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Biogenic amine content in various types of tofu: occurrence, validation and quantification

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Abstract

In the present work, the biogenic amines tryptamine (TRP), putrescine (PUT), histamine (HIS), tyramine (TYR) and spermidine (SPD) were determined in 32 various types of tofu that were obtained from different states in Malaysia. Three main types of tofu; soft tofu, firm tofu and processed tofu, were analysed in the present work. The biogenic amine contents in the respective types of tofu were analysed by a reversed-phase HPLC with a DAD detector after the aqueous extraction and derivatisation with dansyl chloride. The LOD values ranged from 0.019 mg/L for PUT to 0.028 mg/L for TYR. While, the LOQ values ranged from 0.063 mg/L (PUT) to 0.096 mg/L (TYR). The recovery values for all the five amines ranged from 80.3% to 120.5% with RSD ≤ 3.1%. The total levels of biogenic amines found varied, ranging from 1.5 mg/kg to 687.9 mg/kg, with mean values (p < 0.05) in descending order of 44.6, 12.6, 9.1, 4.8 and 4.7 mg/kg for PUT, TYR, SPD, HIS and TRP, respectively. PUT and TRP were the most prevailing biogenic amines and they were found respectively in 90.62% of the tofu analysed. Significant positive correlations (r = 0.266 to 0.874, p < 0.05) were found between some individual biogenic amines and protein content in all the three types of tofu. However, negative correlations (r = -0.246 to -0.832, p < 0.05) were observed between biogenic amines and moisture content, and between biogenic amines and water activity in all the three types of tofu. Significant and strong correlations (r = 0.525 to 0.999, p < 0.05) were found between most of the individual biogenic amines and the total biogenic amines. Those tofu exceeding the legal limits may affect the health of sensitive individuals.

Introduction

Recently, biogenic amines have become a great concern because of their effects on the food quality and human health. Chemically, biogenic amines are low molecular weight organic bases with aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenyethylamine), and heterocyclic (histamine, tryptamine) structures (Ruiz-Capillas and Jiménez-Colmenero, 2004). These amine compounds can also be classified as 'natural polyamines' and 'biogenic amines' depending on the way they are synthesised. Natural polyamines are the amines that are produced naturally by animals, plants and microorganisms (Silla Santos, 1996). This group of amines consists of mainly spermidine and spermine. Putrescine and cadaverine are sometimes considered as natural amines since they are found at low concentration naturally in both plants and microorganisms (Ruiz-Capillas and Jiménez-Colmenero, 2004). This group of amines plays an important role for the proper functions of cells (Toro-Funes *et al*., 2015). On the other hand, biogenic amines are the amines that are produced by microbial activity. This group of amines includes tyramine, histamine, trytamine, β-phenylethylamine, putrescine and cadaverine. They are present in food as a result of bacterial enzymatic decarboxylation of amino acids or through amination or transamination of aldehydes and ketones (Zolou *et al*., 2003).

The presence of biogenic amines in foods may induce toxicological risks and health problems. In

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food, biogenic amines can be formed through the fermentation processes or bacterial decarboxylation of amino acids (Guan *et al*., 2013). Thus, foodstuff produced by fermentation or exposed to microbial contaminant during the processing or storage stages may contain biogenic amines. Among these biogenic amines, histamine and tyramine are of great concern due to their toxicological effects. The diamine putrescine and cadaverine are toxic only in large concentrations, and they can potentiate the effects of simultaneously ingested histamine and tyramine. While the polyamine spermine and spermidine have been reported to react with nitrite to form carcinogenic nitrosoamines (Zhai *et al*., 2012). High concentrations of biogenic amines have been reported by several researchers in various types of foods, such as fish (Mah *et al*., 2002), meat (Papavergou *et al*., 2012), cheese (Valsamaki *et al*., 2000), vegetables (Moret *et al*., 2005) and wines (Proestos *et al*., 2008).

Tofu is a protein rich food made from soybeans and commonly consumed in East Asian and Southeast Asian countries. It is high in protein, calcium, magnesium and iron. It has a low calorie count, very little flavour or smell, and can be served in a variety of ways due to its ability to absorb new flavours through spices and marinades. Tofu can basically be categorised into two main categories: 'fresh tofu' - produced directly from soy milk, and 'processed tofu' - processed in certain ways from fresh tofu. Fresh tofu can be further categorised into soft tofu and firm tofu. Soft tofu is made by coagulating hot soy milk with sea salts such as magnesium chloride after which the resulting curds are pressed into a soft white block. The process of making firm tofu is quite similar to soft tofu, except for firm tofu contains less water because more water is pressed out of the tofu. Processed tofu are usually made by adding egg, meat or seafood to the tofu or they can be made by further frying to create unique textures or flavours or to prolong their shelf life (Hui and Özgül Evranuz, 2015).

Several authors have reported the occurrence of biogenic amines in non-fermented and fermented soybean products. The reports on fermented soybean products are of a wide variability; soy sauce (Guidi and Gloria, 2012), sufu (Guan *et al*., 2013), soybean paste (Shukla *et al*., 2010), miso (Tsai *et al*., 2007), tempeh (Nishimura *et al*., 2006) and natto (Kim *et al*., 2012). However, the reports on non-fermented soybean products such as fresh tofu, hard tofu, soymilk and soy sprouts are relatively scarce (Nishimura *et al*., 2006; Toro-Funes *et al*., 2015). To the best of our knowledge, no comprehensive studies have been carried out on the biogenic amine contents for various types of tofu. Therefore, the aim of the present work was to investigate the biogenic contents for various types of tofu using a validated HPLC method with DAD detection. In the present work, the tofu samples were categorised into three main categories based on their physical appearances; soft tofu, firm tofu and processed tofu.

