Microbiological Evaluation and Quantitative Analysis of Histamine Content in Some Egyptian Cheese Using High-Performance Liquid Chromatography (HPLC)

Ahmed H. Saad[1], Kamelia Abd Ellatif [2] Shaimaa Mohamed Tony[3], and Alaa Abd Algwad[1]*

1 Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Suez Canal University, Egypt

2 Free Veterinarian

3 Animal health research institute (AHRI), Agriculture research center (ARC), Food Hygiene Department, Egypt

Abstract

The histamine levels in Mish and Egyptian Ras (Rumy) cheese were estimated in 100 samples, 50 of each Mish and Rumy cheese from Ismailia City, Egypt, by HPLC. The findings revealed the histamine content in the Mish samples varied from 1.8 to 21.05 mg/kg, with a mean of 7.99 ± 1.107 mg/kg, while in Rumy samples, it ranged from 21.72 to 53.86 mg/kg, with a mean of 29.12 ± 1.769 mg/kg. Consequently, the estimated daily intake of histamine (EDI) from Mish would fall within the range of 0.021-0.242 mg/day, while for Rumy, it would be between 0.25 and 0.62 mg/day. Though all examined samples contained histamine but were below the permissible limits stated by the Egyptian standard, 2008 (200 mg/kg), only 20% of Rumy samples exceeded the BIOHAZ, 2011 allowable limits (50 mg/day) and contained ≤53.86 mg/kg. The examined Mish and Rumy samples contained *Enterobacteriaceae,* coliforms, *lactobacilli,* and total yeasts and molds at the rates of 18%, 17%, 100%, and 94% and 38%, 12%, 100%, and 100%, with means of $15.5\times10\pm86.9\times10^4$ 86.9×10^3, $28.3\times10\pm68.9\times10^3$ 68.9×10^2, $63.3\times10\pm80.4\times10$,^{6} ^{5} and $91.2\times10\pm14.6\times10^2$ $14.6\times10^2$and ($17.8\times10\pm55.8\times10^5$ 55.8×10^4, $93.3\times10\pm12.8\times10^3$ 12.8×10^3, $73.7\times10\pm75.1\times10$,^{6} ^{5} and $68.6\times10\pm10.1\times10^3$ 10.1×10^3), respectively. In conclusion, although the estimated levels have no obvious adverse effect, it doesn't eliminate the potential for histamine poisoning. Commitment to good hygiene practices in milk selection and manufacturing environments can effectively control the presence of microorganisms responsible for biogenic amines production.

Keywords: biogenic amines, cheese, histamine, HPLC

Introduction

Cheese is the most highly consumed dairy product worldwide. In Egypt, there are diverse varieties and kinds of cheese that are consumed in the daily diet, particularly by children, due to its richness in protein, fat, and minerals, for instance calcium and magnesium, which at the same time make them liable to microbial contamination, which may lead to inferior subsequences such as conveying diseases to humans, spoilage of the products, and production of lethal substances. ***(Ma et al., 2020)***. One of them is the biogenic amines which are considered natural toxicants produced by certain microorganisms or due to some amino acid's metabolism or decarboxylation. ***(Omer et al., 2021)***. Biogenic amines are nitrogen compounds with low molecular weights that are active and stable for heating, freezing, and smoking. The biogenic amines include histamine, tyramine, cadaverine, agmatine, putrescine, β-phenylethylamine, spermidine, and spermine. When they are produced in normal doses, they contribute to critical physiological functions such as regulation of gene expression, intracellular signal pathways, cell growth, and tissue renovation, but at high levels, they may pose adverse toxicological properties. ***(Nguyen and Chuyen Van, 2021)***. Histamine and tyramine are considered the most toxic and safety-relevant biogenic amines, and histamine production significantly appeared in cheese more than in other dairy products. Its levels vary depending on the manufacturing measures, intrinsic and extrinsic factors, and microbial contamination during the production process, therefore their presence reflects the quality and hygiene during the processing. ***(Ferrante and Mercogliano, 2023)***. The consumption of high doses of histamine leads to histamine intoxication, which is an allergic reaction characterized by flushing, hypertension, tachycardia, and gastrointestinal disorders. ***(Nguyen and Chuyen Van, 2021)***. However, histamine intoxication cases have been recorded in several countries, and there is limited information regarding its content in dairy products, specifically cheese, in Egypt ***(El-kholy et al., 2020)***. Therefore, this study aimed to estimate the histamine levels in Mish and Egyptian Ras cheese (Rumy cheese) marketed in Ismailia city, Egypt, where the Mish is an Egyptian traditional cheese produced by allowing the salted cheese to ferment for months or even years, while Rumy cheese is an Egyptian traditional hard cheese resembling the Ras cheese that is ripened to 6 months or more to gain its characteristic consistency and flavor ***(Ma et al., 2020)***. The daily intake of histamine, which would

be taken through consuming cheese, was also estimated and its hazards to the consumer's health were illustrated.

