Screening biogenic amines and fish-based food (keropok lekor) extracts in induction of inflammation using Principal Component Analysis

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Abstract

Background: Food-borne biogenic amines (BAs), namely, histamine, putrescine, cadaverine, tyramine, spermine and spermidine are known for their contributions as fish-based food freshness biomarkers to determine level of contamination. The remaining food-borne BAs (phenylethylamine, tryptamine and agmatine) effects on promoting inflammation are yet to be investigated. The effect of these compounds on induction of inflammation in macrophages was investigated using Principal Component Analysis (PCA) from independent (BAs at 1, 10 and 100 µg/ml, food extract and its standard mixture solution) and dependent variables cell viability, nitric oxide (NO) and tumor necrosis factor-α (TNF-α) secretion. Nine individual BAs and keropok lekor extracts were exposed to RAW 264.7 macrophages for 18-24 hr at 37°C with 5% carbon dioxide environment. Cell viability, NO and TNF-α secretion were determined using MTS assay kit, Greiss Reagent System and Enzyme-linked Immunosorbent Assay (ELISA) kits, respectively. Results: Q2V values were not equal to Q2 (an estimate of the predictive ability of the model) values for individual variables because the eigenvalues values were more than 0.5, indicating a good model. All variance (R2VX) values were > 0.9, suggesting goodness of fit. Conclusions: PCA is thus proven as an effective tool to discriminate between inflammogenic and non-inflammogenic food-borne BAs.

Background

Inflammation has been associated with various chronic and acute diseases. As prevention, a method to screen foods which can cause inflammation before we eat is warranted. Food-borne biogenic amines (BAs) [histamine (HIM), putrescine (PUT), cadaverine (CAD), 2-phenylethylamine (PHM), tyramine (TYM), tryptamine (TPM), spermine (SPM), spermidine (SPD) and agmatine (AGM)] had been shown to be present in various foods such as fishes,
seafood-based products either raw, cooked or fermented, cheeses and beverages [12, 13, 15] and is associated to Scombroid fish poisoning (SFP) at high doses. SFP causes allergy-like reactions and inflammation seems to be involved in the manifestation of symptoms such as urticaria.

Inflammation associated with food toxicity and food intolerance in particular had been described previously [14]. The toxicity of some BAs is determined through acute and sub-acute studies. Nonetheless, the toxicity of BAs on in vitro RAW 264.7 macrophages is yet to be determined. Currently, in vitro cell culture work is an alternative to in vivo toxicity study in animal models to minimizing animal usage in laboratory experiments. Mammalian cell culture has been used since they mimicked the cells of human at specific organs. BAs has been claimed to cause allergy-like reactions but their involvement in induction of pro-inflammatory mediator secretion is poorly understood. The relationship between cytotoxicity and potentiality to induce pro-inflammatory secretion remains to be determined.

A food model is needed to validate the PCA results, thus, keropok lekor had been chosen. Keropok lekor or fish sausage is a traditional Malay fish snack popular among the locals in Malaysia. It is made from fish and sago flour and seasoned with salt and sugar. It comes in two main forms: lekor (long and chewy) and keping (thin and crispy). There are various BAs found in keropok lekor [10] which may cause the inflammation during oral itchiness, a problem common after eating keropok lekor.

In this study, a method to screen inflammation-causing food compounds and extracts was developed through an experimental flow in Figure 1. The cell viability and pro-inflammation mediator secretion effects of different biogenic amines individually, as well as in the keropok lekor extracts using the RAW 264.7 macrophages cell line was determined. BAs and keropok lekor extract were used to develop and verify the method to
screen food potentially causing inflammation using RAW 264.7 macrophage cell line and multivariate statistics of Principal Component Analysis (PCA). There is no method yet as to screen food compounds possible to cause inflammation either orally or gastrointestinal. RAW 264.7 macrophages were used as a vehicle to respond to food compounds and their viability and pro-inflammatory mediator secretion data were analyzed using PCA. From the score plot, the pattern of distribution of data was studied and custom pattern for food compounds causing inflammation was determined along with patterns exhibiting apoptosis or necrosis presentation in the RAW 264.7 macrophages.

