean pixel areas and mean gray values. The coefficients of determination of the linear regression equations using the concentration of biogenic amines as influenced by pixel area and mean gray values were determined at α=0.01(two-tailed). The program used for statistical analysis was SPSS 18.0 for Windows.

RESULTS AND DISCUSSION

The determination of biogenic amines in TLC plates is dependent on the formation of Ruhemann's purple from the reaction of ninhydrin and biogenic amines. The molecular structures of the biogenic amines shown in Table 1 can form complexes with ninhydrin (Patil, Devdhe, Kawder, Kulkarni, Nagmoti, Patil, & Kale, 2012) and produce a purple color. In alkaline medium, ninhydrin is converted to o-carboxyphenyl glyoxal which reduces ninhydrin to 2 hydroxyindan-1,3-dione. The primary amino group of the biogenic amines reacts with ninhydrin to give diketohydrindylidenediketohydrindamine.

Table 1.

Profile of Biogenic Amines

Biogenic Amine	Molecular Weight (g/mol)	Chemical Name	Chemical Formula
Cadaverine	202.2	1,5-diaminopentane	
Spermidine	145.3	N-(3-aminopropyl) -1,4-butanediamine	
Tyramine	137.3	4-(2-aminoethyl)-phenol	
Putrescine	88.2	1,4-diaminobutane	

Source: Shukla, S., Kim, J-K., & Kim, M. (2011). Occurrence of biogenic amines in soybean food products, soybean and health (Hany El-Shemy, Ed.). Retrieved from http://www.intechopen.com/books/soybean-and-health/occurrence-of-biogenic-amines-in-soybean-foodproducts

Image Processing of Biogenic Amine Spots

The total procedure took forty minutes for the development of the chromatogram and image analysis. Biogenic amine spots visualized with 0.2% ninhydrin with 2% acetic acid in methanol showed minimal tails and blots in all chromatograms. Biogenic amine spots appeared purple because of the formation of Ruhemann's purple as discussed earlier. Visualization using 0.2% ninhydrin in methanol and iodine vapors were also performed according to the methodology of Vallejos et al. (2011) but the results showed poor resolution.

After processing the images, interfering colors were removed through background subtraction in order to produce a better resolution of the visualized spots. The image was converted to 8 bit format and analyzed using ImageJ software using the particle analysis pathway to determine the mean gray value and pixel area of the spot. An 8-bit format displays a 256 gray level of the image (Ferreira & Rasband, 2012).

The color of the 8 bit image was inverted to black and white to compute the pixel area of each spot using the "wand tool" of ImageJ software. The wand tool creates a selection by tracing an object with uniform color or threshold objects. The images of visualized spots of