Material and Methods

1. Collection of samples

A total of one hundred samples of Mish and Rumy cheese, 50 of each, were randomly collected from the local markets in Ismailia City, Egypt, from September to November 2024. The collected samples were transported aseptically to the Food laboratories in the National Research Center at Dokki for histamine estimation.

2. Estimation of Histamine according to *Pinho et al., (2001)*

2.1. Reagents preparation

- **Dansyl chloride solution**: 500 mg of dansyl chloride was dissolved in 100 ml acetone.
- **Standard solutions of tested histamine**: 25 mg of pure histamine-2HCl dissolved in 25 ml of distilled water.

2.2. Extraction of samples

25 g of each cheese sample was blended with 125 ml of 5% Trichloro acetic acid (TCA) for 3 minutes by using a warning blender then filtrated through a filter paper Whatman No1 and 10 ml of the filtrate was transferred into a suitable glass tube with 4 g of NaCl and 1 ml of 50 % NaOH. The previous filtrate was extracted three times (2 min for each) by using 5 ml n-butanol: Chloroform (1:1 v/v), the upper clear layer was transferred to a 100 ml separating funnel by using a disposable Pasteur pipette then 15 ml of n-heptane was added in a separating funnel and extracted three times with 1.0 ml portions of 0.2 N HCl. The HCl layer was collected in a Stoppard glass tube. Using a water bath at 95°C with a mild flow of air, the solution was evaporated just to dryness.

2.3. Formation of dansyl amines

100 µl of the sample extract was transferred to a 50 ml vial and dried under vacuum. 0.5 ml of saturated NaHCO3 solution was added to the remaining sample extract. The vial was stopped and thoroughly combined, to prevent spattering. 1 mL of dansyl chloride solution was added and completely mixed using a vortex mixer then the resulting combination was incubated for 45 minutes at 55°C. Extraction of dansylated Histamine was performed using 5 ml of diethyl ether 3 times again, the vial was stopped and shacked using a vortex mixer, and the ether layer was collected in a culture tube using a disposable Pasteur pipette. The combined ether extracts were evaporated carefully at 35°C in a dry bath with the aid of circulation air. The resulting dry material was dissolved in 1 ml methyl alcohol and 10 µl of this mixture was injected into HPLC.

2.4. HPLC apparatus conditions

High-performance liquid chromatography (HPLC) used for dansyl amines determination, equipped with quaternary pump

model G 1311A, UV detector (Model G 1314A) set at 254 nm wavelength, autosampler (model G1329A VP-ODS) and Shim pack (150 × 4.6 mm) column (Shimadzu, Kyoto, Japan) was used for Histamine separation. Chemstation Software was used to combine and record data.

3. Estimation of the histamine daily dietary intake according to Ma et al., (2020)

The estimated daily intake (EDI) for the histamine because of the daily consumption of cheese amongst the Egyptians was calculated through this equation:

EDI = Ci x IR

Where Ci is the concentration of histamine in the examined cheese and IR is the average daily cheese consumption rate in Egypt which was appraised as 11.51 g/day according to the annual consumption rate of cheese in Egypt that is recorded by the global per capita consumption of cheese as 4.2 kg ***(Statista Research Department (SRD), 2019)***.

4. Microbiological evaluation

4.1. Enumeration of Enterobacteriaceae on VRBGA according to ***APHA (2012)***

4.2. Enumeration of total Coliforms by MPN according to ***FDA (2020)***

4.3. Enumeration of total lactobacilli on MRS media according to ***De Man et al. (1960)***

4.4. Enumeration of total Yeasts and molds on Malt Extract Agar media according to ***APHA (2012)***

5. Statistical analysis

SPSS software package version 22 was used for statistical analysis. The histamine level values in examined samples were expressed as the mean ± standard error.

