Biogenic amine formation and distribution in Roumy cheese, an Egyptian-type hard cheese during the ripening period

Nahed Shehata and Hanan Rdwan

Department of Home Economics, Faculty of Specific Education, Zagazig University, Zagazig, Egypt

المجلة العلمية المحكمة لدراسات وبحوث التربية النوعية

المجلد الثالث– العدد الأول– مسلسل العدد (٥)– الجزءالثاني، يناير ٢٠١٧

رقم الإيداع بدار الكتب ٢٤٢٧٤ لسنة ٢٠١٦

ISSN-Print: 2356-8690 ISSN-Online: 2356-8690

موقع المجلة عبر بنك المعرفة المصري https://jsezu.journals.ekb.eg

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Department of Home Economics, Faculty of Specific Education, Zagazig University, Zagazig, Egypt

Abstract:

Content of biogenic amines (BA) including tryptamine (Try), phenylethylamine (Phe), histamine (His), tyramine (Tye), putrescine (Put), cadaverine (Cad), spermidine (Sper) and spermine (Spr), chemical quality indices (amino nitrogen, AN; total volatile fatty acids, TVFF and malonaldehyde, MDA) and microbiological traits (total bacterial count, TBC; lactic acid bacteria, LAB and Molds/yeasts, M&Y) were evaluated within the ripening interval of 0- 24 weeks in Roumy cheese, an Egyptian-type hard cheese. The average concentrations of Try, Phe, His, Tye, Put, Cad, Sper, and Spr were recorded 1.89, 1.69, 2.02, 14.78, 1.92, 2.90, 0.54 and 0.42 mg/kg, respectively. With the elongation of ripening period, all of these amines were recorded and gradually increased by different ratios individually. At the end of ripening, after 24 weeks, Try, Phe, His, Tye, Put, Cad, Sper and Spr recorded 0.98, 88.90, 4.80, 511.89, 86.55, 26.34, 2.32 and 2.88 mg/kg, respectively. These represent increase of -48.15, 5157.53, 137.20, 3363.40, 4407.81, 808.28, 329.63 and 585.71% for these compounds above the baseline, respectively. The same behavioral was recorded for the chemical quality indices (AN, TVFF and MDA) and microbiological traits (TBC, LAB and M&Y). The distribution study indicated that substantially higher ($P< 0.01$) contents of individual amines and the sum of BA were found in the outer-part samples in comparison with the core ones in the Roumy cheese samples. Also, the correlation analysis revealed that determination of chemical plus microbiological quality indices on the one hand and BA content on the other hand, gave the consistent results. In nutritional and toxicological point of view, Tyramine content increased linearly ($P < 0.01$) with increasing time of ripening in Roumy cheese, and after 12 weeks of ripening (369.50 mg/kg) exceeded a toxicological limit (100 mg/kg of the consumed food). Individuals suffering from food intolerances and food allergies, and patients receiving monoaminoxidase (MAO) inhibitors, should avoid consumption of such ripened cheese.

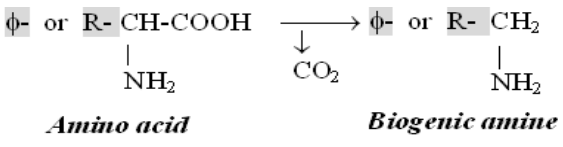
Key words: Biogenic amines, amino nitrogen, total volatile fatty acids, malonaldehyde, microbiological traits, outer-part, core, toxicological limit.

Introduction:

Roumy cheese is one of the main types of cheese in Egypt which derived from the Greek kefalotyri cheese (http://ifood.tv/african/african-cheese/about). It is the main hard cheese in Egypt and belongs to the same family as *Pecorino Romano* and *Manchego*. Roumy cheese is made from cows' milk, or from a mixture of cow and buffalo milk with no starter culture is used (**Hofi *et al.*, 1970**). The milk is natural, with full cream. After 3–4 months the cheese will develop an open texture and a sharp, pungent flavor (**Javier *et al.*, 1990**). Roumy is available in 10 kilograms (22 lb) disks or often slices with variable weight in vacuum packing. There are 100 calories in an ounce serving, with high amount of saturated fat (28%).

At the traditional method of Roumy cheese production, the ripening process is prolongated to more than six months to obtain the best desirable color, flavor and consistency of Roumy cheese. Cheese ripening is a complicated process. It involves several biochemical changes including the fermentation of lactose, the degradation of the protein (proteolysis), and the hydrolysis of fat (Lipolysis) (**Ragab, 2003 and Paul, 2004**). This process results in gradual change in cheese curd from toughness to mellowness and in the development of aroma and taste that constitute the typical cheese flavor.