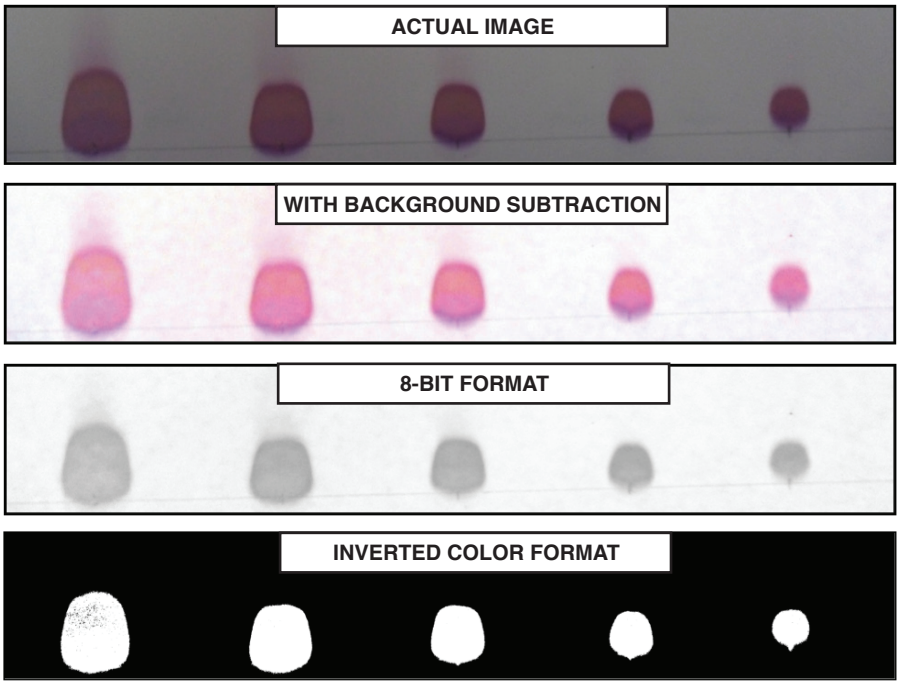


Figure 1. Images of TLC chromatogram of spermidine trihydrochloride before and after processing with ImageJ program.

spermidine trihydrochloride after processing with ImageJ software are shown in Figure 1. Image processing is performed to obtain an image with adequate resolution for image analysis.

Rf Values of Biogenic Amines

The Rf values of cadaverine dihydrochloride, putrescine dihydrochloride, tyramine hydrochloride and spermidine trihydrochloride are shown in Table 2. Based on the results, the tyramine hydrochloride has the highest Rf value (0.852) while spermidine trihydrochloride has the lowest value (0.06). Using the metholodogy in the study, there is poor separation between the spots of spermidine trihydrochloride and putrescine dihydrochloride since the spots overlap. Table 3 shows that among the four biogenic amines, tyramine has also the highest Rf value even if the mobile phase used is ethanol-based (Vallejos et al., 2012) while the Rf value of putrescine dihydrochloride is still low. The results show that the composition of the mobile phase influences the rate of migration of

biogenic amines. For instance, Khan (2006) reported that methanol, acetone, chloroform, ethyl acetate, benzene, hexane and ether do not produce adequate differential movement of spermidine, cadaverine and spermine in silica-coated TLC plates. The mobile phase used in the study however produced adequate separation of cadaverine dihydrochloride and spermidine trihydrochloride.

Table 2.

Rf Values of Biogenic Amines Standard Solutions and Mixtures.

Biogenic Amines	Individual Solutions	Mixture 1	Mixture 2	Mixture 3
Cadaverine dihydrochloride	0.168 ± 0.044	0.169 ± 0.012	0.166 ± 0.034	0.169 ± 0.02
Putrescine dihydrochloride	0.092 ± 0.002	*	0.092 ± 0.003	—
Spermidine trihydrochloride	0.060 ± 0.007	*	—	0.061 ± 0.008
Tyramine hydrochloride	0.852 ± 0.003	0.856 ± 0.031	0.857 ± 0.006	0.852 ± 0.005

Mixture 1: 10μg of cadaverine dihydrochloride, putrescine dihydrochloride, spermidine trihydrochloride and tyramine hydrochloride in methanol
Mixture 2: 10μg of cadaverine dihydrochloride, putrescine dihydrochloride, and tyramine hydrochloride in methanol
Mixture 3: 10μg of cadaverine dihydrochloride, spermidine trihydrochloride and tyramine hydrochloride in methanol

*overlapping spots

Pixel Areas, Mean Gray Values and Circularity of Visualized Biogenic Amine Spots

Figure 2 shows the differences in the shapes, sizes and color intensities of the four biogenic amine spots. Tyramine hydrochloride seemed to appear circular while cadaverine dihydrochloride, putrescine dihydrochloride and spermidine trihydrochloride appeared more elliptical. The color densities of cadaverine dihydrochloride and

Table 3.

Comparison of Mobile Phase and Rf Values of Biogenic Amines.

Biogenic Amines	Mobile Phase	Rf Values	Reference
Cadaverine dihydrochloride	Acetone: ammonium hydroxide (95:5)	0.24	Valls et al., (2002)
	Ethanol: ammonium hydroxide (80:20)	0.059	Wackes et al. (2005)
Putrescine dihydrochloride	Acetone: ammonium hydroxide (95:5)	0.17	Valls et al., (2002)
	Chloroform: toluene: triethylamine (60:28:12)	0.17	Chakradhar & Naik (2007)
	Ethanol: ammonium hydroxide (80:20)	0.046	Wackes et al., (2005)
Tyramine hydrochloride	Acetone: ammonium hydroxide (95:5)	0.72	Valls et al., (2002)
	Ethanol: ammonia (80:20)	0.75	Vallejos, et al., (2011)
Spermidine trihydrochloride	Chloroform: toluene: triethylamine (60:28:12)	0.33	Chakradhar & Naik (2007)

spermidine trihydrochloride appear evenly distributed while tyramine hydrochloride appears denser towards the solvent front. Putrescine dihydrochloride appears broader than the rest of the spots. However, the borders of spermidine trihydrochloride appear denser compared to the other biogenic amines.

