# FLOW INJECTION SPECTROPHOTOMETRIC DETERMINATION OF HISTAMINE IN FISH MEALS

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## Abstract

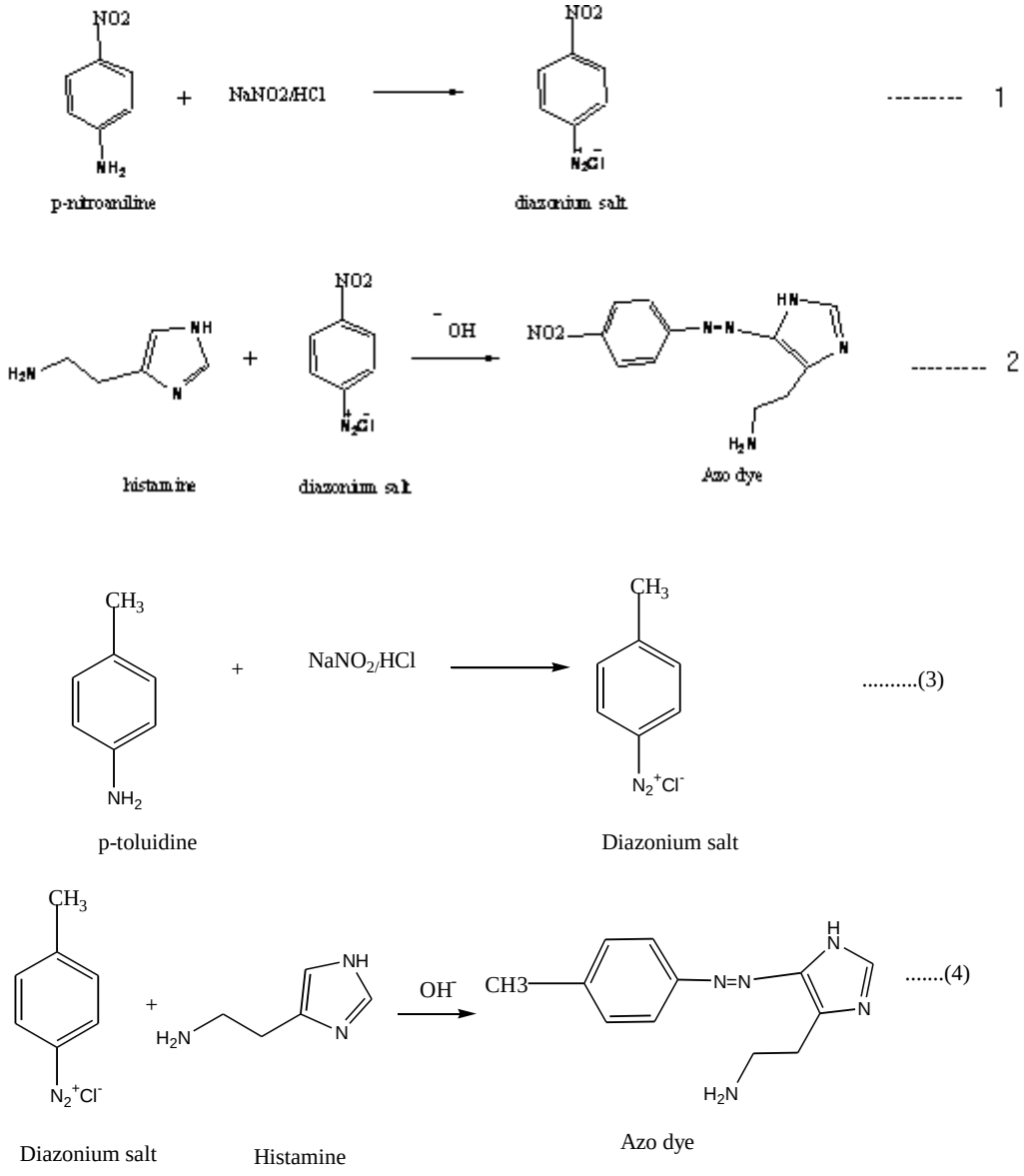
The study describes flow injection spectrophotometric methods for the determination of histamine in fish and fish meal samples. The methods were based on diazotization of p-toluidine and p-nitroaniline and subsequent coupling with histamine in alkaline medium. The calibration graph with diazotizated p-toluidine gives a linear range from 3.0 to 25.0 µg/ml with correlation coefficient 0.9967, while diazotization of p-nitroaniline and coupling with histamine gives a linear range from 0.5 to 22.0µg/ml with correlation coefficient 0.9985and detection limits 1 µg/ml and 0.3 µg/ml respectively. The effect of common amino acids and some ions on the determination of histamine was examined. Amberlite resin (weak cation exchanger) was used for the removing of histamine from interferences. The recommended FI procedures were successfully applied to the quantitation of histamine in 5 commercial fish meal products after appropriate sample treatment.

