# Experimental Design Application for Measuring Histamine in Tuna fish Samples by Phenyl isothiocyanate Derivation method using Ultra-performance liquid chromatography

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# Abstract

Histamine is an important biogenic amino acid was measured in tuna fish samples by ultra- performance liquid chromatography using a Phenyl isothiocyanate derivative. Minitab software was used to design the experiment and investigate the effective factors during the process, which includes screening and optimization steps. A partial factorial design was used in the screening stage and a central composite design was used in the optimization. Effective parameters in Histamine derivation were examined in the screening step including triethylamine volume, phenyl isothiocyanate volume, reaction temperature, reaction time and mobile phase pH. Then, in the optimization, effective parameters were identified and finally, the calibration curve was drawn from a concentration of 0.5–10.0 µg.ml-1 for Histamine and a correlation coefficient of 0.994 was obtained for Histamine. The method detection limit was 0.36 µg.ml-1 and the LOQ was 1.19 µg.ml-1. The relative standard deviation of the method was obtained for concentrations of 1.0–100.0 µg.ml-1 in the range between 1.06 to 2.21%. The recovery method was obtained from 90.8 to 103.1% for measuring Histamine in real fish samples.


# 1. Introduction

Meat groups, including fish, are an important part of our daily diet, providing a large portion of essential amino acids, vitamins and minerals, which are essential for growth and health (Omidi et al. 2021). New trends in the food industry are to produce healthy products and improve food quality. As a result, food analysis is receiving more attention in search of trace compounds that can affect human health (García-Villar, Saurina, and Hernández-Cassou 2006).

Biogenic amines are low molecular weight compounds (Ekici and Omer 2018) and biologically active compounds(Ramos, Brandão, and Rodrigues 2020) that are mainly formed by the decarboxylation metabolism of amino acids (Önal 2007). Most of the amino acids in fish meat are threonine, histidine, arginine, asparagine, phenylalanine, cysteine, glutamine and glycine. The presence of these amino acids in the meat of this species of fish has increased their acceptance of consumption(Type 2018). Measurement of biogenic amines in fish and fish products is toxicologically important because biogenic amines are often associated with food quality and are indicated as a sign of lack of freshness or poor storage conditions or fermented products. Although many biogenic amines are found in fish or fish products, only Histamine is legal for human consumption (Herrero et al. 2016). The amount of biogenic amines is also considered as an indicator of the microbiological contamination of food, so it is important to control the level of biogenic amines in food and also biogenic amines are produced in foods that have high levels of protein (Vinci and Antonelli 2002).

Histamine is one of the most important biogenic amino acids and (Cicero et al. 2020) in food analysis and is a known cause of food poisoning such as allergies (Ito et al. 2009) and the ability to produce headaches, hypotension, digestive problems (Ordóñez et al. 2016) Erythema and urticaria of the skin, sweating, facial flushing and hot flushes of the body, palpitations, nausea, vomiting(Sahebnasagh, Karimi, and Mohajeri 2014). Histamine, which is produced from a carboxylation of the amino acid

histidine (which is abundant in fish) (Norian, R.; Mahmoudi 2013), is a hydrophilic molecule (Fig. 1) composed of an imidazole ring and an amino group linked by two methyl groups (Elia et al. 2017).

Histamine poisoning is widespread worldwide and has been more prevalent in countries such as Japan, the United States and the United Kingdom than in other countries (Chaidoutis et al. 2019). Histamine produced in fish is resistant to heat and has no effect on reducing it in the process of canning, cooking and freezing (Mohtadinia, J.; Zakerzadeh, M.; Goudarzi, M.; Rahmanpour Arjmand, H.; Khadem Haghighian, H. 2014). It is very important to measure biogenic amines. There are several methods for measuring Histamine levels in fish. Such a technique must be selectively, highly sensitive, and highly efficient, and must involve the process of optimizing sample preparation conditions and chromatographic parameters (Salazar and Castro 2017).

Derivatization is essential for these kinds of detection techniques because most BAs lack chromophores and fluorescent groups(Ishimaru et al. 2019). Due to the fact that the most used method for determining biogenic amines is high power liquid chromatography and this method requires a derivation process, different methods of high-performance liquid chromatography have been proposed. For example, researchers use high-power liquid chromatography based on the extraction of Trichloroacetic or Perchloric acid and the derivation of biogenic amines before or after their separation in a chromatographic column(Salazar and Castro 2017). As shown in Fig. 2 (Kim et al. 2019), the amine in Histamine group and a PITC phenyl group react readily to give a stable product (PTC-HA), Since the several factors affect the extraction methods, the use of experimental design allows us to systematically achieve higher efficiency(Sharif et al. 2014). Experimental design and condition optimization is the most important application of chemometrics in analytical chemistry.

Therefore, the design of the experiment helps us to determine the best model of the relationship between the variables as well as the optimal conditions of the experiment. When the number of factors is large, a step-by-step approach is not appropriate and cannot show the interactions between the factors. When different factors at different levels are involved in this experiment, it is important to create an effective and efficient test design to achieve the desired results. Using experimental design to evaluate the factors influencing the extraction process reduces the number of experiments required to optimize the process, thus minimizing time, cost, and environmental pollution. Therefore, experimental design is used for screening and optimization of process parameters(Mousavi, Tamiji, and Khoshayand 2018).

In recent studies different derivation methods have been reported to measure Histamine, such as before and after column derivatives, followed by measurement of Histamine derivative using high performance liquid chromatography. Derivatives before and after columns typically with reagents such as Phenylisothiocyanate (PITC), Orthophthaldehyde and Densyl chloride are used(Hogan et al. 2012; Miyamoto et al. 2004; Wang et al. 2013). PITC reacts easily with primary and secondary amines at alkaline medium. PITC is volatile and allows excess reagent to be removed in a vacuum, thus minimizing the possibility of reagent interference.