The mean gray values of the biogenic amine spots were characterized using the surface plot function, plot profile function and "analyze particles" function instead of using the "gel analysis" pathway because of a greater repeatability in computing the pixel area of the spots. Figure

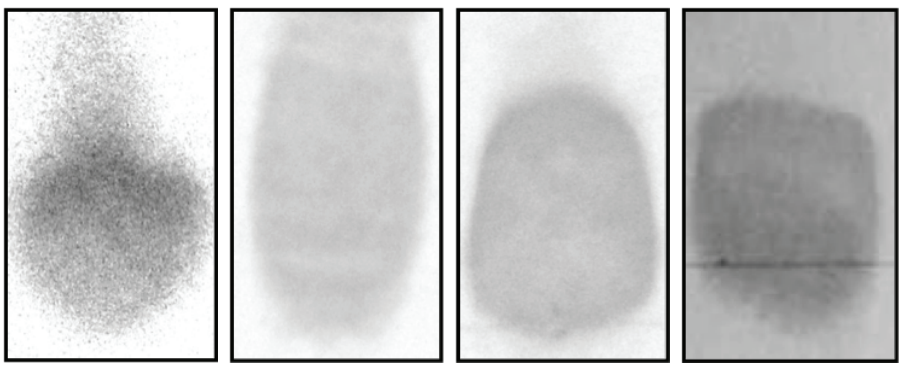


Figure 2. Appearance of the spots in TLC chromatograms.
A = Tyramine hydrochloride B= Cadaverine dihydrochloride
C= Spermidine trihydrochloride D= Putrescine dihydrochloride