***KEYWORDS:*** *Flow- Injection, Determination, Histamine, Fish*

## Introduction

Histamine is an important biogenic amine, produced by decarboxylation of histidine amino acid. Biogenic amines are a group of biologically active organic compounds normally produced by decarboxylation of free amino acids. Biogenic amines are present in a variety of foods and have been widely documented as occurring in fish and fish products, meat, wine, cheese and fermented foods. The presence of biogenic amines in these foods is an indication of food spoilage which is dependent upon the availability of free amine( IblImmuno-Biological Laboratories,2008) . Histamine is mainly found in food which was subject to microbiological and biochemical deterioration due to processing, ripening or storage (Romero et. al, 2002). Histamine in food is normally harmless since humans have several control functions concerning endogenous biogenic amines as well as exogenous amines which are either ingested or of bacterial origin. Ingestion of food containing small amounts of histamine has little effect on humans, but in large doses histamine can be toxic(Den Brinker et. al, 2002, IblImmuno-Biological Laboratories,2008)

There are various methods available for the determination of histamine which includes spectrophotometry (Lowery, 1954, Tadao et.al ,1982, Tadao et.al,1984, Stoner,1985 and Pantag,2005) , flow injection analysis (Hungerford et.al,1990, Niculescu et.al ,2000, Sekiguchi et.al,2001 and Campo,2006) ,flourimetry(Luten, 1981, Gutibrrez et.al, 1987, Ekici et.al,2004, Ekici and Coskun,2004 and Yoshida et.al, 2004) high performance liquid chromatography (Gouygou et.al, 1987, Handley,1998, Kuruma and Sakano, 1999, Paleologos and Kontominas, 2004, Saccani et.al, 2005, Garci-Villar et.al,2005 and Tefera et.al ,2006) and electroanalytical method (Sarada et.al,2000, Zeng et.al,2000 and Oguri et.al, 2002). The aim of the present study is to determine histamine in fish meal by flow injection analysis method. The method depends on diazotization of p-toluidine and p- nitro aniline with nitrous acid then coupled with histamine in alkaline medium, which clarified in the following reactions:




## Materials and Methods

### Reagents and solutions

All chemicals used were of analytical reagent grade and distilled water was used throughout the study.

Histamine stock solution (100 µg/ml) (Riedel de Haen): prepared by dissolving 0.0165g of histamine dihydrochloride in 100 ml d.w. Working standard solution (25 µg/ml) was prepared by a proper dilution of the stock solution.

Sodium nitrite (0.5%) (Fluka) solution: 0.50 g of the compound was dissolved in 100 ml d.w.

Sulphuric acid (0.25M) (BDH) solution: prepared by dilution of concentration sulphuric acid (98%) with distilled water.

Potassium hydroxide (BDH) solutions: 2.801 g of KOH was dissolved in 100 ml for preparation of 0.5M of the compound while 5.602 g of the compound was dissolved in 100 ml for preparation of 1.0M solution.

p-Toluidine (0.2%) (BDH) solution: 0.20 g of the compound was dissolved in 100 ml of 0.5M HCl.

p-Nitroaniline solution (0.1%) (BDH) (For FIA system): 0.10 g of p-nitroaniline was dissolved in 100ml of 0.25M H2SO4.

Trichloroacetic acid solution (2.5%) (Fluka): 2.50 g of the compound was dissolved in 100ml d.w.

Buffer solution (pH 4.63): prepared by mixing equal volumes of 0.2M acetic acid and 0.2M sodium acetate.

Hydrochloric acid (0.2M) (Backer and Thomas) solution: prepared by appropriate dilution of concentrated hydrochloric acid (36%).

**Apparatus**

Spectral measurements were carried out on a Cecil Ce3021 uv/vis spectrophotometer using 1 cm quartz cell. Absorbance measurements were carried out using LKB Ultrospec II single beam uv/vis spectrophotometer, using 1 cm match glass cells.

Peristaltic pump (Watson–Marlow, multi channel) was used for propelling carrier solutions.

Injection valve was a 6 – way loop valve (Omnifit) with various sample loops.

Recorder (Philips PM 8251 A) one line recorder.

A 30µl and 10mm path length quartz flow cell was used in the flow injection system.

**Column preparation**

A 1.0 g of amberlite resin (weak cation exchanger), CG-50, chromatographic grade type 1, 100 – 200 mesh, (BDH) was mixed with 10 ml of buffer solution and poured into a chromatographic column (i.d. 12 mm). The ends of the column were plugged with glass wool (Kose and Hall, 2000).

**Preparation of Fish Samples**

Fish or fish meal product (10.0 g) was homogenized with blender and treated with 100 ml of 2.5% trichloroacetic acid (TCA), filtered with filter paper (Whatman no. 1).A volume of extracted sample (containing not more than 2.0 mg of histamine) was neutralized with 1M KOH to pH 7.0 and filtered using filter paper. The filtrated sample was treated with 5.0ml of chloroform for oil removing. The chloroform layer was discarded after separation with a separating funnel.The column was washed with the buffer solution and the retained histamine was eluted from the column with 0.2M hydrochloric acid solution (Kose and Hall, 2000).

**Results and Discussion**

Figure (1) shows FI manifold used for histamine determination. A multi channel peristaltic pump was used for three streams: propelling p-toluidine or p- nitroaniline solution, sodium nitrite solution and potassium hydroxide solution. The histamine sample was injected through the injection valve into the merged p-toluidine or p- nitraniline and $NaNO_2$. Three reaction coils($RC_1$), ($RC_2$) and ($RC_3$) were used in the system with different lengths. The merged streams were passed through a quartz flow cell (30 µl, 10 mm path length) in a spectrophotometer connected to recorder.