In this study, a phenyl isothiocyanate derivative was used for derivation. PITC derivative and experimental design were used to achieve the optimal point. In the screening stage, in order to select the effective parameters, a partial factorial design with two replications was used and the relevant diagrams were examined. In the optimization stage, a central composite design was used for the optimal point of the process and the effect of different parameters on Histamine derivation was investigated separately.

## 2. Materials And Methods

# 2.1. Materials

For chromatographic analysis, from high purity water (HPLC grade solvents, Merck,> 99%), acetonitrile (ACN grade (HPLC gradient grade, Merck,> 98%)), methanol (Sigma Aldrich,> 98%), acetate buffer (0.1 M, prepared with ammonium acetate (Carlo,> 98%) and acetic acid (Merck,> 96%) were used. For the extraction process, 0.1 N perchloric acid solution prepared from (HClO4) (Chem-lab, 70%) and n-Hexan (Sigma Aldrich, 95%) was used. For the derivatization process, Triethylamine (Merc,> 99%), Phenyl isothiocyanate (Merc, 98%) and instrumental grade Ethanol (Merc,> 99%) were used. To prepare the Histamine standard 1000 mg / l, 169 mg of the Histamine dihydrochloride standard (Sigma Aldrich,> 99%) was weighed and dissolved in 100 ml of 0.1 M Hydrochloric acid. The Acetate buffer solution was dissolved to 250 ml by weighing 0.96 g of Ammonium acetate salt and dissolving it in HPLC Water and adding 150 µl of Triethylamine to the solution. Then the pH of the solution was adjusted between 5.8 and 6.8 with acetic acid.

# 2.2. instrumentation

Isolation and measurement of samples was performed using an Aquity model UPLC Waters equipped with a multi-wavelength ultraviolet detector. An Empower software was also used to process the chromatographic data. The column used was type C18 (100 2 2.1 with a particle size of 1.7 µm). Acetate buffer and a mixture of water, methanol and acetonitrile (60:20:20) were used to prepare the mobile phase and a flow rate of 0.3 ml / min was selected. The pH of the mobile phase was adjusted and measured using a WTW pH meter equipped with a glass-calomel composite electrode (0.3 M potassium chloride). Minitab software version 19 was used to design the experiment in order to determine the effective factors and obtain the optimal point.

# 2.3. Sample preparation

The tuna fish sample was ground and weighed 5.0 grams. Then, 10 ml of 0.1 mol. $L^{1-}$ perchloric acid was added and sample was shaken slightly for 10 minutes using a shaker. After that 5 ml of n-Hexane was added to the sample and stirred again for 5 minutes. The sample was then centrifuged at 4500 rpm for 10 minutes. The sample was separated after centrifugation in three phases included the upper phase of N-hexane, the middle phase of acid and the lower phase of fish tissue. The acidic phase (middle phase) was filtered using a 0.22 micron syringe filter and 100 µl of it was transferred to a 2 ml vial, and dried with nitrogen gas. Then, 28 µl of solution including triethylamine, water and ethanol (2: 2: 1) was

added and dried. 35 µl of diluted phenyl isothiocyanate (with a ratio of phenyl isothiocyanate, triethylamine, water and ethanol 7: 1: 1: 1) was added and allowed to react for 27 min at 0 ° C. This procedure was continued at 50 ° C and then the samples dried with nitrogen gas. In the next step, the sample was diluted with 500 µl of ammonium acetate buffer and injected into the UPLC machine.

## 2.4. Mobile phase preparation

Ammonium acetate buffer (with a concentration of 0.05 mol/l) and organic solvents were used to prepare the mobile phase according to the agenda. For better separation of the analyte from the matrix, the organic mobile phase was optimized for water, methanol and acetonitrile (60:20:20). First, the mobile phase was placed in an ultrasonic bath for homogenization and degassing, and then transferred to the solvent bottles of the device. The experiments were performed at a mobile phase flow rate of 0.3 ml/min. Before injecting the sample into the column, the mobile phase was allowed to pass through the column for about 30 minutes to 45 minutes at a flow rate of 0.3 ml per minute until the column reached equilibrium (the baseline was smoothed).

## 2.5. Optimization of the conditions

In this present work, in order to provide the best sensitivity and therefore the best detection limit, the parameters affecting the measurement of Histamine were investigated. In the study of effective factors, a partial factorial design was used to identify important factors in extraction. After identifying the important factors, the central composite design was used for optimization.

## 2.6. Experimental design in partial factorial design

For screening, a partial factorial design method (at the level of 1/2) with 2 replications and as a result of 32 experiments, the most effective parameters in the Histamine derivative reaction were selected. All the factors under consideration, their levels and ranges are listed in Table 1.

Table 1
Examined factors and related levels

| Variable | Component | Low level (-) | High level (+) |
|---|---|---|---|
| $X_1$ | Volume of TEA (µL) | 10.0 | 30.0 |
| $X_2$ | Volume of PITC (µL) | 10.0 | 30.0 |
| $X_3$ | Derivation temperature (°C) | 25.0 | 50.0 |
| $X_4$ | Derivation time (min) | 7.0 | 27.0 |
| $X_5$ | pH of acetate buffer | 5.8 | 6.8 |

In this section, the factors that are expected to influence the testing process are examined and are given in Table 1. A screening table design was designed using the mini-tab software version 19. As mentioned earlier, by using partial factorial design, the number of experiments will be 32 when 5 factors are

examined with 2 replications. All experiments were performed below the levels specified in the partial factorial design.