Materials and methods

Chemicals and reagents

All chemicals and solvents used were of analytical and chromatographic grade. Spermidine trihydrochloride (SPD), tryptamine hydrochloride (TRP) and acetone were purchased from Merck (Germany). Histamine dihydrochloride (HIS), tyramine hydrochloride (TYR), glutamic acid monosodium monohydrate and dansyl chloride were purchased from Alfa Aesar (USA) while putrescine dihydrochloride (PUT) was purchased from Acros Organic (Belgium). Hydrochloric acid was purchased from Fisher Scientific (USA). Sodium hydrogen carbonate and sodium hydroxide were respectively purchased from Systerm (Malaysia) and R&M Chemicals (UK). Acetonitrile was purchased from Fluka (Germany), and deionised water was produced from the Milli-Q system (Millipore, USA).

Preparation of standard solutions

A stock solution (1,000 mg/L) comprising of a mixture of TRP (30.7 mg), PUT (45.8 mg), HIS (41.4 mg), TYR (31.6 mg) and SPD (43.9 mg) was prepared in 0.1 M HCl in a volumetric flask (25 mL). Standard solutions of concentrations 0.1, 1, 5, 15, 30, 60, 150 and 300 mg/L were prepared by diluting the stock solution and then used to obtain the calibration curves. These standard solutions were stored at 4°C prior to use.

Food samples

The tofu samples analysed in the present work were purchased from supermarkets, wet markets and night markets from the states of Kuala Lumpur, Selangor, Melaka and Johor, Malaysia from May to July in 2016. The tofu samples were of various varieties and categorised according to the ways they were processed; soft tofu, firm tofu and processed tofu. A total of 32 samples were obtained: 9 samples of soft tofu, 6 samples of firm tofu and 17 samples of processed tofu. The soft tofu assessed were of various types which consisted of smooth tofu (a soft tofu), organic tofu (soft tofu prepared from organic soybeans), premium tofu (a brand of soft tofu), silken tofu (a brand of soft tofu), Japanese tofu (Japanese soft tofu), tau fu fa (tofu pudding) and block tofu (soft tofu in block form). The firm tofu assessed were yong tofu (firm tofu with fish in it), square tofu (firm tofu in square form) and traditional tofu (firm tofu in rounded square form). The processed tofu assessed were fried square tofu (deep fried square tofu), tofu puff (smaller size deep fried tofu), egg tofu (egg within tofu), smooth egg tofu (egg within smooth tofu), bean curd (a fried tofu), salted bean curd (salted bean curd), seaweed tofu (a fried seaweed tofu), 5-spice tofu (spices within), seafood tofu (a fried seafood tofu), smelly tofu (a deep fried smelly fermented tofu), tofu-skin wrap fish (fish within fuzuk), fried tofu skin (fried fuzuk), tofu crackers (a dried tofu biscuit), tofu skin (non-fried fuzuk), spring roll with fish (fish within fuzuk in roll form) and bean curd prawn (prawn within fuzuk). ‘Fuzuk’ is a tofu skin made from soybeans. It is a layer of film or skin forms on the liquid surface during the boiling of soy milk. The films are collected and dried into yellowish sheets known as tofu skin or ‘fuzuk’. It is technically not a proper tofu but still widely used in Chinese and Japanese cuisines. All samples were wrapped and stored in a refrigerator at 4°C prior to analyses.

Preparation of food samples

Firstly, 5 g tofu was homogenised using mortar and pestle and then extracted with 25 mL 0.1 M HCl. The resulting mixture was centrifuged at 3,000 rpm (Hermle Z200A, Germany) for 15 min. The homogenate was then refrigerated (4°C) overnight to precipitate the fats and proteins. The supernatant was filtered using a 0.2 µm syringe filter to remove the solid residue. Finally, 25 µL extract was derivatised.

Derivatisation

The derivatisation of biogenic amines in the tofu samples was carried out according to Saaid *et al*. (2009) with slight modifications. Briefly, 25 µL extract was mixed with 200 µL saturated solution of $NaHCO_3$, 20 µL 2 M NaOH and 2 mL dansyl chloride (10 mg dansyl chloride in 1 mL acetone). The mixture was placed in a water bath at 70°C for 15 min. Next, 1 mL glutamic acid (50 mg glutamic acid in 1 mL water) was added to remove unreacted dansyl chloride. After 1 h, the mixture was diluted to 5 mL with acetonitrile. The solution was filtered using a 0.2 µm syringe filter and the filtrate was injected into the HPLC system.

Spiking

Spiking was conducted to understand the food matrix effects on the biogenic amine analysis. It was carried out by spiking the selected tofu samples with three concentrations (5, 15, 30 mg/L) of the standard solutions containing the biogenic amines. The spiking analysis was conducted after the sample extraction. Each spiking analysis was conducted in triplicate. The percentages of recovery and RSD were calculated.

Instrumentation and chromatographic conditions

The quantitative analysis of biogenic amines was conducted using a HPLC unit (Agilent Technologies, California, US) equipped with Agilent 1260 Infinity quaternary pump, Agilent 1260 Infinity diode-array detector, Agilent 1260 Infinity standard autosampler injector and a 20 µL sample loop. Separation was achieved using an Agilent ZORBAX Eclipse XDB 5 µm ODS column (150 mm × 4.6 mm). The mobile phase used was acetonitrile:water (67:33 v/v). The flowrate was set at 1.0 mL/min. This mixture was filtered using a membrane filter and degassed in an ultrasonic bath for 15 min before use, and the detector was set at 254 nm. The biogenic amines analysed were tryptamine (TRP), putrescine (PUT), histamine (HIS), tyramine (TYR) and spermidine (SPD).

Determination of protein content, moisture content and water activity

The protein contents of the tofu samples were determined using the Kjedahl method (Yeğin and Üren, 2008). Briefly, 0.2 g tofu samples were accurately weighed and digested in the Kjedahl digestion block (Buchi Speed Digester K425, Switzerland) for 2 h. Next, 30 mL distilled water and 10 mL 32% sodium hydroxide were added in the distillation step (Buchi Distiller K350, Switzerland). The distillate collected was then titrated with 0.2 M hydrochloric acid using methyl red as indicator. Nitrogen-protein conversion factor of 5.71 was used to calculate total protein content in the tofu samples.