Results And Discussions

Data dimensions were reduced and information was retained by replacing the original correlated variables the systemic variation of the uncorrelated principal components (PCs). The link between the original correlated variables and the uncorrected PCs data were described by correlation circle. The correlation depends on the distance of a variable to the axis and circle in which closeness shows higher correlation to corresponding PC.

In interpreting the chemokine and cytokine data, adapted PCA segregated those combinations of actions leading to inflammatory and non-inflammatory consequences by using multivariate projection methods. The data on the score plot was separated into four quadrants with suggested four distinguished characteristics: (i) cell non-viability and inflammation suggests necrosis; (ii) cell non-viability without inflammation suggests apoptosis; (iii) cell viability without inflammation suggests cell proliferation; and (iv) cell viability with inflammation suggests cell repair upon injury (Figure 2).

The projection of data showed that the further data from the graph origin are mostly from biogenic amines with concentrations of 10 and 100 µg/ml [Figure 3(B) & 3(C)]. On the other hand, the biogenic amines with concentration of 1 µg/ml are plotted nearby origin where the control (cells only) are located, indicating absence of effects on neither cell
proliferation, NO secretion nor TNF-α secretion. Positive control was projected at extreme position, possessing high NO production.

In score plot mapping for 1 µg/ml individual biogenic amines [Figure 3(A)] it was found that HIM, PHM and AGM of 1 µg/ml (H1, PH1 and A1), BA standard aqueous solution (BK) and *keropok lekor* extracts (K) show strong correlation with Negative control while TYM, TPM and SPD of 1 µg/ml (TY1, TP1 and SD1) show correlation with Positive control. For 10 µg/ml individual biogenic amines of HIM, PHM and AGM (H10, PH10 and A10), K and BK show strong correlation with Negative control while TYM and TPM of 10 µg/ml (TY10 and TP10) show correlation with Positive control [Figure 3(B)]. For 100 µg/ml individual biogenic amines of HIM and AGM (H100 and A100), K and BK show strong correlation [Figure 3(C)] with Negative control while TPM, SPM and SPD of 100 µg/ml (TP100, SM100 and SD100) show correlation with Positive control [Figure 3(C)].

The loading plots for all data, 1 µg/ml, 10 µg/ml and 100 µg/ml biogenic amines were similar with NO and TNF-α on the same side of y-axis depicting positive correlation (Figure 4). On the other hand, NO and TNF-α were on the opposite side of y-axis with cell proliferation (cell viability increment) depicting negative correlation with cell proliferation. The results were in parallel with previous studies where both NO and TNF-α levels are reported to be in high concentration during inflammation while cell proliferation was low during inflammation. The positive control for inflammation in the score plot (Figure 3) falls on the same quadrant with NO in the loading plots (Figure 4) suggesting high NO secretion from the positive control cells. The negative control for inflammation in the score plot (Figure 3) falls on the same quadrant as cell proliferation in the loading plots (Figure 4) suggesting high cell proliferation in the negative control cells.

Variance (R²VX) is explained for the components analyzed and for all the principle components (Table 3). Q²VX showed the total variation of X which is predicted by the
respective component estimated through cross-validation. Upon performing PCA with cross
validation, Q2 and the limit are obtained (Table 3). The limit, which was calculated
depending on the number of components for PCA, showed the value of 0.502–0.505 for all
data. The limit setting increases the account for loss in degrees of freedom by subsequent
component. Q2V values are not equal to Q2 values for individual variables because the
eigenvalues are < 1.5. Q2V (cum) values were more than 0.5, indicating that the models
for the variables are good. All R2VX (Table 3) values are > 0.9, suggesting goodness of fit
since it is correlated to the multiple correlation coefficient.
N3 significance level (Table 3) means that the component is not significant because it
lacks degrees of freedom. Improvement can be made by addition of N numbers of data
and K numbers of variables such as interleukin-12, prostaglandin (PGE$_2$) and
phospholipase (PLA$_2$) which has their own roles in progression of inflammation. Huge
variances may come from cell viability data which comprised of triplicates measurement
from seven batches of cells. Differences of measurement exist between batches of
macrophage cells but they are more representative by triangulation of data.
PCA correlation quadrants depicted positive correlation between the secretion of TNF-α
and NO (Figure 4). These results are found to be parallel with the findings which had
identified them as mediators of inflammation [18]. In contrary, TNF-α and NO secretion
were negatively correlated with cell proliferation, in which they are parallel with previous
findings where NO was shown to inhibit T-cell proliferation [11] and TNF-α alpha showed in
vitro anti-proliferative effects in normal and transformed cells [16].
These results showed similar positive correlations between NO and other cytokines such
as TNF-α and PGE$_2$ [8, 9]. In the score plots (Figure 3), the mapping of controls and
biogenic amines and keropok lekor extracts were done in the axes spanned by the first
two principal components, namely PC1 and PC2. PC1 includes variables with the largest variation while PC2 includes variables with second largest variation and it is orthogonal to PC1.