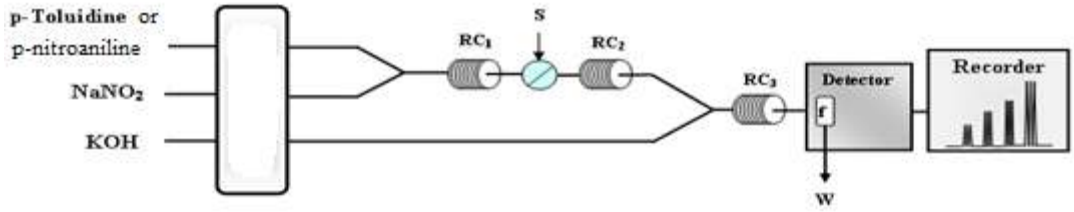



**Figure (1):** FI manifold used for determination of histamine by p-toluidine and p-nitroaniline reagents. P (pump), RC (Reaction coils), S (Sample injector) and W (Waste).

**Optimization of The Flow Systems**

Physical and chemical parameters were checked to obtain optimum conditions for histamine determination. This study was carried out by altering each variable in turn while keeping the others constant.

**Effect of Flow Rates**

Different flow rates were studied using a tube with various i.d for propelling the solutions. It was found that the optimum flow rates were 0.9, 0.8 and 0.4ml/min for propelling the solutions p-toluidine, sodium nitrite and KOH respectively as shown in Figure 2and 3show that the 0.8, 0.7 and 0.8ml/min were the optimum flow rates for propelling the solutions p-nitroaniline, sodium nitrite and KOH respectively. The mentioned values were used in the subsequent experiments for determination of histamine using p-nitroaniline.

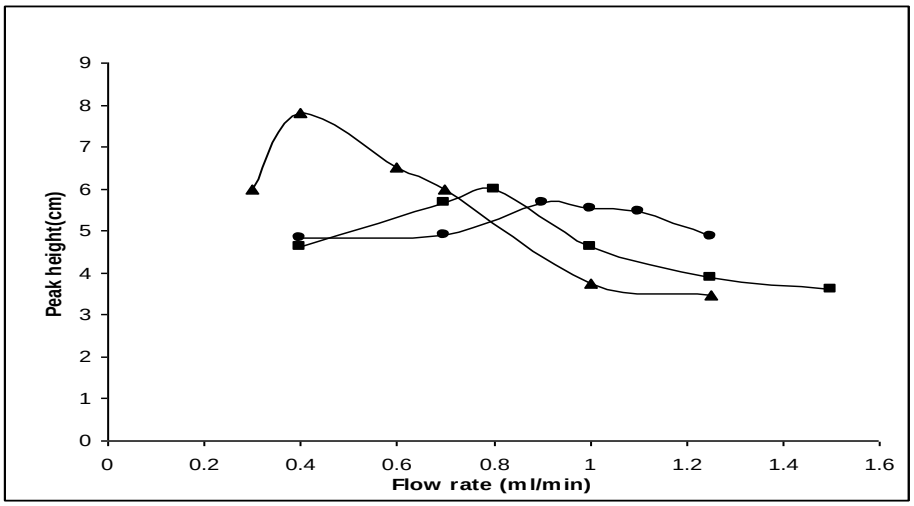



**Figure (2):** Effect of flow rate on peak heights (●) p-toluidine reagent (■) sodium nitrite (▲) potassium hydroxide

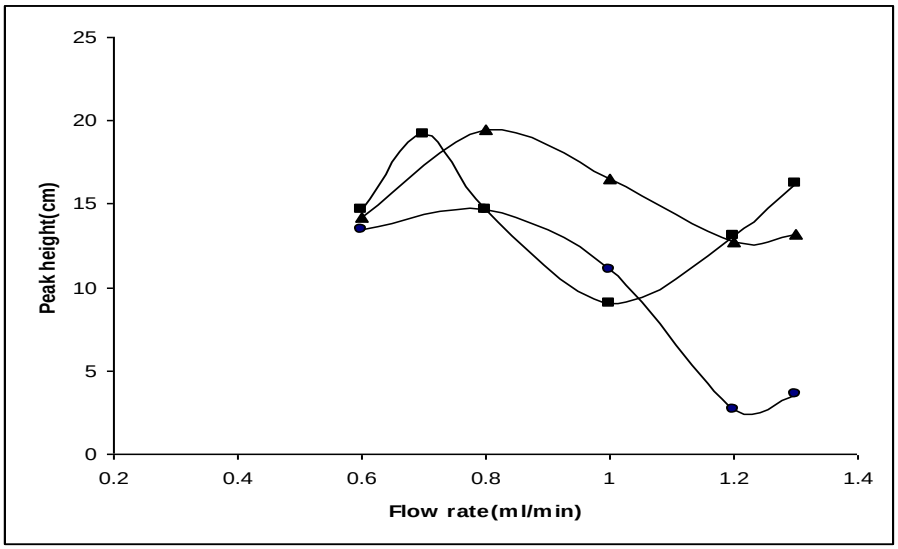



**Figure (3):** Effect of flow rate on peak heights (●) p-nitroaniline reagent (■) sodium nitrite (▲) potassium hydroxide