The moisture content was determined according to Poveda *et al*. (2016) with slight modifications by oven drying method (Binder Drying Oven FDL115, Germany). Briefly, 10 g tofu samples were accurately weighed in aluminium weighing dishes and dried in the oven at 105°C for 4 h. The heating and weighing steps were repeated a few times until constant successive weights were achieved.

The water activities of the tofu samples were analysed using a water activity analyser (AquaLab Lite, USA). All analyses were done in triplicates.

Statistical analysis

All statistical analyses were performed using SPSS v.21 software, which included the calculations of mean, standard deviation and Pearson correlations between biogenic amines and protein content, moisture content and water activity. All data were expressed as mean ± SD for three determinations for each sample.

Results and discussion

Method validation

The analytical method was validated by determining the linear range, repeatability and reproducibility, limits of detection (LOD) and quantitation (LOQ), precision and recovery. Results of the analyses are summarised in Table 1. The linearity of the calibration curves were obtained by injecting five concentrations of standard solutions containing the biogenic amines (0 - 300 mg/L) and analysed in triplicate. High correlation coefficients (R2: 0.9930 - 0.9990) were obtained for the concentration ranges being studied, indicating good linearity between peak area and analyte concentration.

The LOD for each amine was determined from the minimum concentration required to give a signal to noise ratio of three, while the LOQ were determined with a signal to noise ratio of ten (Shukla *et al*., 2010). The LOD values obtained were 0.021 mg/L for TRP, 0.019 mg/L for PUT, 0.021 mg/L for HIS, 0.028 mg/L for TYR and 0.026 mg/L for SPD, whereas the LOQ values were 0.072, 0.063, 0.075, 0.096 and 0.087 mg/L for TRP, PUT, HIS, TYR and SPD, respectively. These values are comparable to those reported in the literature (Proestos *et al*., 2008).

The repeatability of the method were assessed by triplicate injection of each concentration of the standard solutions containing the biogenic amines. The analyses were conducted on the same day for intraday repeatability and over seven days for interday repeatability. Good repeatability was obtained for both the retention time (RSD ranged from 0.01% to 0.70%) and peak area (RSD ranged from 0.04% to 1.79%). Satisfactory recoveries were obtained for the spiking analyses. The recovery values for all the five amines ranged from 80.3% for SPD to 120.5% for HIS with RSD ≤ 3.1 (Table 1).

Biogenic amines in tofu samples

The peak identification was based on the comparison of the retention time between the samples and standard biogenic amine compounds. It was further confirmed by spiking the standard biogenic amine compounds to the samples. Quantification of the biogenic amines in the samples was based on the external standard method using calibration curves fitted by linear regression analysis. The separation of the five biogenic amines in the samples was achieved within 22 min. Typical chromatograms of the derivatives of biogenic amines in standard solution and tofu samples (one for each category) are shown in Figure 1. The retention times of the biogenic amines were stable and consistently reproducible. All the five biogenic amines were well resolved and completely eluted in less than 22 min.

The tofu samples analysed in this study and the levels of biogenic amines found are shown in Table 2. The five biogenic amines analysed (PUT, HIS, TYR, SPD and TRP) were detected in the tofu samples. It was found that the total biogenic amine levels in all the tofu samples varied greatly, ranging from 1.5 mg/kg up to 687.9 mg/kg. The levels ranged from 20.0 mg/kg to 79.9 mg/kg for soft tofu, 1.6 mg/kg to 687.9 mg/kg for firm tofu and 1.5 mg/kg to 395.1 mg/kg for processed tofu. The wide variations were also observed for individual amines, from <LOQ – 63.6 mg/kg for TRP, <LOQ – 306.2 mg/kg for PUT, <LOQ – 65.3 mg/kg for HIS, <LOQ – 179.7 mg/kg for TYR and <LOQ – 73.4 mg/kg for SPD. The variation of biogenic amines could be contributed by the following factors: (i) the types and quality of ingredients used, (ii) processing, fermentation and storage conditions (such as techniques, temperature and duration), and (iii) the presence of potential contaminant microorganisms with decarboxylase activity (Guan *et al*., 2013, Toro-Funes *et al*., 2015).

When considering the occurrences of individual amines in all the three types of tofu, it was found that PUT and TRP were the most commonly found biogenic amines, both covering respectively, 90.6% of the tofu samples analysed. While, TYR, SPD

Table 1: Analytical characteristics of the HPLC method and recoveries of biogenic amines when spiked to a specified tofu.

BA	Linear range (mg/L)	R^2	LOD (mg/L)	LOQ (mg/L)	Intraday (RSD) (*n* = 3)		Interday (RSD) (*n* = 3)		% Recovery (RSD) (*n* = 3)		
					t_R	Area	t_R	Area	5 mg/L	15 mg/L	30 mg/L
TRP	0.1-300	0.9990	0.021	0.072	0.70	1.51	0.01	0.01	94.2 (0.3)	89.8 (0.5)	84.0 (3.1)
PUT	0.1-300	0.9963	0.019	0.063	0.01	0.25	0.17	1.79	109.6 (0.8)	96.9 (0.8)	99.3 (0.1)
HIS	0.1-300	0.9979	0.021	0.075	0.02	0.04	0.06	0.04	120.5 (0.3)	97.0 (0.3)	100.2 (0.4)
TYR	0.1-300	0.9930	0.028	0.096	0.03	0.26	0.15	0.22	102.4 (0.0)	95.3 (0.1)	97.2 (0.2)
SPD	0.1-300	0.9972	0.026	0.087	0.06	0.28	0.14	0.31	85.7 (1.0)	80.3 (0.1)	87.1 (0.4)

R^2, square of regression coefficient; $t_{R,}$ retention time; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.