PCA for *keropok lekor* extracts (K) and standard mixture mimicked biogenic amines content in *keropok lekor* (BK) was plotted on the score plot in the same quadrant as the negative control for inflammation (Neg). These results showed that K and BK possessed strong correlation with L-NAME as negative control (Neg) and did not exhibit inflammation-inducing properties in RAW 264.7 cell culture. The loading plot showed that NO and TNF-α were at the same side of y-axis exhibiting positive correlation while both NO and TNF-α showed negative correlations with cell viability increment. Although the toxicity of histamine, putrescine and cadaverine had been extensively studied, their combined effects remain unclear. From the PCA results, it was shown that the BA standard mixture (BK) and *keropok lekor* extract (K) did not induce pro-inflammatory mediator secretion which may be due to their total BA concentrations not exceeding 100 mg/kg food, which is the maximum permissible concentration of histamine in fish and fish products [1, 3].

Histamine, putrescine, cadaverine, 2-phenylethylamine, agmatine, *keropok lekor* extract and its biogenic amines mixture solution did not induce inflammation in RAW 264.7 macrophage *in vitro* model. This finding did not support previous report on histamine involvement in inflammation and usage of histamine, putrescine and cadaverine in Biogenic Amines Index (BAI = histamine + putrescine + cadaverine + tyramine) [6] and Quality Index [QI = (histamine + putrescine + cadaverine) / (1 + spermidine + spermine)] [17] for fish freshness. However, tyramine, tryptamine, spermine and spermidine induced inflammation in RAW 264.7 macrophages. This finding is parallel to *in vivo* toxicity study. *Keropok lekor* extract and its BA mixture solution did not induce inflammation in RAW 264.7 macrophages. NO and TNF-α showed positive correlations with each other while
both NO and TNF-α showed negative correlations with cell viability.

Food intolerance and food toxicity caused by ingesting biogenic amines may pose risk to bowel diseases in susceptible consumers. In various bowel diseases such as IBD, IBS and Crohn’s disease, mechanism is believed to be multifaceted including cytotoxicity, pro-inflammatory mediator secretion and free radical formation [2, 4]. Various chemokines and cytokines have been associated with numerous pathophysiological conditions which are related to the induction of inflammation [5].

In this study, in interpreting the chemokine and cytokine data, PCA was adapted, segregating those combinations of actions leading to inflammatory and non-inflammatory consequences by using multivariate projection methods. The data was analyzed by determining the clusters of chemokine and cytokine that discriminate the potential inflammatory properties upon exposure to biogenic amines and keropok lekor extracts.

Conclusions

This experiment shows that the individual BAs, and keropok lekor extracts have different effects on RAW 264.7 macrophages resulting in induction or non-induction of pro-inflammatory mediator secretion, i.e. either resulting in necrosis (cell non-viability and inflammation), apoptosis (cell non-viability but without inflammation), cell proliferation (cell viability and without inflammation) and suggesting the last option of cell repair (cell viability but with inflammation). PCA is thus suggested for screening of inflammation-causing food compounds and can be extended to other pro-inflammatory mediators such as interleukin-12 (IL-12) or phospholipase A₂ (PLA₂).