## Effect of the Length of the Reaction Coils

In the FIA systems; the height of the response peak highly depends on the residence time of the sample zone, i.e., on the tube length, flow rate and sample volume (Mousavi et.al, 1998). Figure 4 and 5 show the influence of the lengths of reaction coils on the peak height. Each reaction in both systems requires optimum lengths for maximum peak height achievement. The best response was achieved at lengths 5-cm, 40-cm and 40-cm for p-toluidine system, while 40-cm, 20-cm and 30-cm for p-nitroaniline system. Therefore, these reaction coil lengths were used for further studies.

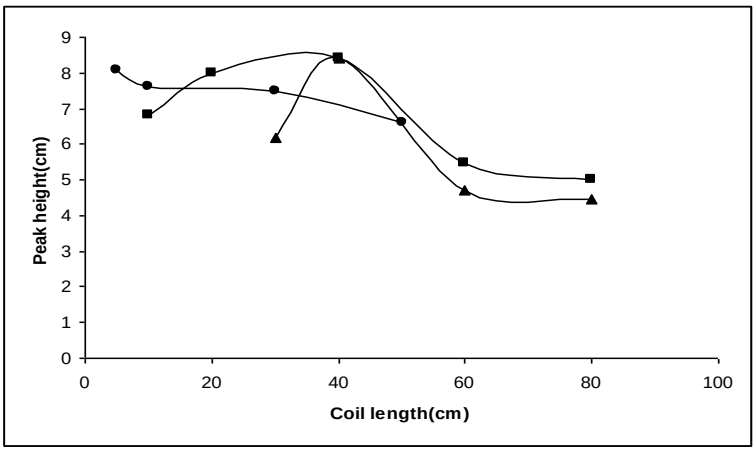



**Figure (4):** Effect of the length of reaction coils on peak heights (●) first reaction coil (■) second reaction coil (▲) third reaction coil

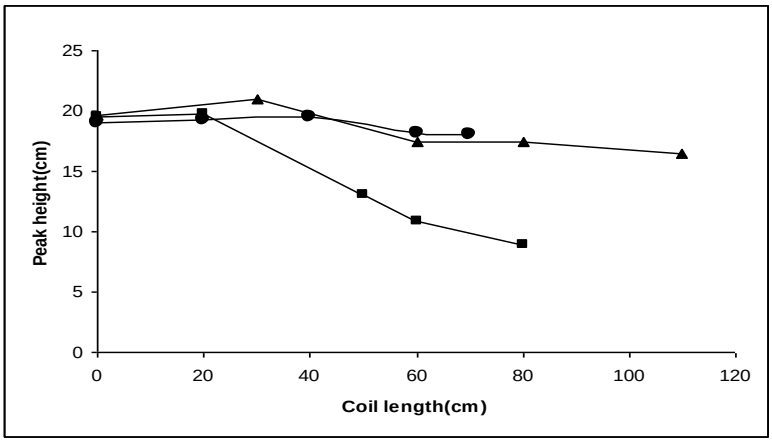



**Figure (5):** Effect of the length of reaction coil on peak heights (●) first reaction coil (■) second reaction coil (▲) third reaction coil

## Effect of the Sample Volume

The effects of the sample volume on the results are shown in Figure ( 6). Various sample volumes were injected through the injection valve. The sensitivity of proposed methods increased significantly with increasing the volume of the sample introduced into the flow system up to 175 µl for p-toluidine and 200 µl for p-nitroaniline. Therefore, these sample volumes were adopted.

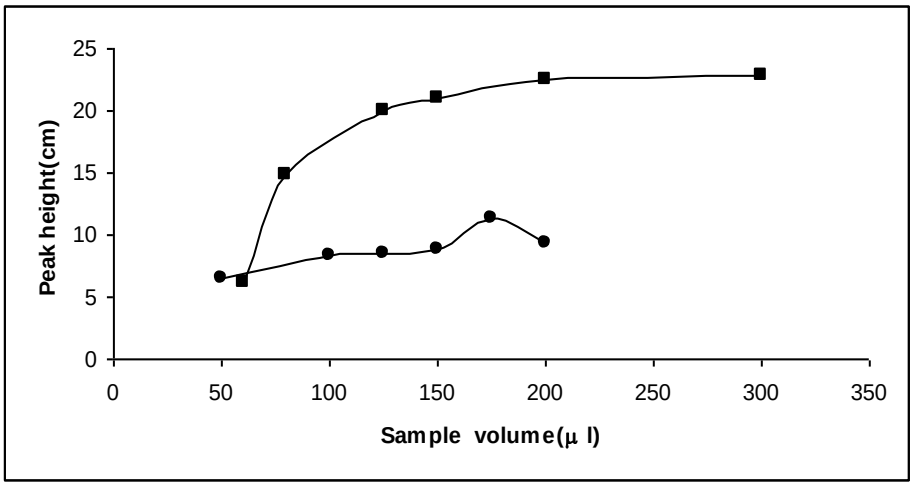



**Figure (6):** Effect of sample volume on peak height (●) p-toluidine reagent (■) p-nitroaniline reagent

**Effect of the Reagents Concentrations**

The effect of the concentration of the reagents (p-toluidine and p-nitroaniline) upon the analytical response of the flow systems were examined in the concentration ranges 0.1 – 0.7% for p-toluidine and 0.025 – 0.1% for p-nitroaniline. Maximum peak heights were obtained using 0.5% for p-toluidine and 0.1% using p-nitroaniline as shown in Figure 7. Therefore, these concentrations were employed in subsequent experiments.