## 2.7. Optimization of effective factors in measuring Histamine

Based on the results of the screening step, the factors of volume of triethylamine, volume of phenylisothiocyanate and pH of acetate buffer were selected as effective factors in measuring Histamine. Then, the central composite design of the environment was used to obtain the optimal points of these effective factors. In the central composite scheme, each factor is examined at five levels. Tables 2, 3 and 4 lists the effective factors and the amount of their levels. Minitab software was used to design the experiments and to study the interactions between the important factors as well as the optimal amount of these factors to create the central composite design experiments. The value of $\alpha = 2$ was also considered.

Table 2
Level of effective factors in central composite design

| Variable | Factor | Star-low | Low | Center | High | Star-high |
|---|---|---|---|---|---|---|
| X1 | Volume of TEA (µL) | 0.0 | 10.0 | 20.0 | 30.0 | 40.0 |
| X2 | Volume of PITC (µL) | 0.0 | 10.0 | 20.0 | 30.0 | 40.0 |
| X5 | pH of acetate buffer | 5.5 | 5.8 | 6.3 | 6.8 | 7.1 |

Table 3
Experimental design and result of two replicates fractional factorial design.

| Run | X1 | X2 | X3 | X4 | X5 | Rep.1 | Rep.2 |
|---|---|---|---|---|---|---|---|
| 1 | - | - | - | - | + | 1516587 | 1535295 |
| 2 | + | - | - | - | - | 1278189 | 1326803 |
| 3 | - | + | - | - | - | 1262651 | 1257260 |
| 4 | + | + | - | - | + | 1648938 | 1653018 |
| 5 | - | - | + | - | - | 1247589 | 1253090 |
| 6 | + | - | + | - | + | 1493366 | 1434994 |
| 7 | - | + | + | - | + | 1766258 | 1511406 |
| 8 | + | + | + | - | - | 1485589 | 1442371 |
| 9 | - | - | - | + | - | 1237996 | 1241386 |
| 10 | + | - | - | + | + | 1293711 | 1585721 |
| 11 | - | + | - | + | + | 1629924 | 1579818 |
| 12 | + | + | - | + | - | 1319766 | 1367022 |
| 13 | - | - | + | + | + | 1549014 | 1559712 |
| 14 | + | - | + | + |  | 1365839 | 1522397 |
| 15 | - | + | + | + | - | 1273253 | 1150053 |
| 16 | + | + | + | + | + | 1806759 | 1817546 |

Table 4
Coefficients obtained from partial factorial design

| Factors | Effect | Coefficient | T value | P value |
|---|---|---|---|---|
| Volume of TEA (µL) | 79421 | 39711 | 2.82 | 0.012 |
| Volume of PITC (µL) | 95621 | 47811 | 3.40 | 0.004 |
| Derivation temperature (□C) | 59072 | 29536 | 2.10 | 0.052 |
| Derivation time (min) | 11657 | 5829 | 0.41 | 0.684 |
| pH of acetate buffer | 271926 | 135963 | 9.66 | 0.000 |

# 3. Results And Discussion

In order to determine the most effective factors in measuring Histamine, the design of a partial factorial experiment of 5 factors with two replications using Minitab software was used. Table 3 shows the factorial design matrix and the sub-peak chromatogram values (responses) for Histamine.

P value: This part was used to check the accuracy of the model. To determine the effectiveness of the factors with 95% confidence level, P≤0.05 was considered as the limit point. As a result, factors with a value less than 0.05, factors affecting the process and factors with a value greater than 0.05 are considered as ineffective factors. According to the value of P, the factors affecting the response are of triethylamine, phenyl isothiocyanate and pH of acetate buffer.

Figure 3 is the residual diagrams used to evaluate the adequacy of the model. In Fig. 3, using the Normal probability diagram, the normality of the data can be detected. In this method, by specifying the data on the graph and passing the best line through the data, the normalcy of the data can be realized.

Figure 3 shows the Versus fits diagram for constant data variance. Residual changes should not have a specific structure and be scattered. Figure 3 Histogram If the remaining changes are gaussian, the data can be said to have a normal population and according to the figure, we can say that the data are normal.

The Residual observation order diagram in Fig. 3 examines the independence of the data with respect to time and the independence of the data with respect to the mean and variance. Also, this diagram should not show a specific structure. In this experiment, the horizontal axis shows the order of 32 experiments that were performed randomly.

Figure 4 shows the diagrams of the interaction between the factors. When the lines between the invoices are parallel, there is no interaction between the invoices. If the lines related to the factors are non-parallel, the greater the angle between the lines, the greater the intensity of the interaction. The lowest interaction between factors is between TEA-pH, PITC-pH, Time- pH and Temperature - pH and the highest interaction is between PITC- pH.

In Fig. 5, the Main Effects diagrams of the vertical axis are the average response, and the larger the angle between the factor lines and the horizontal line of the response axis, the more effective that factor is. According to the figure, we conclude that when the factors are at their highest level, we will have the highest response.

# 3.1. Optimization of factors affecting Histamine measurement

Based on the results of the screening stage, important and effective factors on Histamine measurement were selected and the optimal values of these important factors were determined by central composite design. In all experiments performed on the central composite design, ineffective factors were considered constant. The values of these factors were selected according to their coefficients shown in Table 4. Thus, the variables that had positive coefficients used its upper level and the variables that had negative

coefficients used its low level in the optimization stage. Table 5 shows the central composite design matrix and the sub-peak chromatogram values for Histamine.

The experimental results of the designed experiments were analyzed by ANOVA method. In order to obtain an experimental model for predicting the response, the results of the analysis are shown in Table 6.