Methods

Materials

Chemicals
Histamine dihydrochloride, putrescine dihydrochloride, cadaverine dihydrochloride, 2-phenylethylamine hydrochloride, tyramine hydrochloride, tryptamine hydrochloride, spermine trihydrochloride, spermidine tetrahydrochloride and agmatine sulphate were of analytical grade (Sigma-Aldrich, St. Louis, MO, U.S. A.) HEPES, sodium bicarbonate and dimethyl sulfoxide (DMSO) were also purchased from Sigma-Aldrich (St. Louis, MO, U.S. A.).

**RAW 264.7 macrophage cell line**

The RAW 264.7 macrophage cell line was purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were grown in Dulbecco’s Modified Eagle Medium DMEM (high glucose) with 10% foetal bovine serum (FBS) and supplemented with 7.2 g/L HEPES, 4 mM glutamine, 3.0 g/l NaHCO₃, 100U/ml penicillin and 100 μg/ml streptomycin. Cell cultures were maintained in a humidified 5% CO₂ incubator at 37°C. Cells were harvested at 80% confluence and seeded at a density of 40,000 cells/well in 24-well microtitre plates and cells were grown to 80% confluence overnight (18–24 hr) before expose to different investigated biogenic amines or keropok lekor extracts.

**Growth medium**

DMEM (supplemented with 4500 g/L glucose, sodium pyruvate, glutamine and pyridoxal phosphate), penicillin/streptomycin and inactivated foetal bovine serum (FBS) were of cell culture grade (Gibco®, Life Technologies, NY, U.S. A.). Inactivation of FBS was made by incubating the thawed FBS at 56°C for 30 min.

**Keropok lekor samples**

**Sampling**

Approximately 1 kg of rod-shaped *keropok lekor* (RKL) and 1 kg of sliced *keropok lekor* (SKL) samples were purchased in duplicates from a small vendor in Sri Serdang town, in
the state of Selangor. Samples were purchased during three different visits (n = 6) to the vendor. Both types of *keropok lekor* were placed in a cool box filled with ice packs to keep the samples chilled during the transportation to our laboratory at the Nutrition Unit, Institute for Medical Research, Kuala Lumpur. Upon arrival at the laboratory, the samples were kept at -20°C prior to sample preparation for analysis.

**Sample aggregation**

After thawing, the *keropok lekor* samples were weighed. The utensils and laboratory apparatus used for sample preparation were swabbed with 70% ethanol. RKL was cut into small pieces with scissors into a plastic basin and approximately 300 to 400 g of sample was minced to coarse powder using the heavy-duty blender (Waring; U.S. A.). Mincing was repeated for all cut samples. The minced sample was made into a circle-shaped heap and it was then divided into four equal parts. Of these, only two parts positioned diagonally were taken and mixed homogenously. It was shaped into a circle and divided again into four equal parts. Following this, two parts of the samples positioned diagonally were taken and mixed homogenously. This final mixture was divided into two parts and kept in two different polystyrene plastic containers. These homogenous samples were then used for further analysis. The same procedures were repeated for the SKL.

**Preparation of keropok lekor extracts**

Two types of *keropok lekor*, namely RKL and SKL were sampled. The differences between them are RKL is moulded as long small rod with diameter of 2 cm and length of approximately 30 cm and sold in rods and SKL is moulded as long big rod but kept chilled and sliced into 1-2 mm thick slices upon hardening and sold as slices. Ten grams of minced RKL and minced SKL were weighed and put inside a sterile stomacher bag. Six hundred twenty-five microliters of 1000 ppm internal standard (IS) was pipetted into the sample in each stomacher bag. The concentration of the IS was estimated to be 0.5 ppm in
1 ml of the final keropok lekor sample: methanol (30:70) solution. Seventy millilitres of deionized water were added into the bag and homogenized using a stomacher pummel (Colworth 400 AJ Seward BA6021, U. K.) for 3 min at room temperature. The mixture was then transferred into two 50 ml centrifuge tubes and was centrifuged at 2000 × g for 20 min. The supernatant was then filtered through filter paper No. 541 (Whatman, GE Healthcare, U. K.) into a 100-ml volumetric flask and was filled to 100 ml with deionized water. The solution was mixed well and aliquoted for quantification of BA using Liquid Chromatography-Mass Spectrometry (LC-MS) and kept at –20°C prior to exposure study on RAW 264.7 macrophages.