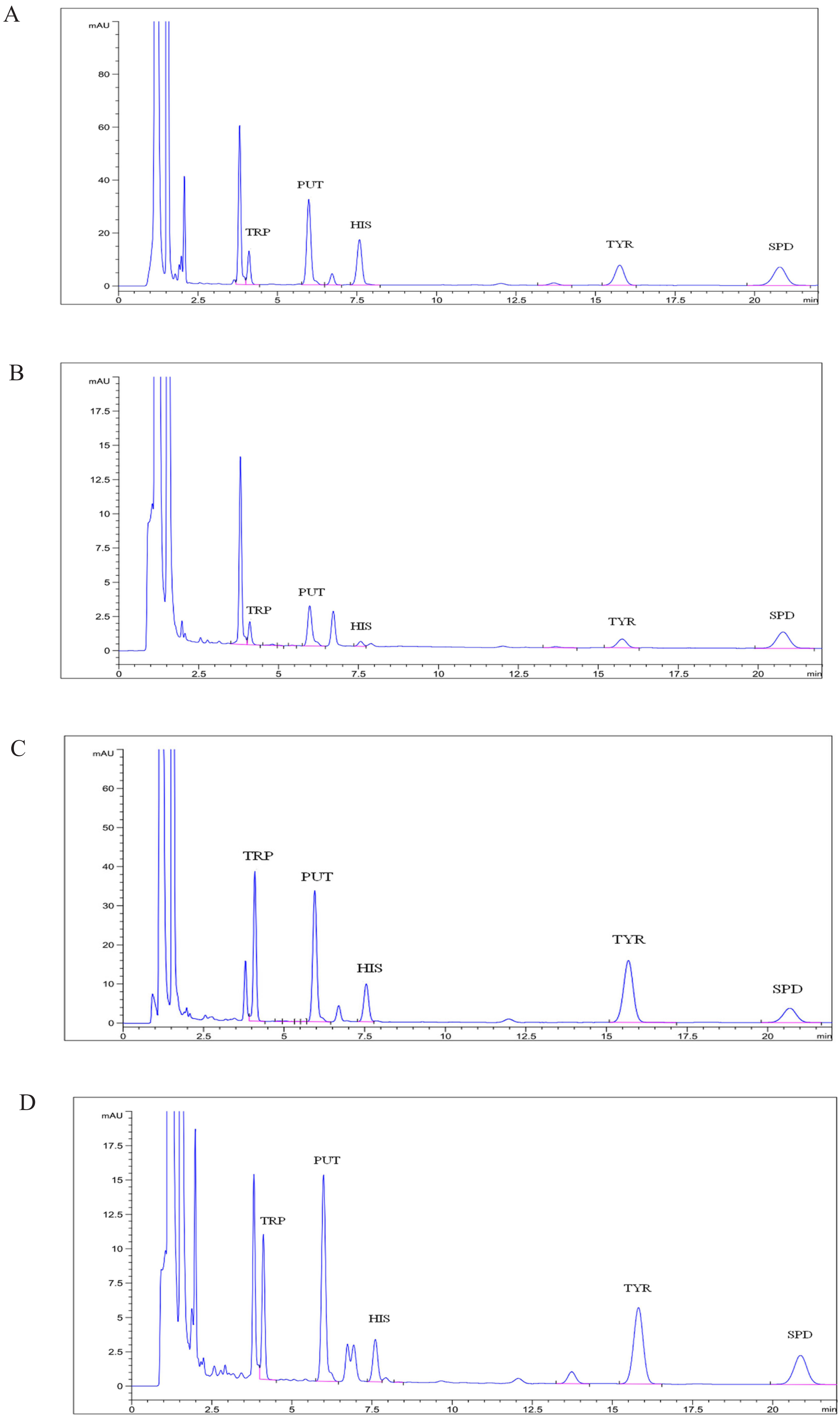

Figure 1: Typical HPLC chromatograms of the derivatives of (A) standard biogenic, and extracts of (B) soft tofu (sample no.9), (C) firm tofu (sample no.11) and (D) processed tofu (sample no.30).

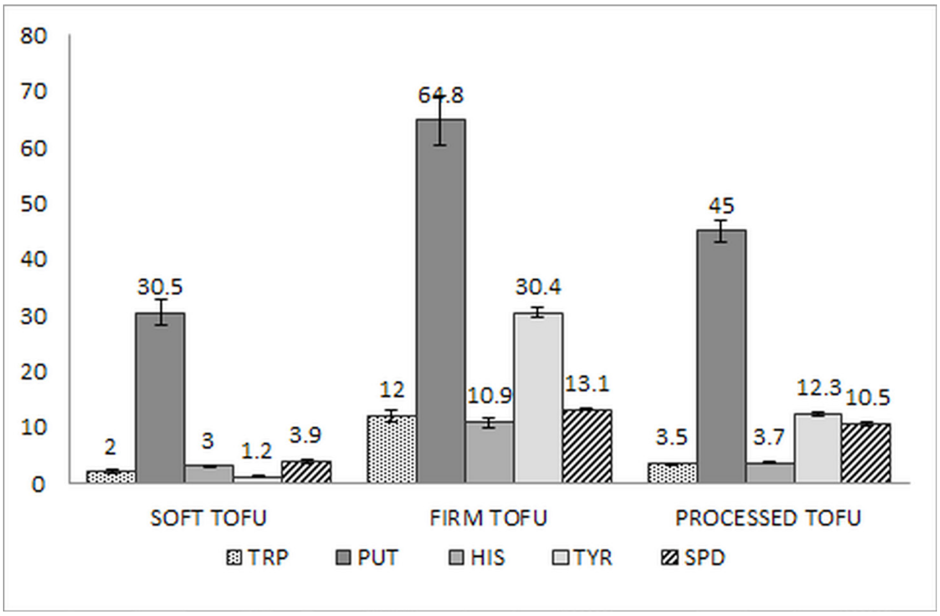

Figure 2: Mean biogenic amine contents (mg/kg) of soft tofu, firm tofu and processed tofu.

and HIS were found in 59.4%, 56.2% and 34.4% of the tofu samples analysed, respectively. Among the five biogenic amines analysed, PUT was the most abundant amine found in all the tofu samples, followed by TYR, SPD, HIS and TRP with the mean values of 44.6, 12.6, 9.1, 4.9 and 4.7 mg/kg, respectively ($p < 0.05$) (Table 2). The mean values ($p < 0.05$) for the individual biogenic amines in the three types of tofu are shown in Figure 2.