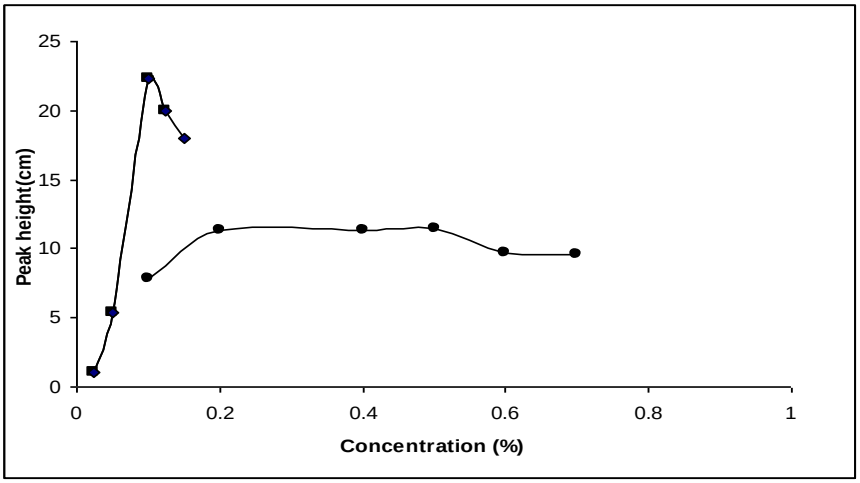



**Figure (7):** Effect of the concentration of reagents on peak height (●) p-toluidine (■) p-nitroaniline

**Effect of the Concentration of Acids**

The influences of concentration of acids (HCl for preparation of p-toluidine and $H_2SO_4$ for preparation of p-nitroaniline) on the peak height were checked and the results are shown in Figure (8). It was found that 1.0M of HCl and 0.25M of $H_2SO_4$ were optimums for diazonium ion production. Low concentrations of acids were not produce adequate quantities of diazonium ions while high concentrations cause salt formation of aryl amines (Smith and March,2001).

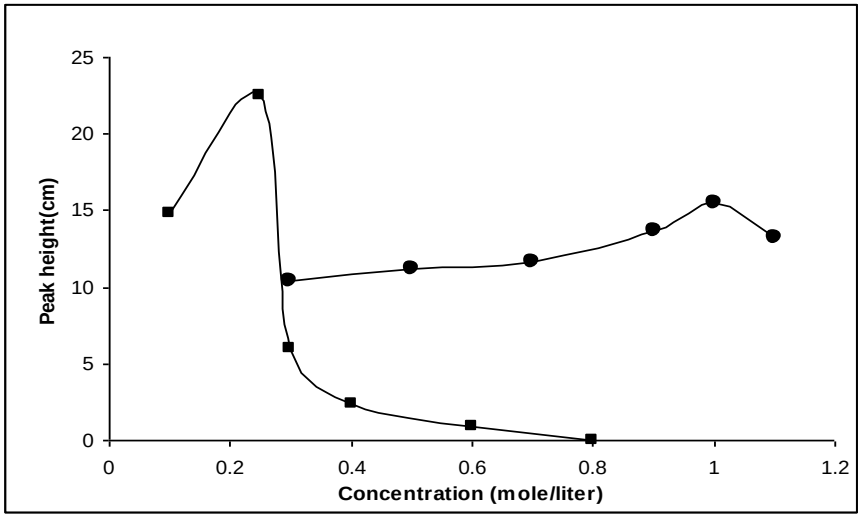



**Figure (8):** Effect of acids concentrations (●) HCl and (■)$H_2SO_4$ on peak heights

**Effect of The Concentration of Sodium Nitrite**

The effect of the concentration of sodium nitrite on the color intensity (as peak height) was examined. The concentration of sodium nitrite that exhibits the greatest peak heights was found to be 0.5% for both reagents. Therefore, this concentration was chosen as the optimum. Upon further increasing of the concentration of sodium nitrite, the peak heights gradually decreased due to the formation of bubbles in the flow system Figure (9).