On the other side, the formation of undesirable and poisoning compounds such biogenic amines is one of the problems connected with proteolysis of some fermented food such as cheese. Biogenic amines are low molecular weight organic bases that possess biological activity. They can be formed and degraded as a result of normal metabolic activity in animals, plants and microorganisms. They are usually produced by the decarboxylation of amino acids according to the following reaction:



After fish, cheese is the next most commonly implicated food item associated with histamine poisoning. The first report case occurred in 1967 in the Netherlands and involved Gouda cheese (**Doeglas *et al.*, 1967**). Since then several cases have been reported in other countries including USA, France, Canada etc. Usually, cheese does not contain amines, but sometimes very high levels are found (**Joosten and Northolt, 1987**). During ripening cheese undergoes marked changes in body smoothness and flavor. These changes are associated largely with proteolysis, resulting in an increase in free amine acid content (**Joosten and Olieman, 1986**). Some of these amino acids can undergo further breakdown by specific bacterial decarboxylases to form carbon dioxide and amine. Factors affecting amines formation in cheese have been reviewed by many authors. These factors include storage temperature, air, pH, salting, the availability of free amino acids and the presence of fermentable carbohydrates such as glucose (**Baranowski *et al.*, 1985; Joosten, 1988; Halasz *et al.*, 1994; El-Aswad, 2001; Ragab, 2003 and Saleh, 2010**).

It is well to know that ready to eat food item, hard cheese, is representing one of the food could be subjected to deterioration in ripening period due to contamination, hydrolysis and oxidation of its content lipid (lipolysis) and protein (proteolysis) by enzymatic as well as microbial reactions. Otherwise, fat of cheese becomes rancid as a consequence of oxidation and oxidative rancidity takes place by autooxidation and microorganisms (**Hofi *et al.*, 1975 and Paul, 2004**). The effect of malonaldehyde, one of the major products of the oxidation of polyunsaturated fatty acids, on human health has been reported by many authors that is mutagenic and carcinogenic (**Shamberger *et al.*, 1974**). Also, microbiological parameters represent a good indicator of keeping quality of different food items. The microbiological quality of fresh and ripening Roumy cheese distributed in Egypt had been studied extensively (**Hofi *et al.*, 1970; Javier *et al.*, 1990**) although there are no enough available data on their correlation with biogenic amines formation during ripening. Therefore, the present study aims to investigate the formation of biogenic amine in Roumy cheese, an Egyptian-type hard cheese during the ripening period. Also, the protein quality (proteolysis), fat quality (lipolysis) and microbiological traits of Roumy cheese as affected by ripening for 6

months at room temperature in correlation with biogenic amines formation will be in the scope of this study.

Materials and Methods:

Materials:

Fresh cow's milk was obtained by special arrangements from milk suppliers in Sharqia Governorate, Egypt. Local rennet was obtained from Smnoud City, Gharbia, Egypt. Iodized salt, ElNasr Saline's Co, Alexandria was used.

Chemicals:

Biogenic amines standard: Ttryptamine (Try), phenylethylamine (Phe), histamine (His), tyramine (Tye), putrescine (Put), cadaverine (Cad), Spermidine (Sper) and Spermine (Spr) and benzoyl chloride (BCl) were purchased from Sigma Chemical Co. (St. Louis, MO, Company agent, Cairo, Egypt).

Reagents: Distilled water was prepared and filtered through 0.45 µM filter membrane; potassium phosphate monobasic and ethanol were obtained from Sigma chemical Co, St Louis, MO, USA. Acetonitril, HPLC grade (Honil Limited, London, United Kingdom).

Buffers: Potassium phosphate monobasic buffer (0.025 M) was prepared by adjusting the pH to 3 then filtered through a 0.45 µM filter membrane and stored at 4˚C until using.

Others: Pure chemicals and buffers, analytical grade, were purchased fron El-Gomhoria for Trading Drugs Chemicals and Medical Instruments, Cairo, Egypt.

Equipments:

All analysis were carried out with TSP (Thermo Separation Products Inc.) HPLC system consisting of Consta METRIc 4100 series pump, Spectra series AS 100 autosampler, spectra system UV 1000, UV-visible variable wavelength detector, spectra system FL 3000 Fluorescence detector and interfaced with IBM computer equipped with PC 1000 Chromatograph software version 3.5. The column used was a reversed-

phase water Spherosorb ODC-2 (3µM; 150mm × 4.6mm I.d., Alltech USA).