Table: 1. *Gradient solvent program for separation of Histamine by HPLC*
A=0.02N acetic acid B=Methanol C= Acetonitrile

Time /min.	Flow rate ml/min.	Solvent A%	Solvent B%	Solvent C%
0	1	60	20	20
10	1	20	40	40
15	1	15	35	50
20	1	60	20	20
25	1	60	20	20

Results and Discussion

The estimated histamine in the examined Mish cheese samples ranged between 1.8 – 21.05 mg/kg with a mean value of 7.99 ± 1.107 mg/kg, and for the examined Rumy samples ranged from 21.72 to 53.86 mg/kg with a mean of 29.12 ± 1.769 mg/kg, as revealed in **Table (1).** Subsequently, the estimated daily intake of histamine in the Mish and Rumy cheese, according to the obtained results, would be in the range of 0.021 - 0.242 mg/day

and 0.25 – 0.62 mg/day, respectively. ***The Egyptian Standard (2008)*** stated that the maximum permissible limit of histamine in Mish and Rumy cheese is 200 mg/ kg, while the Food and Drug Administration ***(FDA, 2011)*** affirmed the maximum limit to 100 mg/ kg and the Netherlands Institute of Dairy Research declared that the histamine limit is in the range of 100 - 200 mg/kg ***(El-kholy et al., 2020)*****.** The no observed adverse effect level (NOAEL) of the histamine was declared as 50 mg by the European Food Safety Authority ***(EFSA Panel on Biological Hazards (BIOHAZ) 2011)***. Therefore, according to the data observed in **Table (1)**, 100% of the examined Mish and Rumy samples contained histamine, but only 20% of the Rumy samples contained ≤53.86 mg/kg, higher than the BIOHAZ limits.

Higher histamine levels were approximated in Egyptian Ras cheese samples by ***El-Kosi et al., (2009)***, ***Ibrahim and Amer (2010)***, ***Abo El-Makarem and Amer (2016)***, ***Swelam and Mehanna (2017)*** and ***Eldenary, A. et al. (2023)*** with mean values of 1753, 174.3, 645± 0.10, 1149 ± 9.1 and 50.2±0.057 mg/kg, respectively. Meanwhile, ***Shalaby (2016)*** **and** ***Aladhadh et al. (2024)*** revealed a lower histamine value in Egyptian Ras cheese samples having 9.6 and 4.36 mg/kg, respectively.

Rabie et al., (2010) and ***El-Zahar (2014)*** detected higher levels of histamine in Mish with values of 2150 and 310 mg/kg, respectively. Whereas ***Amine et al., (2007), El-Leboudy et al., (2019),*** and ***Ma et al., (2020)*** estimated higher histamine values in both Mish and Egyptian Ras cheese with levels of (195 and 54.2), (170.2 ± 3.34 and 169.3 ± 4.01) and (229.60 ± 5.36) mg/kg, respectively. However, ***Hassan et al. (2020)*** recorded lower histamine levels in both Ras and Mish examined samples at which the levels were 00.379 and 0.193 mg/100 g, respectively, while ***El-Zahar (2014)*** estimated lower histamine levels in Ras cheese samples with mean of 26 ± 2.4 mg/100 g.

Table (2) summarizes the microbiological evaluation of the examined Mish and Egyptian Ras (Rumy) samples. From the examined Mish samples, 18%, 17%, 100% and 94% of them contained *Enterobacteriaceae*, Coliforms, *Lactobacilli,* and Total yeasts and molds with means of $15.5\times10^4 \pm 86.9\times10^3$, $28.3\times10^3 \pm 68.9\times10^2$, $63.3\times10^6 \pm 80.4\times10^5$ and $91.2\times10^2 \pm 14.6\times10^2$, respectively. While 38%, 12%, 100%, and 100% of the examined Rumy samples contained these microorganisms in the same order with means of $17.8\times10^5 \pm 55.8\times10^4$, $93.3\times10^3 \pm 12.8\times10^3$, $73.7\times10^6 \pm 75.1\times10^5$ and $68.6\times10^3 \pm 10.1\times10^3$, respectively.

A higher *Enterobacteriaceae* count in Ras cheese was reported by ***Aladhadh et al. 2024*** was $3.26\times10^{10} \pm 38\times10^{10}$ cfu/ ml. Lower

Coliform and *Lactobacilli* counts in Ras cheese were estimated by ***Eldenary, A. et al. (2023)*** (1.5±0.15 and 4.3±0.43 log) and ***Aladhadh et al. (2024)*** ($4\times10^2 \pm 0.39\times10^2$ and $9.65\times10^6 \pm 13\times10^4$) cfu/g. Meanwhile, ***El-Zahar (2014)*** recorded lower Coliform and *Lactobacilli* counts in both Ras and Mish samples with a means of ($3.93\times10^2 \pm 0.23\times10^2$ and $9.13\times10^6 \pm 0.07\times10^6$) and ($5.53\times10^2 \pm 0.35\times10^2$ and $9.15\times10^6 \pm 0.13\times10^6$), respectively.