Using Table 6, the simplest model for measuring Histamine is suggested as follows:

Response = 2453899 + 29056 X1 + 64170 X2 + 154626 X3–31463 X12–75842 X32

$R^2$ = 96.42% $R^2_{adj}$ = 92.84%

Table 5
Central composite design matrix and results obtained

| Run | $X_1$ | $X_2$ | $X_5$ | Rep. |
|---|---|---|---|---|
| 18 | - | - | - | 2075259 |
| 19 | + | - | - | 2190332 |
| 20 | - | + | - | 2200121 |
| 21 | + | + | - | 2283971 |
| 22 | - | - | + | 2365552 |
| 23 | + | - | + | 2399868 |
| 24 | - | + | + | 2465012 |
| 25 | + | + | + | 2636967 |
| 26 | α- | 0 | 0 | 2302695 |
| 27 | +α | 0 | 0 | 2332546 |
| 28 | 0 | α- | 0 | Not Detection |
| 29 | 0 | +α | 0 | 2441860 |
| 30 | 0 | 0 | α- | 1801031 |
| 31 | 0 | 0 | +α | 2479179 |
| 32 | 0 | 0 | 0 | 2495904 |
| 33 | 0 | 0 | 0 | 2469746 |
| 34 | 0 | 0 | 0 | 2458286 |
| 35 | 0 | 0 | 0 | 2398801 |
| 36 | 0 | 0 | 0 | 2429029 |
| 37 | 0 | 0 | 0 | 2450778 |

A time-lapse model shows that it fails to adequately represent the relationship between factors and response variables. To determine if the model fits the data correctly, we consider the p-value. If its value is more than 0.05, it can be said that the model fits the data well. The amount of lack of fit in this study was 0.092, which is more than 0.05. As a result, it can be said that the model fits the data correctly.

Table 6
Statistical parameters for central composite design performed for Histamine measurement response

| Source of variation | DF | F-Value | P-Value | (F-Value/Total F-Value) |
|---|---|---|---|---|
| Model | 9 | 26.94 | 0.000 | 0.08 |
| Linear | 3 | 58.41 | 0.000 | 0.16 |
| TEA | 1 | 5.47 | 0.044 | 0.02 |
| PITC | 1 | 14.93 | 0.004 | 0.04 |
| pH | 1 | 154.83 | 0.000 | 0.43 |
| Square | 3 | 20.75 | 0.000 | 0.06 |
| TEA*TEA | 1 | 9.97 | 0.012 | 0.03 |
| PITC*PITC | 1 | 4.50 | 0.063 | 0.01 |
| pH*pH | 1 | 57.94 | 0.000 | 0.16 |
| 2-Way Interaction | 3 | 0.43 | 0.739 | 0.00 |
| TEA*PITC | 1 | 0.57 | 0.468 | 0.00 |
| TEA*pH | 1 | 0.00 | 0.959 | 0.00 |
| PITC*pH | 1 | 0.71 | 0.423 | 0.00 |
| Error | 9 | | | 0.00 |
| Lack-of-Fit | 4 | 3.69 | 0.092 | 0.01 |
| Pure Error | 5 | | | |
| Total | 18 | | | |

## Two-dimensional and three-dimensional diagrams resulting from central composite design

Using two-dimensional and three-dimensional diagrams, the relationship between response and factors can be shown. In the 3D diagrams in Fig. 6, one factor is considered at a fixed midpoint and the effect of the other two factors is investigated.

In addition, by drawing two-dimensional diagrams or contours, simultaneous examination of the factors can be done. According to the guide next to the chart, the answers change as the colors change. In fact, contour charts are the top view of 3D charts.

According to the guide provided in Fig. 7, as the green color is highlighted, the response will increased.. According to the diagram shown, by keeping TEA constant in its medium value, we increase it by increasing the pH and PITC to a high level. By keeping the pH constant at its medium value, we increase it

by increasing the TEA and PITC to a high level. We also have an increase in PITC and TEA at a high level by keeping the pH constant at its medium value.

## 3.2. Achieving the optimal end point

After modeling and checking the accuracy and adequacy of the model and reviewing the relevant diagrams, optimization was performed to obtain the maximum response. According to the Optimization Plot diagram (Fig. 8) optimal points was determined. According to the obtained optimum point, the optimized values of triethylamine, phenyl isothiocyanate and buffer pH were 28 µl, 35 µl and 6.9, respectively.

Furthermore, Fig. 9 showed the chromatogram of the control sample and the chromatogram of the Histamine sample. For further evaluation of the method developed, Fig. 9 shows the black chromatogram corresponds to the blank sample and the blue chromatogram corresponds to the standard 1.0 mg. $L^{-1}$.

## 3.3. Method validation

The optimized analytical method was validated in terms of recovery, Standard deviation of reproducibility, Relative Standard deviation of reproducibility, Deviation from the standard of Reproducibility and Relative standard deviation of Reproducibility and recorded in Table 7. Spike solutions were prepared and studied at concentrations of 1.0, 2.5, 5.0, 10.0, 50.0 and 100.0 mg/L. Also, by drawing the calibration curve, the obtained coefficient of determination was 0.9994. LOD method was obtained for histamine 1.19 $\mu g.ml^{-1}$ and LOQ was 0.36 $\mu g.ml^{-1}$.

Table 7
Results of the method study for Histamine

| Spike level ($\mu g.ml^{-1}$) | Mean of recovery | Standard deviation of reproducibility | Relative standard deviation of reproducibility | Deviation from the standard of Reproducibility | Relative standard deviation of Reproducibility |
|---|---|---|---|---|---|
| 1.00 | 90.88 | 0.02 | 1.99 | 0.01 | 1.41 |
| 2.50 | 103.80 | 0.05 | 2.06 | 0.04 | 1.69 |
| 5.00 | 95.80 | 0.09 | 1.80 | 0.06 | 1.22 |
| 10.00 | 96.06 | 0.21 | 2.21 | 0.23 | 2.44 |
| 50.00 | 101.23 | 0.90 | 1.78 | 0.38 | 0.74 |
| 100.00 | 97.47 | 0.03 | 1.06 | 0.29 | 0.30 |