Chemicals and Standards:

Roumy Cheese Making:

Roumy cheese, an Egyptian-type hard cheese samples were processed in laboratory according to the procedure suggested by Yossef (1966). Standardized milk (3.5% fat) was heated to 32ºC and sufficient rennet was added in the proportion of 2.5g per 100 kg milk to complete coagulation in 30- 40 minutes. The coagulum was cut into small pieces then vigorously stirred. The temperature of the vat was then raised to 45ºC over a period of around 40-50 minutes, and gently stirring was maintained throughout. After the curd had settled, the whey drained out (acidity~0.14%), salt was sprinkled over the curd at a ratio of 1% (w/w), and the curd was manually pushed to the sides of the fat. Molds, lined with cheesecloth, were filled with sufficient curd to produce one finished cheese, and manual pressure was applied to expel some of the remaining whey. Light mechanical pressure follows over the next three hours at which point the cheese was reversed in the press and left under pressure for 12 hours. The cheese wheels were then removed from the moulds and cloths and placed in the salting chamber. After draining for a further day at ambient temperature, the surfaces of each cheese wheels were coated with a small amount of dry salt. By the following day, most of this salt will had be absorbed into the cheese, so that the wheels were turned and the dry salting process repeated once again. This dry salting produce was continued for a period of around forty-five day, either every other day or once every three days. After completely salting process, cheese wheels was ripened at 16 ºC for six months in a digital incubator (MS Broedmachine- Incubation Experts Ltd., UK), thereafter samples were taken after 4, 8, 12, 16, 20 and 24 weeks for chemical and microbiological analysis. The average values are given ±SD.

Biogenic amines determination:

Biogenic amines were extracted and derivatized according to the methods of **Moret and Conte (1996)**. In brief, 10 g of cheese samples were homogenized in Polytron homogenizer with 20 ml 0.1 M HCl in

stainless steel pestle (Two extractions). The organic extracts were saturated with NaCl and the pH was adjusted to 11.5 with an automatic titrator. An extraction with butanol was then performed. This was carried out in a test tube on 5 ml of acid extract, with three portions of 5 ml each (Vortex agitation). Derivatization of biogenic amines the derivatization of extracted amines was then performed in a test tube as follows: 2 drops of 1 M HCl was added to 1 ml of organic extract in test tube and had been dried under vacuum. Then 1 ml of 0.1 M HCl, 0.5 µl saturated solution of $NaHCO_3$ and 1 ml benzoyl chloride solution (5 mg/ml) were added. The reaction vessel was incubated at 40^0C for 1 h, then the solution was dried under vacuum, mobile phase was added and HPLC injection followed. The chromatographic conditions were as following: Flow rate, 1ml/min; detection, UV absorption at 265 nm; volume of injection, 20 µl; and temperature, room temperature. The mobile phase composition was an isocratic system of 0.025 M potassium phosphate monobasic (pH, 3) : acetonitril (75 : 25 v/v). Biogenic amine standards were chromatographed singly and in a mixture (See Figure 3). The same volume of cheese extracts was chromatographed under the same conditions. All analysis was run in triplicates.Retention times and absorbance ratios (from two detectors) against those of standards were used to identify the separated phenolic acids and to check their purity. Quantitative determination of each biogenic amine was determined from its respective peak areas and their corresponding response factors.

Amino nitrogen:

The amino nitrogen was determined using the method of **Kolchov (1952)**. In brief, 25 gof fresh sample were soaked over - night at 4°C in NaCl solution using a 250 ml volumetric flask. 50 ml of the filtrate was taken into a 100 ml volumetric flask on the second day, then 1 ml of phenolphthalein indicator, 0.5% and 2 g of $BaCl_2$ were added. After shaking, saturated Ba $(OH)_2$ solution was added until a red color appeared, then excess of $Ba(OH)_2$, about 5 ml, was added. The volume was completed by distilled water to make up the mark, shaken and allowed to stand 15 min. 50 ml were taken from this solution and titrated with 0.2 N HCl to rose color, then 20 ml of neutral formaldehyde solution were added until the solution became colorless and titrated with 0.05 N

NaOH. results were presented as mg amino nitrogen l00 g sample according to the following equation:

Amino nitrogen (mg/100 g sample) =

$$\frac{(V_1 - V_2) \times N \times 0.014 \times 250 \times 100}{}$$

Where: V_1, volume of NaOH (ml) for sample; V_2, volume of NaOH (ml) for blank; N, normal concentration of NaOH; W, weight of Sample

Total volatile fatty acids:

The total volatile fatty acids were estimated according to **DiLallo and Albertson (1961)**. In brief, A 10 ml of samples was transfer to beaker, heated on magnetic stirrers and the pH of the solutions with initial pH below 5.75 was raised with concentrated NaOH solution to values of between 6.5 and 7.0. The samples were titrated with sulfuric acid to pH 5.75, 5.0, 4.3, 4.0 and 3.3–3.5. After boiling, the pH of the samples was raised first to 4.0 and then 7.0. The normality of the sulfuric acid and sodium hydroxide used was between 0.02 N and 0.20 N, and 0.01 N and 0.40 N, respectively. The solutions of sulfuric acid were standardized with primary standard sodium carbonate (Na_2CO_3), while sodium hydroxide solutions were standardized with previously standardized solutions of sulfuric acid. Total volatile fatty acids was calculated as follow:

$$\text{Total volatile fatty acids (TFFA, mg/l)} = \frac{\text{Volume NaOH pH4 to 7} \times \text{N NaOH} \times 50000}{\text{Sample volume}} \times F$$

F= 1 if TVFA ≤ 180 mg/l and F=1.5 if TVFA >180 mh/l

Malonaldehyde content:

Malonaldehyde (MDA) content was determined as described by **Pearson (1970)** as follows: 10 g of the sample were distilled with (47.5 ml of distilled water + 2.5 ml HCI, 4N) for 10 min. 5 ml of the distilled water were added to 5 ml of thiobarbituric acid (TBA) reagent (0.2883 g TBA/100 ml of 90% glacial acetic acid) into stoppered tube, and then heated in a boiling water bath 35 min. After cooling, absorbance was measured at 538 nm using Labo-med., Inc., spectrophotometer. The TBA

value was calculated by multiplying the absorbance (ABS) by the factor (7.8). The results represented as mg MDA/kg sample.

Microbiological examination:

Preparation of sample:

To prepare the sample for bacteriological analysis, the outer 0.5 inch portion from each block of cheese was discarded by means of a sterile knife. One gram from the freshly exposed area of the cheese was accurately weighed into a sterile watch glass, and then transferred to a sterile mortar. One ml of 20% solution of sodium citrate was then added and the cheese was thoroughly ground into a homogenous suspension by means of a sterile pastel. Finally, 8 ml of a sterile distilled water, previously warmed to 37^{o}C, were added and mixed well. The suspension thus obtained was 1: 10 dilution of the cheese which was used for preparing other serial dilutions.

Total bacterial count (TBC):

TBC was determined by plating suitable dilution in duplicates using nutrient agar medium (**Difco Manual, 1966**). This medium consists of beef extract, bacto peptone, agar and sodium chloride by 3, 5, 15, and 5 g/L, respectively and completed by distilled water to1000 ml then the pH adjusted to 7. Plates were incubated at 32ºC for 3 days before counting and recording the results.

Total molds and yeasts (M&Y)

Poured plate method was used according to the method of **APHA (1998)**. One ml of suitable folds serial dilutions of all cheese samples were inoculated onto a plate containing potato dextrose agar (PDA) medium (three replicates). Approximately 15 ml of PDA medium at about 50°C was poured in each plate, then thoroughly mixed and left for solidification. The plates were incubated at 25°C for 5 days. After the incubation period, developed colonies were counted per each plate. The mean count of plates was recorded to represent molds and yeasts count.

Lactic acid bacteria (LAB):

LAB was determined according to the method of **Rogasa *et al*. (1951)** which modified by **Helmy (1956)**. The medium components were

steamed at 100 ^{0}C for 30 minutes. It was found to Keep a long time without deterioration in the refrigerator.

Statistical analyses:

Analysis of variance was performed on data using the General Linear Modles Procedure (GLM) of the Statistical Analysis System (**SAS, 2004**).

Results and Discussion:

Biogenic amines concentration (mg/100 g) in Roumy cheese, an Egyptian-type hard cheese during the ripening period:

The single amine recovery illustrated in Table (1). From such data it could be noticed that the percent of recovery for all detected amines were ranged 69.69 to 96.30 %. The highest recovered amine was recorded for tryptamine while the lowest one was tyramine. All of these data are in accordance partially with that obtained by **Moret and Conte (1996), Ragab (2003) and Saleh, (2010)** who mentioned that the differentiation in recovery percent noticed amongst the all detected amines could be attributed to the different in their selective extraction. The selective extraction of single amine from acid solution is a process influenced by many factors such as: the type of acid, the type of organic solvent, the salt used for saturation, the pH at which amine extraction is carried out, the time and the type of stirring, etc.

Table (1): Single amine recovery of Egyptian-type hard cheese.