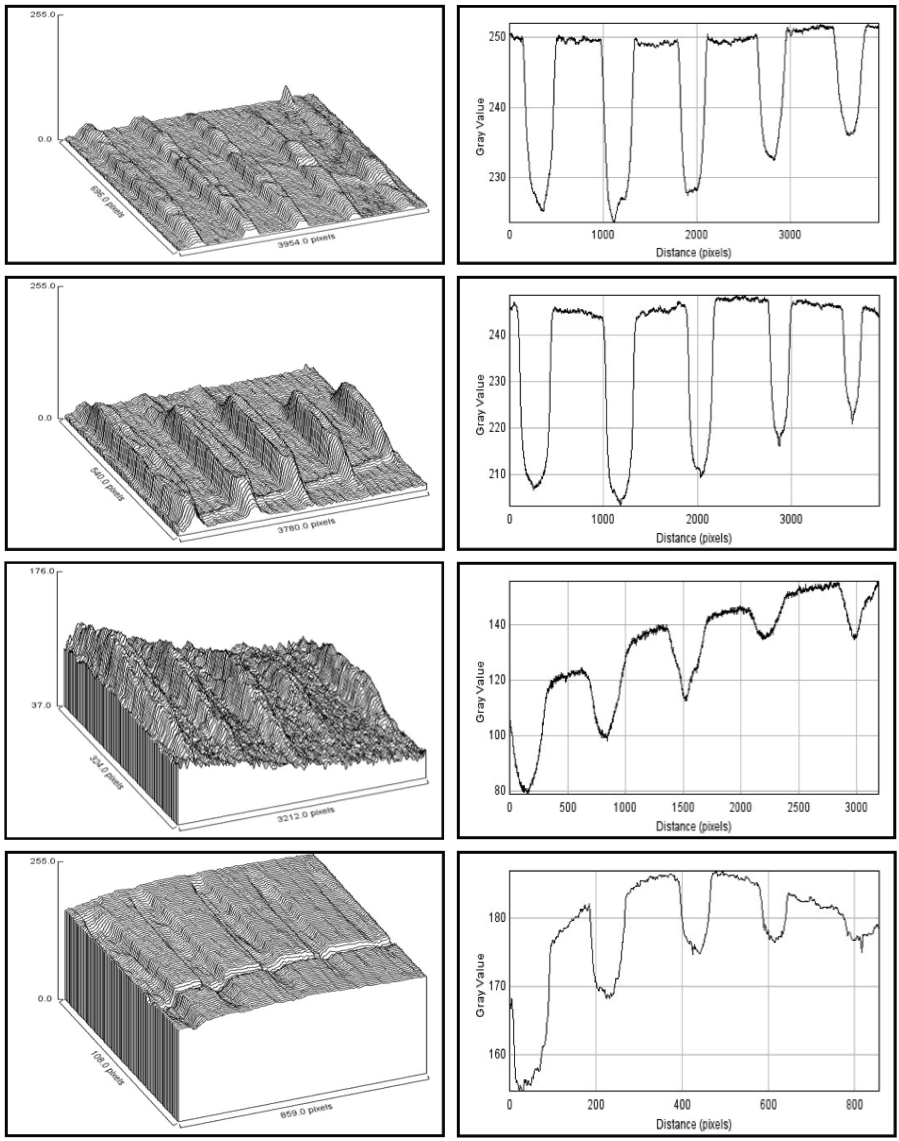


Figure 3. Surface plot and mean gray values of biogenic amines.
A = Cadaverine dihydrochloride C = Tyramine hydrochloride
B = Spermidine trihydrochloride D = Putrescine dihydrochloride

3 shows the surface plots and mean gray values (integrated densities) of cadaverine dihydrochloride, putrescine dihydrochloride, spermidine trihydrochloride, and tyramine hydrochrloride with concentrations ranging from 2μg to 10 μg per spot.

The surface plots and line graphs illustrated the consistency of the color intensities on the surface of the silica-coated TLC plates in terms of gray values. It can be observed that the pixel area and color intensity increases as the concentration of the biogenic amine standards increases. However, it could also be observed that the baseline density is influenced by the appearance of the chromatogram, as seen in tyramine hydrochloride.

Based on the results, the ImageJ program can detect the relative color densities of the spots of the TLC chromatograms. Density of the spot is illustrated by a hyperbolic appearance of the line graph. The relative area of the spots is illustrated by a wide base. Cadaverine dihydrochloride showed the greatest mean gray value. A higher gray value indicates that the actual color intensity is lighter (Ferreira & Rasband, 2012). However, the surface of the spot is not consistent as revealed by the surface plot. The lowest mean gray value was observed in tyramine hydrochloride.

The contrast of each spot with its background influences the baseline of the surface plots and line profiles. Both tyramine hydrochloride and putrescine dihydrochloride had a darker background in the chromatogram, resulting in varying levels of initial baselines of the surface plots and line profiles. The pencil mark also influenced the appearance of the surface plots and line profiles. This was observed in the chromatogram of tyramine hydrochloride and putrescine dihydrocloride, resulting in a linear depression on the surface plot.

Linearity of Regression Equations of Pixel Areas and Mean Gray Values vs. Concentration

The use of image analysis based on mean gray values relies on the quality of the image produced. Using the program, the chromatograms were transformed into pixels and subsequently processed, providing a quantitative evaluation of the biogenic amine spots. Furthermore, the concentrations of each biogenic amine in the study were correlated with pixel areas and mean gray values. The linear regression equations of the different biogenic amines were constructed using the two parameters.

Table 4 shows that the linear regression equations and mean circularity of the biogenic amine spots. The linear regression equations using pixel areas showed varied slopes and intercepts and higher coefficients of determination (R^2>0.940, ϱ<0.01, two-tailed) compared

Table 4.

Linear Regression Equations and Mean Circularity of Biogenic Amines.