## 4. Conclusion

The proposed method described by UHPLC is a sensitive, accurate and robust method for determining Histamine as a one of the most important biogenic amines that causes Histamine poisoning. This method involves a simple derivative and requires only a small amount of extract. It enables to determine the optimal conditions for separation and increase the length of the column by using the low temperature of the column and the lower pH of the mobile phase. To investigate the effect of five factors using the general isolation factor as a response for analysis, a fractional factorial was used and a quadratic model as a function of important factors was fitted to the response using a central composite design. In the experimental design step, first screening was used to examine the effective parameters and then these parameters were used in the optimization stage to find the optimal point. Also, a phenyl isothiocyanate derivative was used to measure Histamine. According to the obtained optimum point, the optimized values of triethylamine, phenyl isothiocyanate and buffer pH were 28 µl, 35 µl and 6.9, respectively. Also, the detection limit of this method is 0.36 and the LOQ was 1.19 $\mu g.ml^{-1}$. Proposed method was successfully used for determination of Histamine in tuna fish samples.

# Declarations

### Conflict of Interest Statement

All authors declare there is no financial/commercial conflicts of interest.


### Funding declaration

No funds, grants, or other support was received.


### Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Author contributions

All authors contributed to the study conception and design. Data collection and analysis were performed by Maede Ameri. The first draft of the manuscript was written by Maede Ameri and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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# Figures

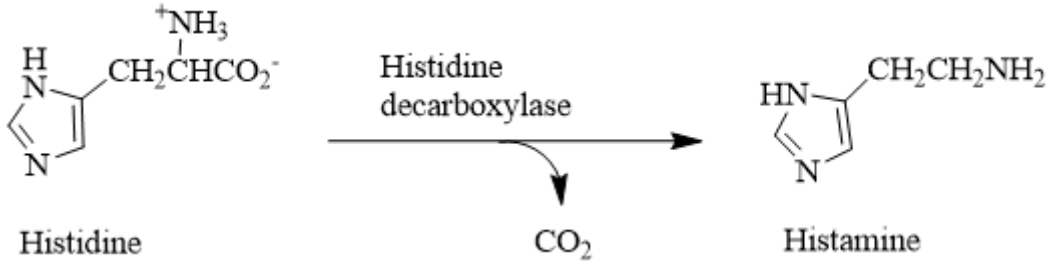



**Figure 1**

Histidine metabolism and Histamine toxin formation during the bacterial decarboxylation process

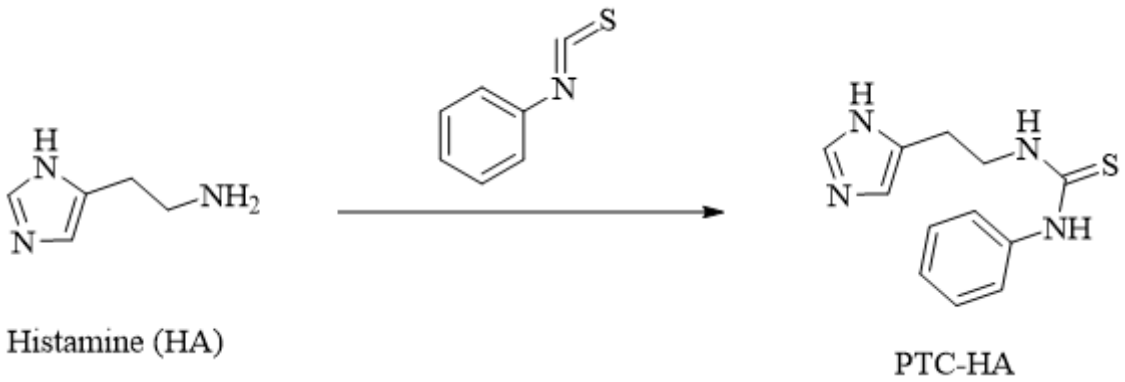
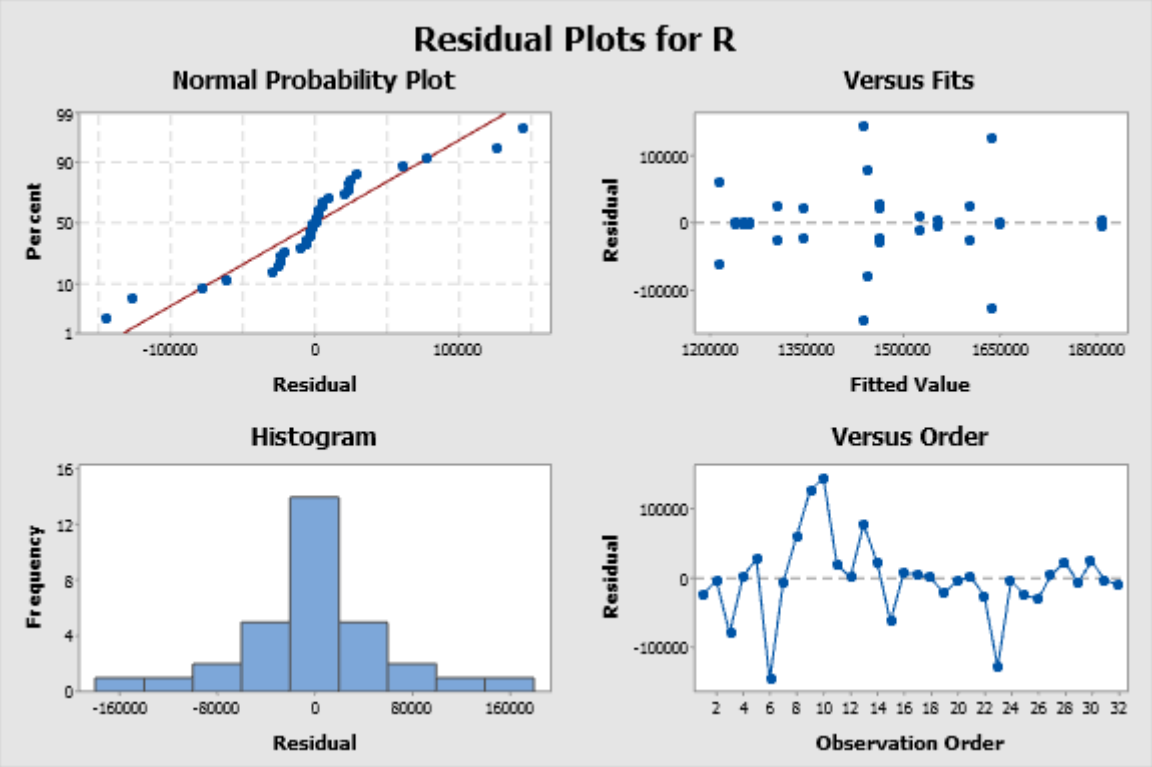