Methods

Quantification of biogenic amines (BA) using Liquid Chromatography-Mass Spectrometry (LC-MS)

Standard preparation

Each BA external standards (ESTDs) were diluted in 10 ml deionized water, producing final solution concentration of 1000 ppm (1000 mg/L). The ESTDs were further diluted to final concentrations of 0.125, 0.25, 0.5, 0.75 and 1.0 ppm and was added with 1, 7-diaminoheptane as internal standard (ISTD) at final concentration of 0.5 ppm in 70% LC-grade methanol. The standard solutions were syringe-filtered through 0.2 µm GHP filter (Pall Corporation, U.S. A.) and 10 µl of each standard was then injected into LC-MS for analysis.

Sample extraction

From the frozen keropok lekor extract prepared, extracts were thawed and four millilitres of the solution was pipetted into a 10,000 molecular weight cut-off (MWCO) centrifugal filtration tube and was later centrifuged at 2000 × g for 15 min. Eighty microliters of the filtrate solution (contained molecules of <10kDa) was pipetted into a microfuge tube and
was added with 220 µl deionized water and 700 µl methanol (liquid chromatography grade). After syringe filtration through 0.2 µm membrane, the solution was injected into the LC-MS for analysis.

**Liquid Chromatography-Mass Spectrometry (LC-MS)**

Liquid chromatography was done using Ultra High-Performance Liquid Chromatography (UHPLC) (Waters, Milford, U.S. A.) through XSELECT HSS-PFP, 3.0 x 100mm (2.5 µm particle) column. A gradient of acetonitrile: deionized water [with 0.1% trifluoroacetic acid (TFA)] was used as stated below (Table 1).

The temperature of column and sample compartment was set at 40°C and 4°C, respectively. The separated compounds were then subjected to the Q-Exactive Quadrapole Mass Spectrometry (Thermo Fischer Scientific, U.S. A.) for molecular mass determination.

**Effects of biogenic amines (BA) individually, and keropok lekor extract on cell viability and pro-inflammatory mediator secretion**

**Preparation of individual BA solutions**

Stock of individual BA solutions were prepared at 10mg/10ml in Milli-Q water. The individual BA and UCA working solutions were then prepared using Milli-Q water at two times more concentrated than the final volume needed for exposure study. DMEM (2×) with 20% FBS was added to the individual BA working solutions in equal volume. The final working solution was obtained with concentration of 1× DMEM with 10% FBS.

**Preparation of BA standard mixture**

The standard mixture mimicking the biogenic amines contents in *keropok lekor* extracts were prepared according to the results obtained using LC-MS (Table 2).

**Exposure study for determination of cell viability and pro-inflammatory mediator secretion**
The cells were treated with test compounds (BA individual solutions, *keropok lekor* extracts, and BA standard mixture solutions mimicking the BA contents in each *keropok lekor* extract (Table 2) at 37°C with humidified 5% carbon dioxide overnight. Aliquots of 150 µl supernatant were collected for determination of nitric oxide and TNF-α secretion. Cell viability test was done on the cells in the remaining media consecutively using MTS assay.

Aliquots of 20 µl of CellTiter 96® AQeuousOne Solution Reagent were added into each well and incubated for 20 min at 37°C. Absorbance was measured at 492 nm using a microplate reader. Cell viability was determined as percentage of viable cells.

**Nitric oxide (NO) assay (Greiss Reaction)**

NO formation was determined by measuring the production of nitrite (NO₂) using the Griess Reagent System (Promega, Madison, USA). Fifty microliters of the culture medium were incubated with 50µl of Sulfanilamide Solution (1% sulfanilamide in 5% phosphoric acid) and 50µl NED Solution [0.1% N-(1-naphthyl) ethylene diamine dihydrochloride], respectively, at room temperature for 10 minutes for each solution. The absorbance was measured at 540nm using a microplate reader. The nitrite content was determined from a standard reference curve obtained using serial dilutions of 0.1M sodium nitrite standard and it is correlated with nitric oxide content.