Biogenic Amine	Regression Equation 1 (Concentration vs. Pixel Area)	R^2	Regression Equation 2 (Concentration vs. Mean Gray Value)	R^2	Mean Circularity
Cadaverine dihydrochloride	y = 17755.1X + 37140.2	0.981*	y = 2225082.15X – 703306.95	0.993*	0.9152 ± 0.01
Putrescine dihydrochloride	y = 726.0X + 113.0	0.948*	y = 1167354003X - 496545674.1	0.866*	0.9864 ± 0.01
Spermidine trihydrochloride	y = 12549.15X + 1069.9	0.988*	y = 2746515.15X -11483.1	0.952*	0.9584 ± 0.02
Tyramine hydrochloride	y = 10291.4X – 23232.4	0.946*	y = 150070.7X - 33682.55	0.884*	0.8574 ± 0.10

*Results are significant at α=0.01 (two-tailed), x=concentration (μg/10μL), n=5

to the equations which utilized mean gray values (R^2>0.880, ρ<0.01, two-tailed).

The largest pixel area was observed in the visualized spots of cadaverine dihydrochloride while the smallest pixel area was seen in putrescine dihydrochloride. The pixel area seems to increase as the concentration of biogenic amines increases. The greatest circularity and mean integrated density were observed in putrescine dihydrochloride while the least circularity was noted in tyramine dihydrochloride.

A higher mean integrated density means that an image is lighter (in terms of mean gray value) while a circularity value of zero means that the spot is elliptical and a value of one means that the spot is perfectly circular (Ferreira & Rasband, 2012). Based from the mean circularity value in Table 4, putrescine dihydrochloride has the greatest value as observed in Figure 2. The difference in the results between direct inspection and image analysis may be attributed to the ability of ImageJ software to exclude parts of the image which do not show consistent color densities. The lowest mean gray value was observed in tyramine hydrochloride, meaning, it produced the darkest color intensity compared to other biogenic amines.

Determination of Biogenic Amines in Sample Fish Paste

Using the methodology described in this study, the biogenic amines in a fish paste extract was determined. The image of the chromatogram was converted to 8-bit format for the determination of mean gray values using the particle analysis of the software. After background subtraction, the TLC spots were not visible. In order to quantify the biogenic amine spots, 10μL of each of the biogenic amine samples (10mg/mL) was added. Using the linear regression equations, the concentrations of each of the biogenic amines were determined. The known concentration of the biogenic amines was subtracted from the detected amounts to estimate the concentration of biogenic amines in the sample.

Using the linear regression equations in Table 4, the mean concentration of biogenic amines in a sample fish paste (n = 5) was determined after image processing. Compared to other analytical methods, thin layer chromatography was characterized to have relatively lower reproducibility and moderate sensitivity (Cserhati & Szogyi, 2012), probably because there are several interfering compounds in the development of the chromatograms. One anticipated

problem in the study was the contamination which might be caused by free amino acids. According to Valls et al. (2002), the Rf values of amino acids using the mobile phase (acetone: ammonium hydroxide, 95:5, v/v) are below 0.10. This necessitates the utilization of alternative methods to validate the presence of spermidine trihydrochloride and putrescine dihydrocloride in the fish paste sample. However, this was not performed in the study since the methanolic extracts of fish pastes were mixed with a known concentration of biogenic amines. The relatively larger standard deviations in spermidine trihydrochloride and putrescine dihydrochloride could be explained by possible interference of amino acids in the extract. The pixel areas and circularity of the spots from fish paste were compared to the characteristics of the spots of the standard solutions to validate that the spots were biogenic amines.

The chromatogram appears diffuse although there is visible separation of distinct spots. In order to visualize the biogenic amines, 10μg of each of the biogenic amines was added in the methanolic fish paste extract and the total concentration obtained using the regression equations were subtracted from the concentration of biogenic amines added to the solutions. Since the spots of putrescine dihydrochloride and spermidine trihydrochloride overlap, two mixtures containing only three biogenic amines were used in order to determine the individual concentrations of spermidine and putrescine. The concentrations obtained without adding the standard solutions were subtracted from the concentrations obtained when standard solutions were added.