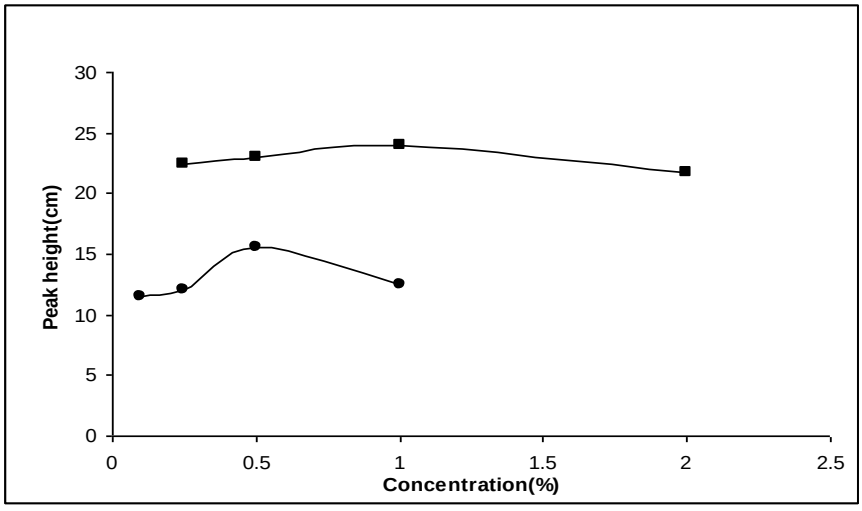



**Figure (9):** Effect of the concentration of sodium nitrite on peak height (●) p-toluidine (■) p-nitroaniline

**Effect of The Concentration of Potassium Hydroxide**

When the diazotized reagents coupled with histamine in alkaline medium, an orange color of the azo dyes was formed, while the diazotizated reagents were colorless in acidic medium. The influence of the concentration of potassium hydroxide was checked by varying the concentration from 0.25 to 1.5M. Figure (10) shows the effect of potassium hydroxide concentration on the peak heights which indicate that 1M of potassium hydroxide gave maximum peak heights in both cases. Low concentrations of the base were not sufficient for producing an intense color while color intensity decreased (peak height) at higher concentrations.

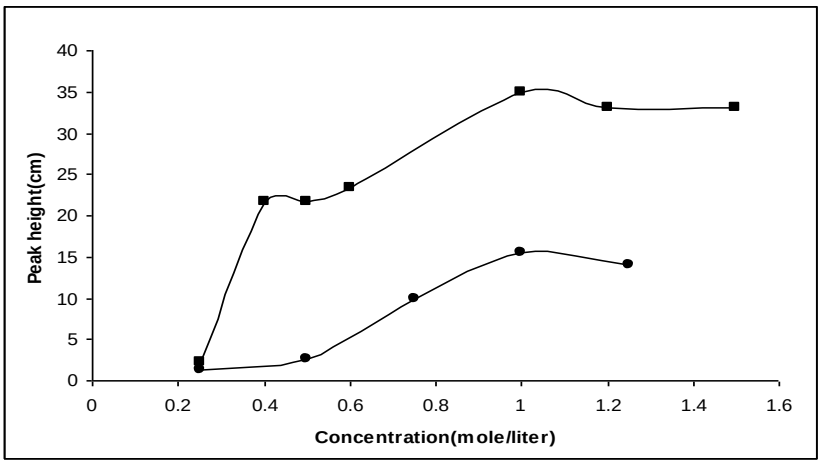



**Figure (10):** Effect of the concentration of potassium hydroxide solution on peak height (●) p-toluidine (■) p-nitroaniline

## Recommended Procedures

**Flow Injection Spectrophotometric Determination Of Histamine Using P-Toluidine Reagent**

Figure (1) shows FI manifold used for histamine determination. A multi channel peristaltic pump was used for three streams: propelling p-toluidine (0.5% in 1.0M hydrochloric acid) solution, 0.5% sodium nitrite solution and 1M potassium hydroxide solution, with flow rates 0.9, 0.8 and 0.4 ml/min respectively. A 175 µl of histamine sample was injected through the injection valve into the merged p-toluidine and NaNO2. Three reaction coils were used in the system with lengths 5- cm (RC1), 40- cm (RC2) and 40- cm (RC3). The merged streams were passed through a quartz flow cell (30 µl, 10 mm path length) in a spectrophotometer connected to recorder.

**Flow Injection Spectrophotometric Determination Of Histamine Using P-Nitroaniline Reagent**

The same flow system shown in Figure ( 1) was used in this work.To propel 0.1% of p-nitroaniline (prepared in 0.25M H2SO4) solution, 0.5% sodium nitrite solution and 1M potassium hydroxide solution a multi-channels peristaltic pump was used with flow rates 0.8, 0.7 and 0.8 ml/min respectively. A 200 µl of histamine was injected through the injection valve into the carrier stream. Three reaction coils were used with lengths 40- cm (RC1), 20- cm (RC2) and 30- cm (RC3). The merged streams were passed through a quartz flow cell (30 µl, 10 mm path length) in a spectrophotometer connected to recorder.

**Calibration Curves**

Using the FI-manifold under optimum conditions for histamine determination in both systems, linear calibration curves over the ranges 3.0 – 25.0 µg/ml using p-toluidine and 0.5 – 22.0 µg/ml using p-nitroaniline were established with detection limits 1 µg/ml and 0.3 µg/ml respectively (Figure 11). Table (1) shows the statistical data of the methods.