Amines	Amount detected in samples (mg/ 100 g)	Amount of Std added (mg/ 100 g)	Total amount detected in samples (mg/ 100 g)	Recovery (%)
Tryptamine	2.22	2.10	4.16	96.30
Phenylethylamine	1.60	1.20	2.54	90.71
Histamine	1.55	1.22	2.65	95.67
Tyramine	3.15	1.70	3.38	69.69
Putrescine	2.03	1.01	2.46	80.92
Cadaverine	1.13	1.17	1.81	78.70
Spermidine	1.00	1.13	1.58	74.18

The biogenic amines concentration in Roumy cheese, an Egyptian-type hard cheese during the ripening period are listed in Table (2) and Figure (1). The average concentrations of tryptamine (Try), phenylethylamine (Phe), histamine (His), tyramine (Tye), putrescine (Put), cadaverine (Cad), spermidine (Sper) and spermine (Spr) were 1.89, 1.69, 2.02, 14.78, 1.92, 2.90, 0.54 and 0.42 mg/kg, respectively. With the elongation of ripening period, all of these amines were recorded and gradually increased by different ratios individually. At the end of ripening, after 24 weeks, Try, Phe, His, Tye, Put, Cad, Sper and Spr recorded 0.98, 88.90, 4.80, 511.89, 86.55, 26.34, 2.32 and 2.88 mg/kg, respectively. These represent increase of -48.15, 5157.53, 137.20, 3363.40, 4407.81, 808.28, 329.63 and 585.71% for these compounds above the baseline, respectively. Also, the rate of increasing was higher noticeable from the start point up to 16, 24, 12, 20, 24, 24, 20 and 20 weeks of ripening for Try, Phe, His, Tye, Put, Cad, Sper) and Spr and less after that, respectively.

In compared with the other studies, the obtained data are in accordance with that observed by **El-Leboudy *et al*. (1995) and Saleh (2010)**. Lower values for tyramine and putrescine in Damiatta cheese were reported by **Mehanna *et al*. (1989)**. Nearly much higher values for Phe, Tyr, Put and Cad were reported by **Ragab (2003) and Saleh (2010)**. Tyramine formation was observed in cheese made from milk contaminated with *lactobacillus* strain and *Streptococcus faecalis* (**Joosten and Northolt, 1987**). However, **Koehler and Eitenmiller (1978)** found that tyramine was present in 90% of cheese samples studied. On the other side, histamine formation was only observed when certain strains of *lactbacilli* contaminated the cheese. It was stimulated under certain circumstances as ripening, storage temperature, salt content, started cultures and nature of milk (**Joosten and Stadhouders, 1987**). Finally, **Parrot and Nicot (1965)** reported that certain diamines such as putrescine and cadaverine enhance the toxic amount of histamine by facilitating passage of the mono amine through the intestinal barrier.

Several studies have reviewed factors affecting amine formation in cheese. These factors include: storage temperature (**Ababouch *et al*., 1991; Stratton *et al*., 1991; Nout *et al*., 1993; and Halasz *et al*., 1994**), air redox potential of the medium (A**rnold and Brown, 1978; and**

Halasz *et al.*, 1994), pH (**Teodororic *et al.*, 1994; Pogorzelski, 1992; and Maijala *et al.*, 1993**), salting (**Yatsunami and Echigo, 1993; Teodorovic *et al.*, 1994**) and microorganisms present (**Joosten and Northolt, 1987 and Joosten, 1987**). From such studies it could be noticed that increasing of storage/ ripening temperature and degree of salting, reducing the redox potential of the medium, and acidic environment, leads to increase the formation of biogenic amines in cheese. This information's gives an explanation for the larger concentrations of biogenic amines reported in repining cheese than the fresh ones.

From the nutritional and toxicological points of view, the sum of all biogenic amines after 24 weeks of ripening was well under the safety limit of 900 mg/kg for the sum of tyramine+ histamine + putrescine + cadaverine according to **Valsamaki *et al.* (2000)**. In this concern, **Taylor (1985)** mentioned that the level of 1000 mg/kg (amine/food) is considered dangerous for health. This level is calculated on the basis of food borne histamine intoxications related to amine concentration in food. The discrepancy in the toxic histamine level in food might be due to the absence or presence of other synergistic biogenic amines like putrescine and acdaverine.

As a potent vasoactive substance, tyramine is considered to be the most toxic biogenic amine, toxicological levels being above 100 mg/kg of the consumed food (**Silla-Santos, 1996**). From this viewpoint, Roumy cheese should be approached with caution when ripened more than 8 weeks. On the other hand, considering that toxic dose for patients receiving non-selective monoamine oxidase (MAO) inhibitors was suggested to be 6 mg of tyramine (**Novella-Rodrigues *et al.*, 2003**), already 20 g portion of Roumy cheese, 12 weeks old, could be dangerous for these patients. In the time interval from the 12th week of ripening, Roumy cheese contained also higher levels of putrescine, a known tyramine toxicity potentiator (**Bover-Cid *et al.*, 2000**).