The high levels of biogenic amines were found in one of the firm tofu (i.e. square tofu) and three of the processed tofu (i.e. tofu puff, tofu-skin wrap fish and tofu skin) but it was not found in any of the soft tofu (Table 2). The highest PUT, HIS, TYR and TRP were detected in the square tofu with the values of 306.2 mg/kg, 65.3 mg/kg, 179.7 mg/kg and 63.6 mg/kg, respectively, while the highest SPD was found in tofu puff (73.4 mg/kg). Other samples had PUT < 50 mg/kg, HIS, TYR and SPD < 30 mg/kg, and TRP < 10 mg/kg. The high levels of biogenic amines are related to improper control of the quality of raw material, prolonged production processes, inadequate production and storage conditions, and the presence of decarboxylating bacteria (Bover-Cid *et al*., 2001). In Malaysia, the production of tofu, which is usually conducted in an open air environment by smallholders, has high chance for the biogenic amines formation. The high humidity and hot weather of Malaysia as an equatorial country may encourage the growth of biogenic amines (Shukla *et al*., 2010). High levels of PUT is generally associated with spoilage (Papavergou *et al*., 2012), while low levels of it could be attributed to its natural occurrence, since PUT is a natural occurring amine found in foods (Shalaby, 1996; Toro-Funes *et al*., 2015). HIS and TYR are the biogenic amines of concern due to their toxicity. The HIS levels found in the present work were generally low (most of them below 30 mg/kg, $p < 0.05$) and no tofu samples exceeded the upper limit of 100 mg/kg as legally set by the EU (Tsai *et al*., 2007). However, one of the firm tofu samples (i.e. square tofu, 65.3 mg/kg) exceeded the more restricted limit, 50 mg/kg as set by the USFDA (Papavergou *et al*., 2012). TYR levels were generally low in most of the tofu samples analysed. High levels of TYR (> 50 mg/kg, $p < 0.05$) were found in one of the firm tofu samples (i.e. square tofu, 179.7 mg/kg) and three of the processed tofu samples (i.e. tofu puff, 61.4 mg/kg; tofu-skin wrap fish, 52.2 mg/kg; and tofu skin, 63.8 mg/kg) and it was not found in any of the soft tofu samples. The square tofu exceeded the legal limit of 100 mg/kg as proposed by Shalaby (1996).

TRP and SPD concentrations found in the present work were low or moderate ($p < 0.05$). Higher levels of TRP were found in square tofu (63.6 mg/kg, a firm tofu), and most of the samples had TRP levels below 10 mg/kg. For SPD, two of the samples, square tofu (73.1 mg/kg) and tofu puff (73.4 mg/kg), had SPD exceeding 50 mg/kg, and majority of the samples showed SPD levels below 10 mg/kg. TRP is the intermediate product of the metabolism of arginine to PUT (Proestos *et al*., 2008). This explains why low levels of TRP (with one exception, i.e. square tofu) were encountered in all the tofu samples. On the other hand, low levels of SPD in most of the tofu could be related to low levels of PUT since PUT is the precursor for the synthesis of SPD (Atiya Ali *et al*., 2011). The consumption of SPD (as an alternative

Table 2: Levels of biogenic amines, protein content, moisture content and water activity detected in tofu samples (*n*= 3).