A closely similar result for the Total Yeasts and Molds in Mish was reported by **El-Zahar (2014)** with mean of $4.82\times10^3 \pm 0.42\times10^3$, whereas lower results were counted by ***Eldenary, A. et al. (2023)*** and ***Aladhadh et al. (2024)*** with mean values of 5.33±0.17 and $5.12\times10^3 \pm 2.2\times10^2$ cfu/g, respectively.

The high microbial values, which were recorded in these studies, indicate the inferior quality of the milk used in the dairy products manufacture as well as the poor hygienic condition of the process through which these products were manufactured, which consequently will develop more serious problems such as biogenic amines and aflatoxin production.

The variety in the histamine values between the studies may be regarded to the milk used in the cheese manufacture, either raw or pasteurized, inoculated by starter culture bacteria or not, total bacterial count, presence of specific bacterial strains, the manufacturing processes, pH values, salting, ripening, fermentation, and storage conditions ***(Zazzu et al., 2019)***. In this study, the histamine content was higher in the Rumy cheese than in the Mish cheese, which may be because of the high salt content in the Mish, which adversely affected the microbial growth and the production condition of the Rumy cheese, including long ripening and storage periods at favorable temperatures for microbial development. This finding matched the observation of ***Liu et al., (2018)*** who found that there is a high positive correlation with the time of ripening, so the histamine content in ripened cheeses is higher than in unripened fresh cheeses because the amount of amino acid increased during the casein proteolysis throughout the ripening. Therefore, histamine production and accumulation in cheese remain an important issue due to their potentially hazardous health effects, especially with the lack of a specific law limit ***(Nguyen and Chuyen Van, 2021)*.**

Consuming high doses of histamine leads to histamine poisoning, which is considered an allergic reaction with symptoms of headache, flushing, itching, tachycardia, blood pressure instability, and gastrointestinal disorders including diarrhea, vomiting, and abdominal pain. These symptoms vary according to individual sensitivity and genetic predisposition and can persist for a few hours or a day, or

rarely for several days ***(FAO/WHO 2012)*.** Histamine poisoning symptoms are intensified when the other biogenic amines are also present in the consumed food. However, the obtained data in this study and the calculated EDI were lower than the ***BIOHAZ (2011)*** limit, which recommended the maximum allowable limit of histamine to be not more than 50 mg per person per meal, revealing no apparent adverse effect in most of the examined samples, this does not eliminate the possibility of the histamine adverse effect onset because of intaking other foods that may contain high levels of histamine, such as other fermented foods and fish, besides the amount of the consumed food that when it is greater it will deliver higher histamine levels ***(Omer et al., 2021)***. Therefore, it is recommended to look for high-quality dairy products and it is mandatory to

Table (2): *Histamine levels (mg/kg) in the examined Mish and Egyptian Ras (Rumy) cheese (N = 50)*

	Mish	**Egyptian Ras (Rumy)**
Minimum	1.80	21.72
Maximum	21.05	53.86
Mean ± SE	7.99 ± 1.11	29.12 ± 1.77

Table (3): *Descriptive analysis for detected microorganisms in the examined Mish and Egyptian Ras (Rumy) cheese samples.*

Dairy Product	**Microorganism**	**Positive samples**	**Min.**	**Max.**	**Mean ± SE**
Mish	*Enterobacteriaceae*	18%	5×10^2	70×10^4	$15.5 \times 10^4 \pm 86.9 \times 10^3$
	Coliforms	17 %	60×10^2	50×10^3	$28.3 \times 10^3 \pm 68.9 \times 10^2$
	Lactobacilli	100%	30×10^6	10×10^7	$63.3 \times 10^6 \pm 80.4 \times 10^5$
	Total yeasts and molds	94%	48×10^2	18×10^3	$91.2 \times 10^2 \pm 14.6 \times 10^2$
Rumy cheese	*Enterobacteriaceae*	38%	10×10^4	53×10^5	$17.8 \times 10^5 \pm 55.8 \times 10^4$
	Coliforms	12%	50×10^3	15×10^4	$93.3 \times 10^3 \pm 12.8 \times 10^3$
	Lactobacilli	100%	45×10^6	11.8×10^7	$73.7 \times 10^6 \pm 75.1 \times 10^5$
	Total yeasts and molds	100%	82×10^2	93×10^3	$68.6 \times 10^3 \pm 10.1 \times 10^3$

Conclusion

The level of histamine in cheese purchased in Egypt, such as Mish and Egyptian Ras (Rumy), reflects the inferior hygienic conditions where the cheese was produced, finally influencing the consumer's health. In this study, histamine was detected in all examined samples but with accepted non-harmful levels in 80%. Despite histamine poisoning, it may occur with a high consumption rate of food containing biogenic amines or with

individual susceptibility to such disorders. Therefore, controlling the biogenic amine formation in cheese during its manufacture and storage should be applied, legalization of the biogenic amine limits, especially histamine, should be enacted, and the consumer's awareness about the danger of biogenic amines and the products that may contain it should be raised.