Table (2): Biogenic amines concentration (mg/kg) in Roumy cheese, an Egyptian-type hard cheese during the ripening period.

Biogenic amine	Ripening time (weeks)													
	0		4		8		12		16		20		24	
Tryptamine (Try)	1.89±	0.19^{c}	3.02±	0.49^{b}	3.40±	0.47^{b}	5.02±	0.42^{a}	5.89±	1.02^{a}	2.12±	0.25^{c}	0.98±	0.19^{d}
Phenylethylamine (Phe)	1.69±	0.11^{f}	2.88±	0.37^{f}	7.80±	1.08^{e}	37.11±	3.13^{d}	58.80±	10.18^{c}	72.40±	8.49^{b}	88.90±	16.95^{a}
Histamine (His)	2.02±	0.20^{bc}	1.20±	0.26^{bc}	3.50±	0.35^{b}	8.64±	0.73^{a}	6.85±	1.02^{a}	7.87±	0.95^{a}	4.80±	0.81^{b}
Tyramine (Tyr)	14.78±	1.52^{f}	44.60±	5.80^{e}	72.80±	10.12^{d}	369.50±	29.15^{c}	588.43±	77.45^{b}	632.21±	74.10^{a}	511.89±	97.62^{b}
Putrescine (Put)	1.92±	0.19^{d}	4.12±	0.60^{d}	8.70±	1.34^{d}	38.78±	3.27^{c}	58.70±	10.17^{b}	77.29±	9.46^{a}	86.55±	14.51^{a}
Cadaverine (Cad)	2.90±	0.39^{d}	3.20±	0.48^{d}	8.25±	1.15^{bc}	10.67±	0.94^{bc}	12.42±	2.20^{bc}	19.20±	3.25^{b}	26.34±	5.02^{a}
Spermidine (Sper)	0.54±	0.05^{c}	0.89±	0.12^{c}	2.48±	0.21^{b}	3.70±	0.31^{b}	6.88±	1.19^{a}	7.32±	0.86^{a}	2.32±	0.40^{b}
Spermine (Spr)	0.42±	0.06^{b}	0.76±	0.13^{b}	2.20±	0.31^{a}	3.04±	0.16^{a}	4.02±	0.84^{a}	3.23±	0.43^{a}	2.88±	0.55^{a}
Sum of BA	26.16±	2.83^{f}	60.67±	7.97^{e}	109.13±	17.17^{d}	476.46±	36.17^{c}	741.99±	91.51^{ab}	821.64±	90.30^{a}	724.66±	118.19^{b}

Each value represents the mean of three replicates ± SD. Means with the different superscript letters in the same row are significantly different at $p \leq 0.05$.

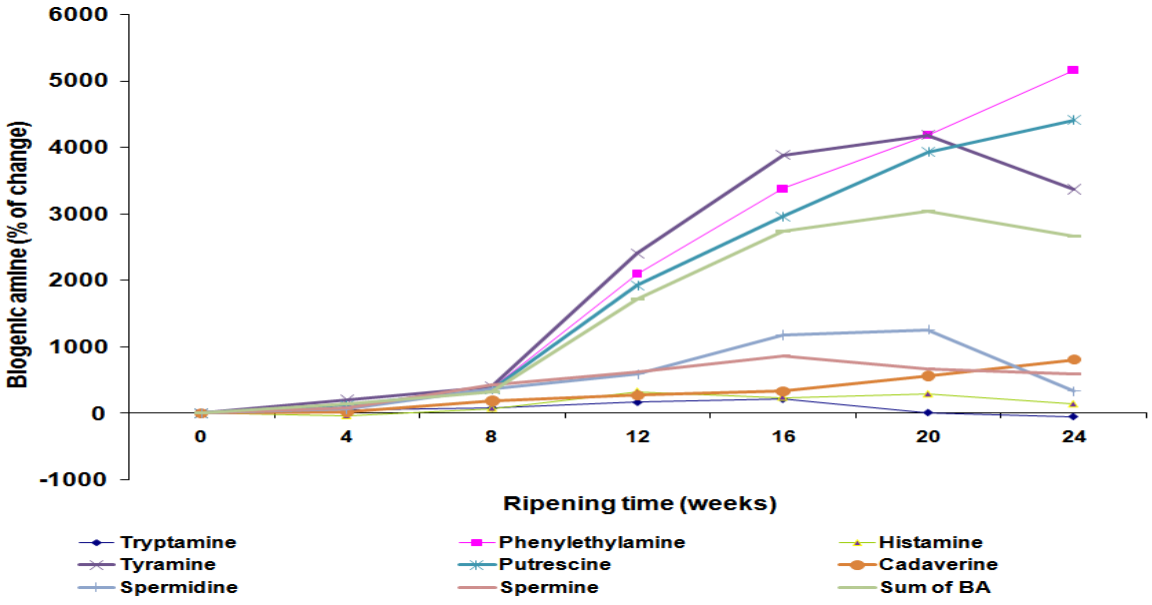


Fig. (1): Biogenic amines concentration (as a% of control) in Egyptian-type hard cheese during the ripening period.