Sample no.	Sample type	Concentration, mg/kg								
		TRP	PUT	HIS	TYR	SPD	Total	Protein content (%)	Moisture content (%)	a_w
Soft tofu										
1	Smooth tofu	<LOQ	35.0 ±1.6	<LOQ	0.6 ± 0.1	0.9 ± 0.3	36.5 ±2.0	1.2 ±0.14	74.4 ± 0.94	0.84 ± 0.01
2	Organic tofu	1.8 ± 0.1	38.0 ± 4.8	<LOQ	0.8 ± 0.2	<LOQ	40.6 ± 5.1	1.5 ± 0.17	71.9 ± 0.47	0.83 ± 0.00
3	Premium tofu	1.5 ± 0.3	24.8 ± 2.1	<LOQ	0.8 ± 0.1	0.9 ± 0.2	28.0 ± 2.7	2.0 ± 1.46	89.5 ± 1.63	0.86 ± 0.01
4	Silken tofu	1.7 ± 0.3	25.5 ± 2.7	3.1 ± 0.4	0.9 ± 0.3	<LOQ	31.2± 3.7	0.9 ± 0.08	78.0 ±1.26	0.83 ± 0.02
5	Japanese pressed tofu	1.7 ± 0.4	24.6 ± 3.1	<LOQ	0.6 ± 0.1	<LOQ	26.9 ± 3.6	2.0 ± 0.16	81.0 ± 1.98	0.85 ± 0.01
6	Tau fu fa	1.7 ± 0.1	37.3 ± 0.9	21.8 ± 1.4	<LOQ	3.4 ± 0.5	64.2 ± 0.9	1.2 ± 0.15	91.2 ± 0.62	0.85 ± 0.01
7	Johor block tofu	4.1 ± 0.2	15.9 ± 1.9	<LOQ	<LOQ	<LOQ	20.0 ± 2.1	4.6 ± 0.31	73.6 ± 3.52	0.81 ± 0.01
8	Melaka block tofu	1.5 ± 0.5	31.0 ± 1.9	<LOQ	<LOQ	5.9 ± 0.7	38.4 ± 3.1	4.4 ± 0.26	74.9 ± 2.50	0.82 ± 0.01
9	KL block tofu	4.3 ± 0.5	42.5 ± 2.2	2.1 ± 0.2	7.2 ± 0.2	23.8 ± 1.0	79.9 ± 4.1	4.8 ± 0.08	72.4 ± 2.06	0.81 ± 0.00
	$\bar{x}$	2.0 ± 0.3	30.5 ± 2.4	3.0 ± 0.2	1.2 ± 0.1	3.9 ± 0.2	40.6 ±3.0	2.5±0.31	78.5 ± 1.66	0.83 ± 0.01
Firm tofu										
10	Yong tofu	1.7 ± 0.1	17.5 ± 1.1	<LOQ	0.5 ±0.1	<LOQ	19.7 ±1.3	2.7 ±0.96	68.3 ±0.51	0.79 ±0.02
11	Square tofu	63.6 ±4.9	306.2 ±19.3	65.3 ±3.4	179.7 ±4.9	73.1 ±0.4	687. 9 ±32.9	4.2 ±0.28	59.2 ±7.32	0.72 ±0.01
12	KL traditional tofu	1.6 ±0.2	<LOQ	<LOQ	<LOQ	<LOQ	1.6 ±0.2	2.1 ±0.00	72.9 ±3.24	0.85 ±0.02
13	Melaka traditional tofu	1.5 ±0.1	18.9 ±2.3	<LOQ	<LOQ	2.2 ±0.4	22.6 ±2.8	2.4 ±0.23	72.1 ±3.13	0.84 ±0.01
14	Johor traditional tofu	1.5 ±0.2	15.0 ±1.0	<LOQ	<LOQ	<LOQ	16.5 ±1.2	2.5 ± 0.29	75.7 ±2.69	0.82 ±0.02
15	Selangor traditional tofu	1.8 ±0.1	31.0 ±2.2	0.3 ±0.0	2.1 ±0.2	3.0 ±0.1	38.2 ±2.6	2.2 ± 0.10	73.3 ±3.16	0.86 ±0.01
	$\bar{x}$	12.0 ±0.9	64.8 ±4.3	10.9 ±0.6	30.4 ±0.9	13.1 ±0.2	131.1 ±6.8	2.7 ± 0.31	70.3 ±3.34	0.81 ±0.02
Processed tofu										
16	Fried square tofu	1.7 ±0.1	22.4 ±1.6	<LOQ	<LOQ	<LOQ	24.1 ±1.7	8.2 ±0.67	33.2 ±5.76	0.76 ±0.01
17	Tofu puff	22.8 ±0.8	213.7 ±8.5	22.9 ±1.6	61.4 ±0.6	73.4 ±2.5	394.2 ±14.0	7.6 ±0.16	37.3 ±7.33	0.75 ±0.00
18	White egg tofu	1.8 ±0.2	<LOQ	<LOQ	1.4 ±0.1	3.8 ±0.2	7.0 ±0.5	2.2 ±0.17	77.9 ±0.96	0.85 ±0.02
19	Yellow egg tofu	1.8 ±0.1	25.8 ±0.7	<LOQ	0.7 ±0.1	2.3 ±0.2	30.6 ±1.1	2.4 ±0.09	77.1 ±1.50	0.84 ±0.01
20	Smooth egg tofu	1.9 ±0.2	23.9 ±1.0	<LOQ	0.8 ±0.1	4.6 ±0.3	31.2 ±1.6	2.0 ±0.13	81.1 ±1.17	0.82 ±0.03
21	Salted bean curd	1.7 ±0.1	21.0 ±0.6	<LOQ	0.6 ±0.0	1.7 ±0.1	25.0 ±0.8	3.1 ±1.62	65.5 ±2.26	0.74 ±0.04
22	Bean curd	<LOQ	14.3 ±1.1	1.2 ±0.1	20.9 ±0.6	3.1 ±0.1	39.5 ±1.9	3.3 ±1.11	68.7 ±3.84	0.85 ±0.01
23	Seaweed tofu	1.5 ±0.3	<LOQ	<LOQ	<LOQ	<LOQ	1.5 ±0.3	6.0 ±0.86	68.9 ±0.53	0.86 ±0.01
24	5-spice tofu	1.6 ±0.1	20.3 ±0.3	<LOQ	<LOQ	1.6 ±0.1	23.5 ±0.5	4.0 ±0.34	60.9 ±2.88	0.75 ±0.01
25	Seafood tofu	1.5 ±0.2	21.7 ±1.0	<LOQ	0.5 ±0.1	<LOQ	23.7 ±1.3	2.8 ±0.16	47.4 ±0.11	0.70 ±0.01
26	Smelly tofu	1.6 ±0.1	38.2 ±1.3	0.4 ±0.0	<LOQ	3.7 ±0.0	43.9 ±1.4	6.9 ±3.47	31.9 ±3.19	0.65 ±0.02
27	Tofu - skin wrap fish	<LOQ	132.9 ±2.9	15.8 ±0.4	52.2 ±1.1	41.4 ±1.2	242.4 ±5.6	2.0 ±0.15	18.7 ±3.65	0.66 ±0.00
28	Fried tofu skin	1.5 ±0.1	22.4 ±0.6	<LOQ	<LOQ	<LOQ	23.9 ±0.7	2.6 ±0.56	2.6 ±0.56	0.59 ±0.01
29	Tofu crackers	1.5 ±0.1	22.4 ±1.4	<LOQ	<LOQ	<LOQ	23.9 ±0.7	4.4 ±0.67	4.4 ±0.67	0.50 ±0.02
30	Tofu skin	16.6 ±0.9	146.7 ±7.5	20.5 ±0.6	63.8 ±1.4	42.4 ±1.3	290.0 ±11.7	3.0 ±1.11	3.0 ±1.11	0.57 ±0.04
31	Spring roll with fish	1.5 ±0.1	23.1 ±1.4	<LOQ	7.5 ±0.3	<LOQ	32.1 ±1.8	6.3 ±1.08	6.3 ±1.08	0.59 ±0.00
32	Bean curd prawn	1.5 ±0.3	16.6 ±1.0	1.6 ±0.2	<LOQ	<LOQ	19.7 ±1.5	3.2 ±0.46	3.2 ±0.46	0.56 ±0.01
	$\bar{x}$	3.5 ±0.2	45.0 ±1.8	3.7 ±0.2	12.3 ±0.3	10.5 ±0.4	75.1 ±2.8	4.1 ±0.75	40.5 ±2.18	0.70 ±0.01
	Final $\bar{x}$	4.7 ±0.4	44.6 ±2.4	4.9 ±0.3	12.6 ±0.4	9.1 ±0.3	75.9 ±3.6	3.4 ±0.06	57.5 ±0.84	0.76 ±0.01