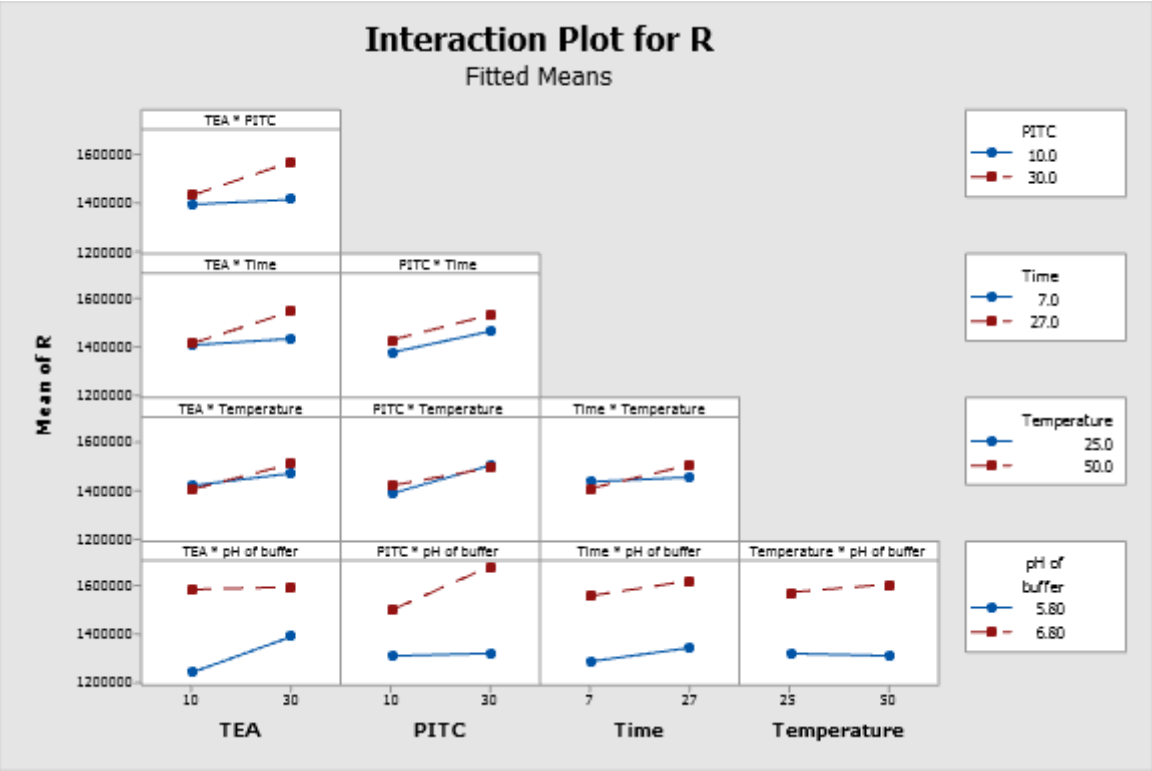

**Figure 2**

Reaction of Histamine with Phenyl isothiocyanate (PITC) reagent




## Figure 3

Remaining diagrams from partial factorial design

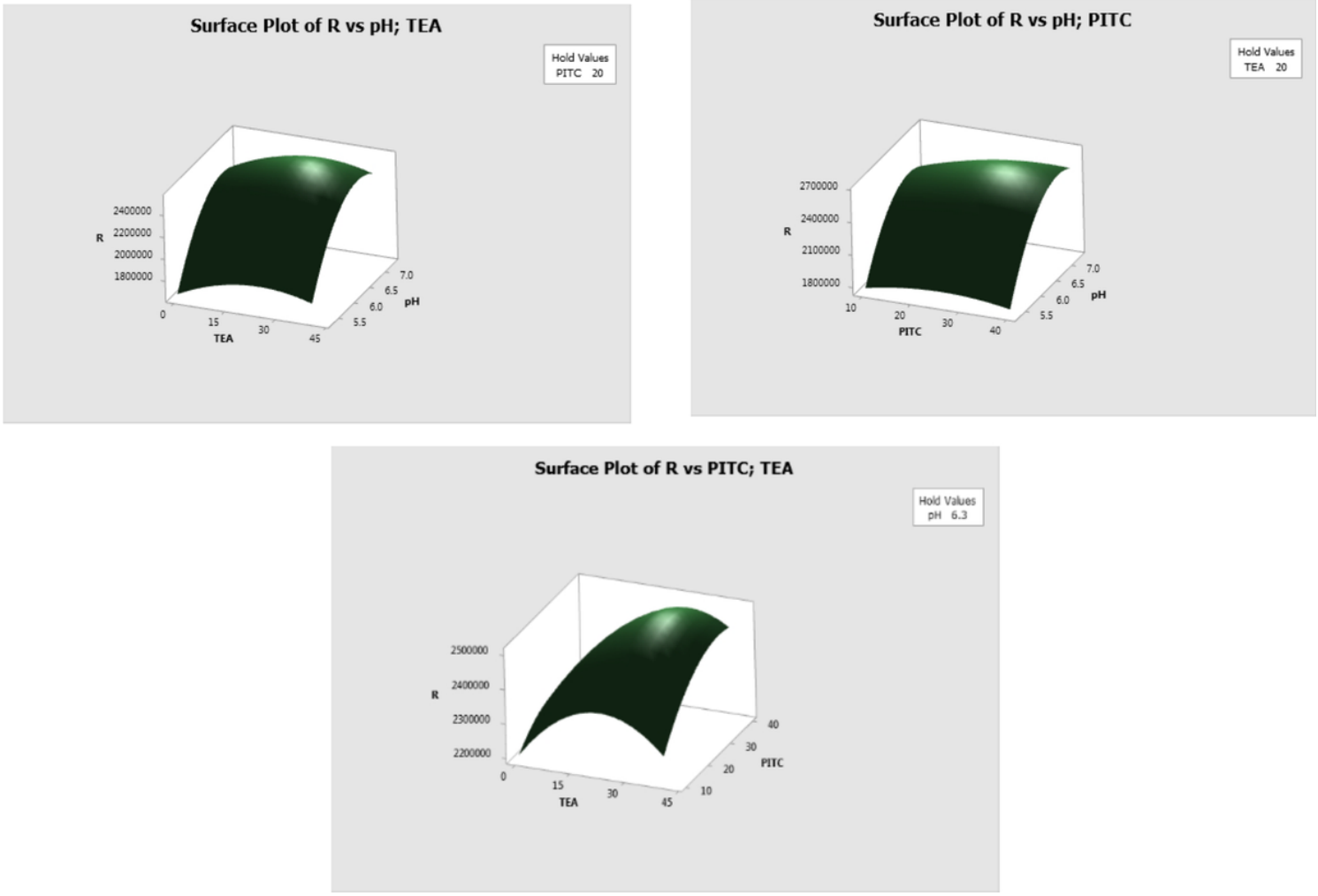



## Figure 4

Interaction diagrams from partial factorial design