**Tumor Necrosis Factor-Alpha (TNF-α) assay**

TNF-α secretion in the supernatant was determined using Mouse TNF-α Instant ELISA kit (Bender MedSystems GmbH, Austria). Fifty microliters of each standards, controls and samples were pipetted into the respective microplate wells in duplicates. The microplate was covered with an adhesive film and incubated at room temperature (RT) (18°C to 25°C) for 3 hr on a microplate shaker at 200 rpm. The incubated microplate was then emptied
and washed six times with Wash Buffer using a microplate washer. Microplate was tapped on paper towels to remove excess Wash Buffer. One hundred microliters of TMB Substrate Solution were pipetted to all wells and incubated at RT for about 10 min. Stop Solution was added when the highest standard had developed a dark blue colour which could be confirmed by the ELISA reader at 620 nm when absorbance of this standard well (Standard 1) reaches optical density of 0.9—0.95.

**Statistical analysis**

All statistical analyses in this study were calculated using Statistical Package for the Social Sciences (SPSS) version 21.0 software (SPSS Inc., Chicago, Il. U.S. A.), GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, U.S. A.; [www.graphpad.com](http://www.graphpad.com)) and SIMCA software (Umetrics, Umea, Sweden). The comparison of means for normal distributed data was determined using Bonferroni’s one-way Analysis of Variance (ANOVA) and for non-normal distributed data, comparison of medians was determined using Kruskal-Wallis with post-hoc Dunn’s Multiple Comparison Test. p<0.05 was accepted as statistically significant.

The analytical data of different assays of cell proliferation, NO and TNF-α from incubations of individual BA, *keropok lekor* extract (K) containing highest BA content, and BA standard mixture solution mimicking the BA content in the selected *keropok lekor* extract (BK) were subjected to principal component analysis (PCA) using Score plots display similarities and dissimilarities between samples while loading plots display the correlation between variables. Mean of data was used to build PCA model and validation was done using cross-validation.

PCA was conducted on the dependent variables (Y), namely: cell proliferation, nitric oxide (NO) and tumor necrosis factor-α (TNF-α) secretion, to discriminate between different properties of compounds/extracts according to various quadrants. Prior to PCA, Partial
Least Squares (PLS), PLS-Discriminant Analysis (PLS-DA) and Orthogonal Partial Least Squares (OPLS) were also carried out to find the best analysis for presentation of data.

List Of Abbreviations

AGM
Agmatine
ANOVA
Analysis of Variance
BA
Biogenic amines
CAD
Cadaverine
CO₂
Carbon dioxide
DMEM
Dulbecco’s Modified Eagle Medium
ELISA
Enzyme-linked immunosorbent assay
ESTD
External standard
FBS
Foetal Bovine Serum
H₂O
Water
HEPES
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

HIM
Histamine

HPLC
High Performance Liquid Chromatography

hr
Hour

IFN-γ
Interferon-gamma

IL-12
Interleukin-12

KLD
Keropok lekor dough

LC
Liquid chromatography

LC-MS
Liquid chromatography-mass spectrometry

L-NAME
L-Nitro-Arginine Methyl Ester

min
Minute

MWCO
Molecular weight cut-off

NaCl
Sodium chloride
NaHCO₃
Sodium bicarbonate

NaOH
Sodium hydroxide

NO
Nitric oxide

PC
Principal component

PCA*
Principal Component Analysis

PGE₂
Prostaglandin E₂

PHM
2-phenylethylamine

PLA₂
Phospholipase A₂

PUT
Putrescine

RKL
Rod-shaped keropok lekor

RT
Room temperature

s
Second
SKL
Sliced *keropok lekor*

SPD
Spermidine

SPM
Spermine

TNF-α
Tumor necrosis factor-alpha

TPM
Tryptamine

TYM
Tyramine

UHPLC
Ultra High Performance Liquid Chromatography

Declarations

Ethics approval and consent to participate: There are no participation from human respondents.