a_w – water activity

source of nitrogen) by microorganisms during fermentation processes may also lead to low levels of SPD (Tang *et al*., 2011).

The biogenic amine levels found in the present work are generally higher than the results reported by Toro-Funes *et al*. (2015) and Nishimura *et al*. (2006) for fresh tofu samples. The differences could be due to varying quality of raw ingredients used, the boiling temperature, processing time, storage duration and conditions (Guidi and Gloria, 2012). The higher temperature and humidity in Malaysia could be an additional factor that contributed to higher biogenic levels in the tofu samples.

The highest biogenic amine contents found in firm tofu could be related to the manufacturing processes of the tofu. Firm tofu which has relatively lower water content and higher protein levels may lead to higher growth of biogenic amine bacteria. The prolonged processes and storage time and the uncontrolled factors such as improper processing, manufacturing practices and storage conditions may contribute to the higher biogenic amine levels in firm tofu especially for the square tofu (Toro-Funes *et al*., 2015).

In the context of processed tofu, there are a variety of factors contributing to the high levels of biogenic amine in these tofu. These include the preparation processes, the additional ingredients used such as meat, egg, fish, prawn and 'fuzuk', and the environmental factors. The ingredients used may contain considerable amounts of protein that may breakdown to form peptides and amino acids (the precursors for biogenic amine formation), which may contribute to the pool of biogenic amines in the end products. Moreover, the preparation processes such as boiling of the raw material, steaming, grinding and deep frying may contribute to the prolonged time and exposure to the growth of biogenic amines.

Thermal treatment or cooking was reported to reduce biogenic amines contents due to the deactivation of the decarboxylases during cooking (Maijala *et al*., 1995; Kebary *et al*., 1999). However, some authors (Silla Santos, 1996; Ruiz-Capillas and Jimenez-Colmenero, 2004) found that biogenic amines are not significantly reduced even after high temperature treatment. This is because certain biogenic amines like SPD, TYR, PUT, when subjected to heat, may give rise to the formation of secondary amines. These secondary amines may produce carcinogenic N-nitrosoamines by reacting with nitrite compounds which were usually added as a curing agent in food preparation (Ruiz-Capillas and Jimenez-Colmenero, 2004). In addition, some halotolerant bacteria, with amino acid decarboxylase activity could overcome or reduce the effects of temperature, NaCl, and other biological and physicochemical factors that induce stress responses in their cells, with the production of some biogenic amines (Tkachenko *et al*., 2001). All these facts may explain why SPD, TYR and PUT were detected at high levels in square tofu (a firm tofu) and three of the processed tofu (i.e. tofu puff, tofu-skin wrap fish and tofu skin) (Table 2). Results also showed that PUT levels in the square tofu and three of the processed tofu contributed to 44.5 – 54.8% of the total biogenic amines found in these tofus (Table 2). This suggests that, the main factors that contributed to the production of higher biogenic amines in these tofus are the environmental factors which are related to prolonged production processes, unhygienic production and storage conditions (Bover-Cid *et al*., 2001) since this amine is associated with spoilage (ten Brink *et al*., 1990). Moreover, biogenic amines (such as TRP, PUT, HIS and TYR) are expected to form if the manufacturing process of tofu was contaminated by biogenic amine-forming bacteria (Guan *et al*., 2013; Toro-Funes, 2015).

Biogenic amines and their relationship with protein content, moisture content and water activity

To understand the correlations between individual amines and the individual amines with protein content, moisture content and water activity, statistical package SPSS, v.21 was used for computing the Pearson correlation coefficients. Bivariate correlations among individual amines with protein content showed significant positive correlations ($p < 0.05$ or $p < 0.01$) between biogenic amines and protein content for the tofu samples analysed. This is especially relevant for firm tofu, whereby all the five biogenic amines (TRY, PUT, HIS, TYR, SPD) showed strong positive correlations with protein content with the coefficient correlations (r) ranged from 0.862 to 0.874 (Table 3). However, there are only three biogenic amines (TRY, TYR and SPD) from soft tofu (r = 0.412 to 0.675), and one (TRY) from processed tofu (r = 0.266) that showed positive correlations with protein. This indicated especially for the case of firm tofu and partly from the soft tofu, that protein as one of the precursors in the synthesis of biogenic amines did show significant contribution to the production of biogenic amines. However for the case of processed tofu, protein had no significant (or limited) contribution to the production of biogenic amines.

In the case of soft tofu, the partial or low correlations could be attributed to the fact that these types of tofu are usually prepared fresh and sold within a short period of time. The exposure

Table 3: Correlations between individual biogenic amines with their respective protein contents, moisture contents and water activities for soft, firm and processed tofu.