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التقييم الميكروبيولوجي والتحليل الكمي لمحتوى الهستامين في بعض أنواع الجبن المصرية باستخدام التحليل الكروماتوجرافي السائل عالي الأداء(HPLC)

احمد حسن على سعد[1]، كاميليا مجدى عبد اللطيف[2]، شيماء محمد توني معوض[3]، آلاء عبده حلمى[1*]

[1] قسم الرقابة الصحية على الأغذية- كلية الطب البيطري بجامعة قناة السويس، مصر

[2] طبيب بيطري حر

[3]قسم صحة الأغذية، معهد بحوث صحة الحيوان، مركز البحوث الزراعية، مصر

الهستامين هو أحد الأمينات الحيوية التي يتم إنتاجها بشكل طبيعي كمنتج ثانوي سام أثناء تحلل الحمض الاميني الهيستيدين أو بسبب أنشطة التحلل البروتيني لبعض الكائنات الحية الدقيقة. هدفت هذه الدراسة إلى تقييم الوضع الميكروبي و مستويات الهستامين في جبن المش و الرومي فتم جمع إجمالي 100 عينة من الجبن، 50 عينة من كلا من جبنة المش والرومي، بشكل عشوائي من مدينة الإسماعيلية بمصر، وتم إخضاعها لتحليل HPLC لتقدير محتوى الهستامين. أظهرت النتائج أن محتوى الهيستامين في عينات جبن المش التي تم تحليلها تراوح من 1.8 إلى 21.05 ملغم/كغم، بمتوسط قيمة 7.99 ± 1.107 ملجم/كجم. وأظهرت عينات الجبن الرومي التي تم فحصها مستويات

هستامين تتراوح بين 21.72 إلى 53.86 ملجم/كجم، بمتوسط 29.12 ± 1.769 ملجم/كجم. اظهر التحليل الميكروبيولوجي احتواء 18% و17% و100% و94% من عينات المش على البكتيريا المعوية و بكتيريا الكوليفورم و بكتيريا انتاج حمض اللاكتيك و كذلك اجمالي الخمائر والعفن و كان متوسط هذه الميكروبات هو$10^3\times86.9$ ± $10^4\times15.5$ و$10^2\times68.9$ ± $10^3\times28.3$ و ± $10^6\times63.3$ $10^5\times80.4$ و $10^2\times14.6$ ± $10^2\times91.2$ وحدة تكوين المستعمرة / جرام على التوالي. وقد تواجدت هذه الميكروبات في عينات الجبن الرومي بنفس الترتيب بالنسب التالية 38% و 12% و 100% و 100% و كانت متوسط تعدادهم كالتالي؛ $10^4\times55.8$ ± $10^5\times17.8$ و$10^3\times12.8$ ± $10^3\times93.3$ و $10^5\times75.1$ ± $10^6\times73.7$ و $10^3\times10.1$ ± $10^3\times68.6$ وحدة تكوين المستعمرة / جرام، على التوالي. وكان الاستهلاك اليومي المقدر من الهستامين من خلال استهلاك المش يقع في حدود 0.021- 0.242 ملجم/ يوم، بينما الاستهلاك اليومي المقدر من الهستامين من خلال للجبن الرومي سيكون بين 0.25 و 0.62 ملجم/ يوم. وعلى الرغم من أن جميع العينات المفحوصة كانت تحتوي على الهستامين، لكنها كانت أقل من الحدود المسموح بها بموجب المواصفة القياسية المصرية لعام 2008 (200 ملجم/كجم)، فإن 20% فقط من عينات الرومي تجاوزت الحدود المسموح بها BIOHAZ لعام 2011 (50 ملجم / يوم) وكانت تحتوي على ≤53.86 ملجم/كجم. و على الرغم من أن المستويات المقدرة ليس لها أي تأثير سلبي واضح، إلا أنها لا تمنع احتمالية التسمم بالهستامين، مع الأخذ في الاعتبار الاختلاف الفردي والعوامل المساهمة الأخرى. و لذلك فإن الالتزام الصارم بممارسات النظافة الجيدة في كل من اختيار المواد الخام وبيئات التصنيع يمكن أن يتحكم بشكل فعال في وجود الكائنات الحية الدقيقة المسؤولة عن الأمينات الحيوية بما في ذلك الهستامين.