Consent for publication: Consent was obtained from the National Institutes of Health Malaysia on 12 July 2019 [Ref. no.: KKM.NIHSEC.800-5/3/1 Jld 60(06)].

Availability of data and material: Related study data & materials are available to be submitted to the publisher upon request.

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Tables

Table 1. Gradient of mobile phase used for the Liquid Chromatography-Mass Spectrometry (LC-MS) for quantification of biogenic amines

| Time (min) | Flow rate (ml/min) | Deionized water with 0.1% TFA (%) | Acetonitrile with 0.1% TFA (%) |
|-----------|--------------------|----------------------------------|------------------------------|
| 0.0       | 0.2                | 98                               | 2                            |
| 2.0       | 0.2                | 98                               | 2                            |
| 10.0      | 0.2                | 0                                | 100                          |
| 11.0      | 0.2                | 0                                | 100                          |
| 12.1      | 0.2                | 98                               | 2                            |
| 20        | 0.2                | 98                               | 2                            |

Table 2. Concentrations of biogenic amines [histamine (HIM), putrescine (PUT), cadaverine (CAD), 2-phenylethylamine (PHM), tyramine (TYM), tryptamine (TPM), spermine (SPM), spermidine (SPD) and agmatine (AGM)] of rod-shaped keropok lekor (RKL) and sliced keropok lekor (SKL) obtained using Liquid Chromatography-Mass Spectrometry (LC-MS)
Table 3. Summary of fit for all data, 1 µg/ml single biogenic amines, 10 µg/ml single biogenic amines and 100 µg/ml single biogenic amines

| Type | Visit | Code | HIM | PUT | CAD | PHM | TYM | TPM | SPM | SPD | AGM |
|------|-------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| RKL  | 1     | 1KB11 | 31  | 10  | 17  | 0   | 16  | 0   | 0   | 1   | 4   |
|      | 1     | 1KB21 | 14  | 9   | 16  | 0   | 10  | 0   | 0   | 0   | 1   |
|      | 2     | 2KB11 | 3   | 9   | 22  | 0   | 5   | 0   | 0   | 1   | 3   |
|      | 2     | 2KB21 | 3   | 10  | 12  | 0   | 12  | 0   | 0   | 0   | 2   |
|      | 3     | 3KB11 | 9   | 10  | 12  | 0   | 6   | 0   | 0   | 2   | 2   |
|      | 3     | 3KB21 | 12  | 10  | 23  | 0   | 6   | 0   | 0   | 1   | 2   |
| SKL  | 1     | 1KN11 | 1   | 6   | 25  | 0   | 4   | 0   | 0   | 0   | 3   |
|      | 1     | 1KN21 | 14  | 8   | 11  | 0   | 6   | 0   | 0   | 0   | 2   |
|      | 2     | 2KN11 | 4   | 6   | 25  | 0   | 0   | 0   | 0   | 0   | 4   |
|      | 2     | 2KN21 | 3   | 5   | 10  | 0   | 5   | 0   | 0   | 0   | 1   |
|      | 3     | 3KN11 | 1   | 4   | 8   | 0   | 2   | 0   | 0   | 0   | 0   |
|      | 3     | 3KN21 | 1   | 5   | 23  | 0   | 2   | 0   | 0   | 0   | 0   |

Table 3. Summary of fit for all data, 1 µg/ml single biogenic amines, 10 µg/ml single biogenic amines and 100 µg/ml single biogenic amines
Figures

Figure 1
Experimental flow chart of compounds’ exposure study to RAW 264.7 macrophages.

Figure 2
Suggested derivation of summary from Principal Component Analysis (PCA) score plot from the study.

Figure 3
Score plot for control cells without treatment (C), positive control for inflammation (Pos), negative control (Neg), keropok lekor extract (K), standard mixture solution mimicked biogenic amines content in keropok lekor (BK) and (A) 1 µg/ml or (B) 10 µg/ml or (C) 100 µg/ml individual biogenic amines, respectively.

Figure 4
Loading plot for (A) 1 µg/ml single biogenic amines, (B) 10 µg/ml single biogenic amines and (C) 100 µg/ml single biogenic amines.