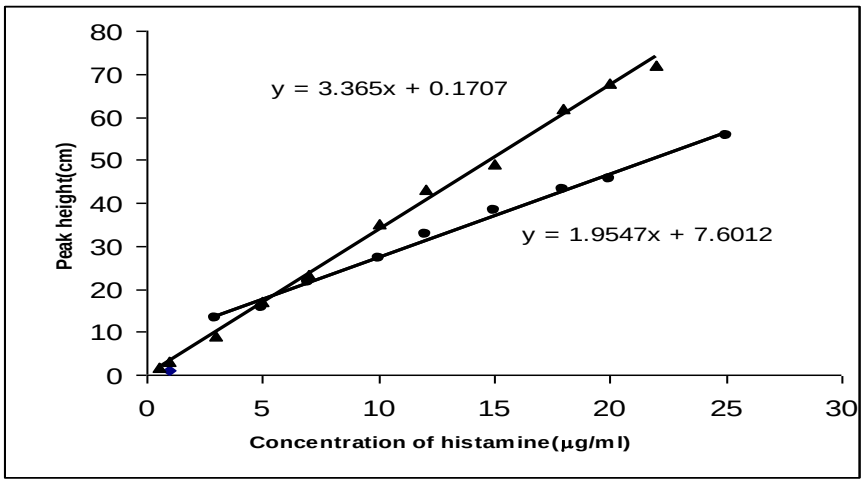



**Figure (11):**Calibration curves of FI- spectrophotomteric determination of histamine using p-toluidine (●) and p-nitroaniline (▲)

**Table (1):**Statistical data of FI-spectrophotomteric determination of histamine using p-toluidine and p-nitroaniline

| Parameter | p-toluidine | p-nitroaniline |
|---|---|---|
| Dynamic linear range(µg/ml) | 3.0-25.0 | 0.5-22.0 |
| D.L(µg/ml) | 1.0 | 0.3 |
| Correlation coefficient | 0.9967 | 0.9985 |
| Regression equation | Y=1.954x + 7.601 | y=3.365x + 0.17 |
| *Sample frequency (sample/hr.)* | 40 | 60 |

## Precision and Accuracy

Under the optimum conditions, the values of relative standard deviation and relative error of three injections for three concentration levels of histamine were calculated as shown in Table (2).

**Table (2):** Precision and accuracy data of FI-spectrophotomteric determination of histamine

| | Conc.(µg /ml) | RSD% | $E_{rel}$ % |
|---|---|---|---|
| **p-toluidine reagent** | 3.00 | 1.56 | +4.25 |
| | 10.00 | 0.72 | -0.60 |
| | 25.00 | 0.10 | -0.86 |
| p-nitroaniline reagent | 0.50 | 5.00 | -3.10 |
| | 10.00 | 1.46 | +3.50 |
| | 22.00 | 0.87 | -2.97 |

## Study of Interferences

The effect of common amino acids and some ions on the determination of histamine was examined. A synthetic sample of histamine (10.0 µg/ml) and the interfering substance was injected to the carrier stream and the analytical signals were compared with that of histamine solution alone. A ≤±5% error was considered to be a tolerable amount of interfering species. The results are shown in Table (3).

**Table (3):** Effect of foreign species on FI-spectrophotomteric determination of histamine.

| p-toluidine | | | p-nitroaniline | | |
|---|---|---|---|---|---|
| **Foreign species** | **Conc.(µg/ml)** | **$E_{rel}$ %** | **Foreign species** | **Conc.(µg/ml)** | **$E_{rel}$ %** |
| Albumine | 5.0 | +2.86 | Albumine | 5.0 | +2.51 |
| Alanine | 40.0 | +2.23 | Alanine | 20.0 | -1.29 |
| Arginine | 5.0 | +1.86 | Arginine | 5.0 | +4.0 |
| Aspargine | 50.0 | +3.62 | Aspargine | 30.0 | +1.01 |
| Aspartic acid | 25.0 | -2.91 | Aspartic acid | 5.0 | -1.39 |
| Cysteine | 25.0 | +4.88 | Cysteine | 5.0 | -3.12 |
| Histidine | 0.5 | +3.90 | Histidine | 0.5 0 | +3.41 |
| Lysine | 5.0 | +4.0 | Lysine | 5.0 | +3.13 |
| Methionine | 10.0 | +3.64 | Methionine | 10.0 | +4.12 |
| Proline | 10.0 | +1.16 | Proline | 5.0 | -2.70 |
| Phenyalanine | 50.0 | +4.45 | Phenyalanine | 5.0 | +4.82 |
| Tryptophane | 3.00 | -3.90 | Tryptophane | 5.0 | -4.86 |
| Tyrosine | 5.0 | +4.85 | Tyrosine | 2.0 | +3.81 |
| $Ca^{2+}$ | 30.0 | +3.81 | $Ca^{2+}$ | 40.0 | +2.90 |

**Removing of Interfering Substances**

Either removal of the interferences or histamine itself should be performed before the application of the method for the determination of histamine in natural samples. Therefore, amberlite resin (weak cation exchanger) was used for the removing of histamine from interferences. A column (12 mm i.d.) packed with 10g of the resin was used for this purpose. Histamine in fish meal samples were analyzed with FIA systems after treatment with column for removing of interferences.