Chemical quality indices of Roumy cheese, an Egyptian-type hard cheese during the ripening period:

Data in Table (3) and Figure (2) were shown the chemical quality indices of Roumy cheese, an Egyptian-type hard cheese during the ripening period. From such data it could be noticed that fresh samples of Roumy cheese, the amino nitrogen (AN), total volatile fatty acids (TVFA) and malonaldehyde (MDA) contents were recorded 0.08 g/100 g, 23.76 ml 0.1 N NaOH/100 g and 0.229 mg/g fat, respectively. With the elongation of the ripening period, all of these quality parameters were gradually increased by different ratios individually. At the end of ripening, after 24 weeks, they recorded 1.42g/100 g, 73.87ml 0.1 N NaOH/100 g and 0.802 mg/g fat, respectively. These represent increase of 1675, 210.91 and 250.22% for these parameters above the baseline, respectively. Such data are in accordance with that obtained by **Mahran (1999)** on samples of pickled cheese collected from the Middle-Delta Governorates of Egypt. Also, **Ragab (2003)** reported that AN, TVFA and MDA were increased in Mish cheese with the elongation of storage period.

Many studies reviewed that several bacterial genus responsible for spoilage of refrigerated dairy products are highly proteolytic and can cause flavor defects (**El-Aswad, 2001 and Paul, 2004**). Proteolytic enzymes produced by psychrotrophic bacteria during growth in milk often remain active after heat treatments, and they may damage the quality of stored, heat treated products. On the other side, the degree of lipolysis in cheese depends on the variety and varies from slight to extensive. Extensive lipolysis in internal bacterially-ripened cheese (e.g. Cheddar, Gouda and Swiss) is undesirable, while in mould-ripened cheese, lipolysis is essential for flavour development (**McSweeney and Fox, 1993 and Paul, 2004**). A number of procedures have been developed to quantify lipolysis including free fatty acids and MDA (**Ragab, 2003**). Several years ago, there is an increasing international concern about the precence and the adverse effects of some toxic compounds such as MDA. It is formed in fresh and ready to eat foods including cheese as a consequence of oxidation of their contents of polyunsaturated fatty acids during storage, processing and cooking (**Gray and Morton, 1981 and Shalaby, 2015**). The effect of MDA on human health has been reported by many authors that is mutagenic and carcinogenic (**Shamberger *et al.*, 1974 and Farmer and Davoine,**

2007). The significance for human health of any of these reported concentrations of MDA in Roumy cheese samples are unknown, but reports that this substance is mutagenic and carcinogenic emphasizes the desirability
of minimizing their occurrence during marketing, storage and

Table (3): Chemical quality indices of Roumy cheese, an Egyptian-type hard cheese during the ripening period.

Parameters	Ripening time (weeks)						
	0	4	8	12	16	20	24
Amino nitrogen (AN, g/100 g)	0.08± 0.02 ^{e}	0.24± 0.09^{d}	0.48± 0.12^{c}	0.56± 0.13^{c}	0.93± 0.25^{ab}	1.19± 0.21^{ab}	1.42± 0.30 ^{a}
Total volatile fatty acids (TVFA, ml 0.1 N NaOH / 100 g)	23.76± 3.02 ^{d}	67.92± 5.32^{c}	77.54± 8.12^{ab}	81.21± 5.98^{a}	81.66± 6.69^{a}	79.53± 7.92^{ab}	73.87± 9.34^{ab}
Malonaldehyde (MDA, mg/g fat)	0.229± 0.09 ^{e}	0.286± 0.09^{e}	0.478± 0.07^{d}	0.513± 0.06^{c}	0.671± 0.10^{b}	0.769± 0.11^{a}	0.802± 0.08 ^{a}

Each value represents the mean of three replicates ± SD. Means with the different superscript letters in the same row are significantly different at $p \leq 0.05$.