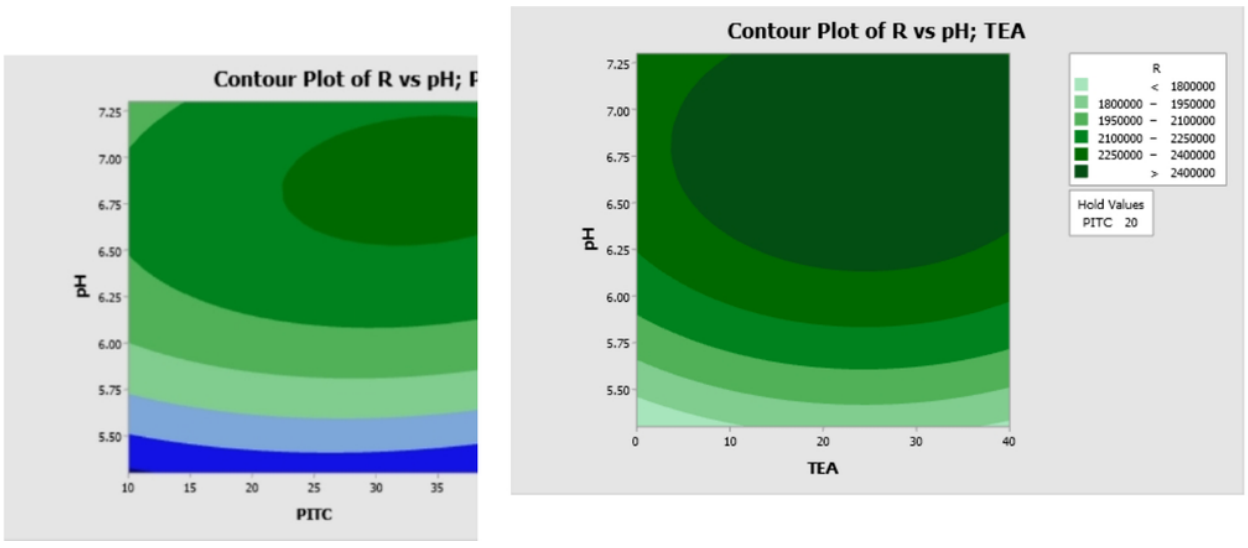

## Figure 5

Main Effects diagrams from partial factorial design



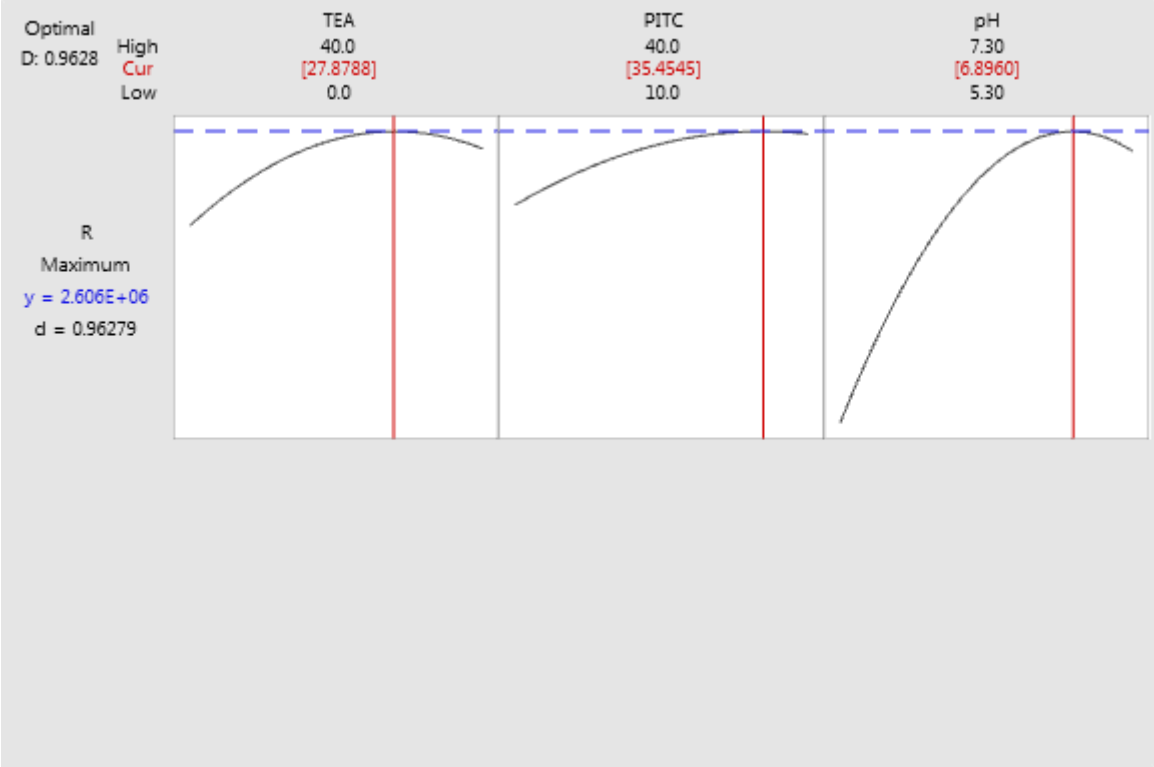

## Figure 6

Surface diagram of simultaneous study of triethylamine, buffer pH and phenyl isothiocyanate



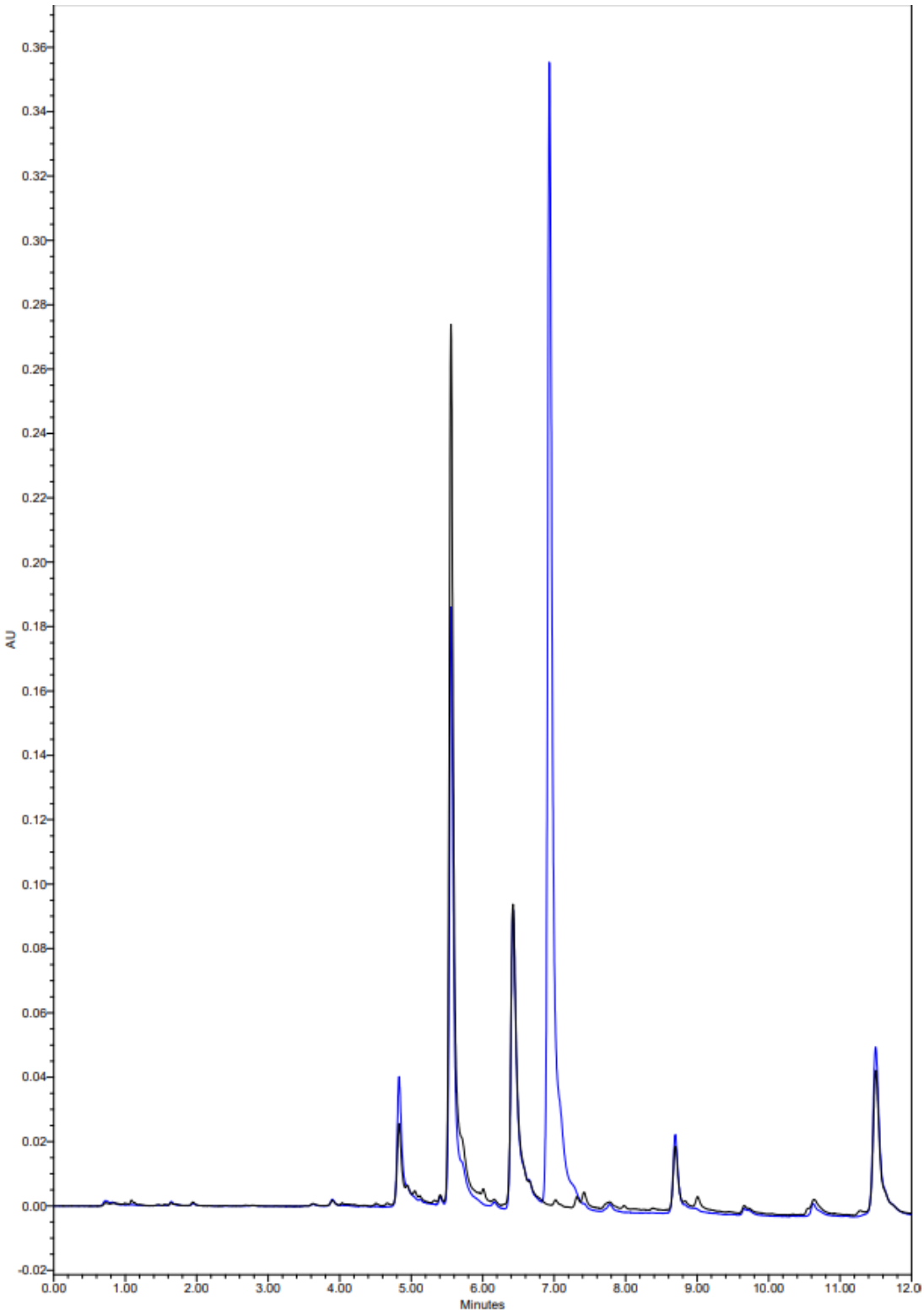

## Figure 7

Contour diagram of simultaneous study of triethylamine, buffer pH and phenyl isothiocyanate




## Figure 8

Achieving the optimal end point by optimization plot diagram




## Figure 9

Chromatogram shown in **black** for the blank and chromatogram in **blue** for the 1.0 mg/L Histamine sample