		Protein content	Moisture content	a_w	TRP	PUT	HIS	TYR	SPD	Total BA
Soft Tofu	TRY	0.675**	-	-0.592**	1	-	-	0.567**	0.565**	-
	PUT	-	-	-	-	1	-	0.525**	0.573**	0.847**
	HIS	-	0.613**	-	-	-	1	-	-	0.512**
	TYR	0.412*	-	-0.345*	0.567**	0.525**	-	1	0.922**	0.719**
	SPD	0.569**	-	-0.427*	0.565**	0.573**	-	0.922**	1	0.820**
Firm Tofu	TRY	0.871**	-0.805**	-0.825**	1	0.996**	0.998**	0.996**	0.996**	0.998**
	PUT	0.874**	-0.806**	-0.827**	0.996**	1	0.996**	0.995**	0.996**	0.999**
	HIS	0.871**	-0.803**	-0.830**	0.998**	0.996**	1	0.999**	0.998**	0.999**
	TYR	0.867**	-0.790**	-0.832**	0.996**	0.995**	0.999**	1	0.999***	0.999**
	SPD	0.862**	-0.785**	-0.822**	0.996**	0.996**	0.998**	0.999**	1	0.999**
Processed Tofu	TRY	0.266*	-	-	1	0.845**	0.833**	0.757**	0.849**	0.853**
	PUT	-	-0.354**	-	0.845**	1	0.974**	0.933**	0.984**	0.995**
	HIS	-	-0.354**	-	0.833**	0.974**	1	0.974**	0.974**	0.989**
	TYR	-	-0.324*	-	0.757**	0.933**	0.974**	1	0.934**	0.960**
	SPD	-	-0.246*	-	0.849**	0.984**	0.974**	0.934**	1	0.990**

**Correlation is significant at the 0.01 level (1-tailed)
*Correlation is significant at the 0.05 level (1-tailed)
- No statistically significant correlation at 0.05 level (1-tailed)

to the contaminant bacteria is therefore reduced. Moreover, additional processes were not applied nor other ingredients added in the preparation of these tofus. For processed tofu, with one exception, the limited correlations between the biogenic amines and protein content could be related to the additional processes and ingredients used in the preparation of these tofu. The amino acids composition of protein vary broadly for different processed tofus due to the differences in ingredients used (such as egg, meat, seafood and 'fuzuk'). The processing conditions (such as steaming, grinding, deep frying and duration) are different for different processed tofus. The variation and the amount of biogenic amines formed were also affected by the availability of different microorganisms possessing different amino acid decarboxylase activities (Loizzo *et al*., 2013) and the amount of microorganisms present (Sun *et al*., 2016). The limited correlations between protein and biogenic amines indicate that the processing methods and environmental factors could be more important factors affecting the formation of biogenic amines (Yeğin and Üren, 2008; Lu *et al*., 2010b, Poveda *et al*., 2016). This is because longer processing time and the uncontrolled environment may incur longer exposure for the contaminant bacteria resulting in the expected formation of biogenic amines.

For moisture content and water activity analyses, the results show that there were no significant correlations between biogenic amines and moisture content, and between biogenic amines and water activity (a_w) for soft and processed tofu. However, strong significant negative correlations (p < 0.05 or 0.01) were found between the biogenic amines and moisture content (r = -0.785 to -0.806), and between biogenic amines and water activity (r = -0.822 to -0.832) for firm tofu. Soft tofu, firm tofu and processed tofu had average a_w levels of 0.83, 0.81 and 0.70, respectively (Table 2). All these tofus which had a_w values close to 0.9 were therefore expected to have bacterial growth since most bacteria can grow at a_w > 0.9 (Koral *et al*., 2013). The low levels of biogenic amines found in soft tofu were again attributed to the freshness and the hygienic conditions of the production of these tofu. For firm tofu, the negative correlations could be related to the fact that water was consumed by microorganisms during the production of biogenic amines which had limited water levels. While for the processed tofu, the relatively low a_w levels as compared to soft and firm tofu could be linked to the extra processes applied in the preparation of these tofus. Evaporation and dehydration may occur because of the additional processes and longer processing time. Similar negative correlations between biogenic amines and moisture content, and between biogenic amines and a_w were also reported by other authors (Lorenzo *et al*., 2008; Komprda *et al*., 2009).

When comparing the relationship between individual biogenic amines, significant positive correlations were found between all the biogenic amines in firm tofu (r = 0.995 to 0.999, p < 0.01) and processed tofu (r = 0.757 to 0.974, p < 0.01) and some of the soft tofu (r = 0.525 to 0.922, p < 0.01) (Table 3). These results indicated that the formation of the biogenic amines in the tofu samples especially the firm and processed tofu were contributed by the same factors (Marcobal *et al*., 2005). Significant positive correlations were also found between individual biogenic amines and the total biogenic amine for all the three types of tofu (with one exception for TRP in soft tofu). The correlation coefficients (r) ranged from 0.512 to 0.820 (p < 0.01) for soft tofu, 0.998 to 0.999 (p < 0.01) for firm tofu, 0.853 to 0.995 (p < 0.01) for processed tofu. It is noticed that positive correlations within biogenic amines were always reported by other researchers in their food samples analyses, indicating the similar factors influenced the formation of biogenic amines (Guidi and Gloria, 2012; Czajkowska-Myslek and Leszczyńska, 2017).

Further studies are however needed to understand the correlations between the free amino acids and biogenic amines; and the relationship between the biogenic amines and bacterial diversity.

Conclusion

The present work demonstrated that majority of the tofu samples analysed were found to contain lower levels of biogenic amines. Only 14.3% of the samples showed relatively high levels (> 100 mg/kg) of total biogenic amine. PUT and TRP were the most abundant biogenic amines detected in most of the tofu samples, with PUT being in the largest proportions in terms of the amount of biogenic amines. Only one sample (3.1%) analysed in the present work contained both HIS and TYR that exceeded the legal limits of 50 mg/kg and 100 mg/kg for HIS and TYR, respectively. However, no sample assessed in the present work contained HIS that exceeded the recommended ‘action level’ of 500 mg/kg. Since tofu is a protein rich food and consumed widely in Asia and most of the western countries especially by vegetarians, vegans and those who consume it as a healthy cholesterol-free condiment, measures must be enforced in the manufacturing processes and storage conditions in order to minimise the biogenic amine content in tofus for safe consumption.

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