**Application of the methods**

The recommended FI procedures were successfully applied to the quantitation of histamine in 5 commercial fish meal products after appropriate sample treatment. The histamine contents in fish meal samples were found to be around 6 mg/100g for fish samples. In order to evaluate the proposed FI methods for histamine determination, comparative determinations of the fish meals using the standard method were carried out based on spectrophotometric procedure (Hardy and Smith, 1976). It was found that the results obtained by both methods compared favorably (Table 4).

**Table (4):** Determination of histamine in fish meals by the proposed methods

| Sample | Sardine(mg/ 100gm) | Tuna(mg/ 100gm) | Mackerel(mg / 100gm) | Salmon(mg/ 100gm) | Shilana(mg/ 100gm) |
|---|---|---|---|---|---|
| Standard method | 4.67 | 5.72 | 4.13 | 5.43 | 4.15 |
| FI-Spectrophotomteric determination using p-toluidine | 5.25 | 5.69 | 4.50 | 5.78 | 4.59 |
| *FI-Spectrophotomteric determination using p-nitroaniline* | 4.66 | 5.73 | 4.07 | 5.46 | 4.00 |

## Conclusion

The proposed methods for determination of histamine in fish meals are simple, sensitive and also with relatively high sampling rate about 40sample/h for p-toloudine reagent and 60sample/h for p-nitroaniline reagent.

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## تقدير الهستامين في عينات السمك بطريقة الحقن الجرياني الطيفي

**الخلاصة**

يتضمن اساس تقدير الهيستامين في عينات السمك بطريقة الحقن الجرياني على ازوتة كل من باراتولودين و باراناپتروانيلين وتكوين ايون الدايوزنيوم ثم ازدوادج الايون مع الهيستامين فى وسط قاعدى . وتظهر صبغتا الأزو المتكونتان اعلى امتصاص عند الطول الموجي 475 نانوميتر لكاشف باراتولودين و 473 نانوميتر لكا شف باراناپتروانيلين. ينطبق قانون بير فى مدى تركيز 3.0-25.0مايكروكرام/مل مع معامل ارتباط 0.9967 لكاشق باراتولودين و 0.5-22.0 مايكروكرام/مل لكاشف باراناپتروانيلين مع معامل ارتباط 0.9985. و كانت حدا الكشف 1 مايكروكرام/مل و 0.3 مايكروكرام/مل للكاشفين على التوالي.

تم دراسة تأثير بعض الاحماض الامينية الشائعة و كذلك بعض الايونات على تقدير الهستامين . استعملت راتنج من نوع امبرلايت(amberlite resin)لآزالة المواد المتداخلة في تقدير الهستامين . الطريقة المقترحة طبقت بنجاح في تقدير الهستامين في عينات السمك بعد المعالجة المناسبة للعينات.

## دیاریکردنی پەیتی هیستامین لە نموونەی ماسی بەرێگای دەرزی لێدانی ڕۆیشتووی شەبەنگی

**پوختە**

**بنچینەی خەملاندنی هستامین بەڕێگای دەرزی لێدانی ڕۆیشتوو پێك هاتووە لە گۆڕینی هەریەك لە پاراتولودین و پارانایتر و ئەنیلین بۆ ئایۆنی دایزونیوم کە لەکەڵ هستامین جووت دەبن لەناوەندی تفتدا وە بۆیەی ئازۆ دروست دەبێت، بۆیەی ئازۆ دروست بووەکان بەرزترین هەڵمژینی درێژە شەپۆلی هەیە لە 475 نانومیتەر بۆ پارانایترۆ ئەنیلین و 473 نانۆمەتر بۆ پاراتولودین یاسای بیر جێ بەجێ دەبێت بە سەریدا لەمەودای 3.0-25.0 مایکرۆگرام /مل بۆ باراتولودین بەهاوکۆلکەی لکاندنی 0.9967 وە 0.5-22.0 مایکرۆگرام /مل بۆ پارانایترۆ ئەنیلین وە بەهاوکۆلکەی لکاندنی 0.9985 . سیستەمی دەرزی لێدانی ڕۆیشتوو دانراوە بۆ خەملاندنی هستامین بە بەکارهێنانی هەردوو ناسەرەوەکان. مەودای ناسینەوە بریتی بوو لە 1.0 مایکرۆگرام /مل بۆ ناسەرەوەی پاراتولودین وە 0.3 مایکرۆگرام/مل بۆ ناسەرەوەی پارانایترۆ ئەنیلین.**

**کاری هەندێك لە ترشە ئەمینیە باوەکان لەسەر دیاریکردنی پەیتی هیستامین وە هەندێك لە ئایۆنەکان ڕوون کراوەتەوە.ڕاتنجی ئەمبرلایت(amberlite resin) بەکارهاتووە بۆ لابردنی کاری هەندێك لە ئاوێتەکان لە دیاریکردنی پەیتی هیستامین.ئەم ڕێگای پێشنیارکراوە بەسەرکەوتویی جێ بەجێ کراوە بۆ دیاریکردنی پەیتی هیستامین لە نمونەی ماسی،پاش چارەسەر کردنی گونجاو بۆ نموونەکان.**