Quantification of biogenic amines was done by utilizing the regression equations obtained using the pixel areas and mean gray values as parameters (Table 4). The image was converted to 8 bit format and then analyzed using Image J software. Based on the results shown in Table 5, the fish paste sample showed varying concentrations of biogenic amines when the linear regression equations using the pixel areas and mean gray values were used. Generally, the values obtained using the mean gray values yielded higher concentrations of biogenic amines and higher standard deviations as compared to the results when the pixel areas were used, probably due to presence of free amino acids in the sample. The biogenic amines were also identified based on the characteristics of the spots such as the circularity and Rf values. Results show that the most abundant biogenic amine in the sample fish paste is putrescine, followed by spermidine, cadaverine and tyramine. The values obtained are higher compared to the results

obtained by Visciano, Schirone, Tofalo, and Suzzi (2012) using fish paste samples in Italy.

Table 5.

Biogenic Amine Content of the Fish Paste Sample.

Biogenic Amine	Average Amount Detected Using Pixel Area (μg/spot)	Average Amount Using Mean Gray Values (μg/spot)	Rf Values
Putrescine	13.20 ± 0.20	14.50 ± 2.10	0.08 ± 0.02
Tyramine	2.83 ± 0.12	3.45 ± 1.22	0.87 ± 0.11
Cadaverine	3.42 ± 1.14	4.13 ± 1.64	0.17 ± 0.07
Spermidine	6.22 ± 2.30	6.85 ± 3.12	0.06± 0.06

Generally, the level of allowable biogenic amine content of food samples vary from one country to another. Based on the biogenic amine levels in the fish paste sample, the concentrations of biogenic amines detected in the fish paste sample are higher compared to the results in other studies. The amounts of cadaverine, putrescine, spermidine and tyramine were higher compared to the allowable limit in foods as set by the US Food and Drug Administration as generally accepted level of biogenic amines which is 5 mg/100g. The amount of tyramine in the fish paste is lower compared to the legal limit set by the Nutritional Codex for the Slovak Republic which is 200 mg/kg or 20mg/100g (Vallejos et al., 2011). Ladero et al. (2010) proposed that the total biogenic amine level should be 750 to 900mg/kg.

The biogenic amines in the sample fish sauce are influenced by its storage condition, salt concentration, preparation and type of biogenic amine-producing microorganisms (Zaman, Abdulamir, Abu Bakar, Selamat, & Bakar, 2009; Chong, Abu Bakar, Russly, Jamilah, & Mahyudin, 2011). Karovicova and Kohajdova (2005) reported that biogenic amine synthesis is possible if there are available amino acids, presence of decarboxylase-positive microorganisms, and other conditions which promote decarboxylase activity and synthesis, and bacterial growth. These factors might have played a role in the formation of biogenic amines in the fish paste samples. Furthermore, the concentrations of biogenic amines in the fish paste sample may imply possible contamination by decarboxylase producing bacteria

during fish preparation and fermentation process.

CONCLUSIONS

Thin layer chromatography coupled with image analysis using the ImageJ program can satisfactorily determine the concentration of biogenic amines in mixtures and food samples using the pixel area and mean gray values of the ninhydrin-visualized TLC biogenic amine spots. However, the results may vary depending on the quality of the chromatograms and the appropriateness of image processing. Cadaverine dihydrochloride and tyramine hydrochloride were adequately separated from spermidine trihydrochloride and putrescine dihydrochrloride using the ammonium hydroxide: acetone mobile phase. Overlapping spots is minimized by ninhydrin solution acidified with acetic acid. The TLC method obtained reproducible Rf values in biogenic amine mixtures and methanolic extracts of fish paste while the linear regression equations utilizing mean gray values and pixel areas showed adequate linearity when used to quantify the biogenic amine concentrations. Using the regression equations, the biogenic amines were quantified in the TLC chromatogram image of fish paste. Thin layer chromatography combined with image analysis provides a cheaper and faster method in quantifying biogenic amines compared to other analytical methods in small laboratory settings since expensive visualizing agents such as dansyl chloride and o-phathaladehyde can be replaced by ninhydrin. Furthermore, ImageJ, which is free and downloadable, can be readily used to analyze an image from a simple digital camera without compromising accuracy and precision of results. Additional studies could be performed to compare this simple method to highly analytical methods such as high performance liquid chromatography or gas chromatography. Furthermore, studies about varied choices of mobile phase, TLC plates and visualizing reagents may be explored.

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