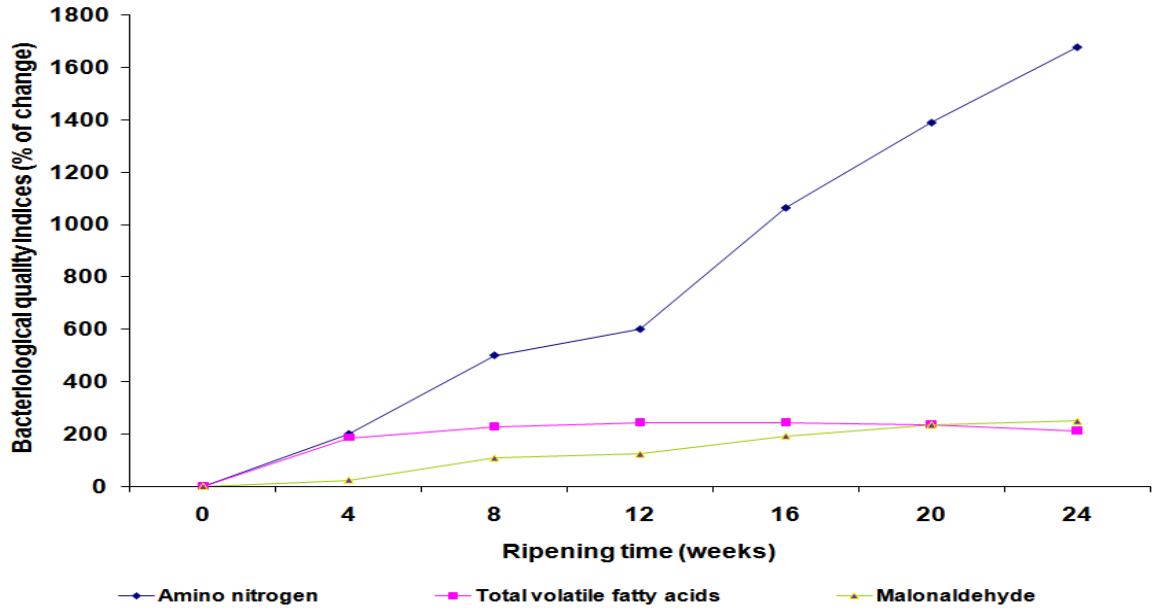


Fig. (2): Chemical quality indices of Egyptian-type hard cheese during the ripening period.

Unfortunately, there are no limits concern the allowed level of TVFA value, and MDA content for fat extracted from Roumy cheese, mentioned in FAO/WHO Codex and the Egyptian Standard Specification. Consequently, it is difficult to evaluate the obtained data regarding the TVFA and MDA levels found in both fresh and repined Roumy cheese, under the present study.

Bacteriological quality indices of Roumy cheese, an Egyptian-type hard cheese during the ripening period:

Data in Table (4) and Figure (3) shows the Bacteriological quality indices of Roumy cheese, an Egyptian-type hard cheese during the ripening period. In fresh samples of Roumy cheese, the total bacterial count (TBC, cfux10^7/g), the total of lactic acid bacteria (LAB, cfux10^7/g), and total moulds and yeasts (M&Y, cfux10^4/g) were recorded 1.8, 0.6 and 0.4, respectively. With the elongation of ripening period, all of these factors were gradually decreased by different ratios individually. At the end of repining, after 24 weeks, they recorded 4.7, 1.9 and 18.2, respectively. These represent decrease of 161.11, 216.67 and 4450% for these parameters under the baseline, respectively. Such data are in accordance with that obtained by **Mahran (1999)** on samples of pickled cheese collected from the Middle-Delta Governorates of Egypt. Also, **Ragab (2003)** reported that TBC, LAB and M & Y were increased in Mish cheese with the elongation of storage period.

Table (4): Bacteriological quality indices of Roumy cheese, an Egyptian-type hard cheese during the ripening period.

Parameters	Ripening time (weeks)						
	0	4	8	12	16	20	24
Total Bacterial Count (TBC, cfux10^7 /g)	1.8± 0.12 ^{d}	2.5± 0.19 ^{c}	3.4± 0.48 ^{c}	4.4± 0.61 ^{ab}	5.6± 0.54 ^{a}	5.4± 0.66 ^{a}	4.7± 0.49 ^{ab}
Lactic acid bacteria (LAB, cfux10^7 /g)	0.6± 0.11 ^{c}	0.9± 0.13 ^{c}	1.3± 0.10 ^{b}	2.5± 0.19 ^{a}	3.1± 0.23 ^{a}	2.8± 0.33 ^{a}	1.9± 0.28 ^{b}
Molds/yeasts (M&Y, cfux10^4 /g)	0.4± 0.06 ^{e}	0.9± 0.08 ^{e}	2.1± 0.17 ^{d}	8.3± 1.54 ^{c}	13.4± 2.08 ^{b}	14.8± 1.89 ^{b}	18.2± 2.34 ^{a}

Each value represents the mean of three replicates ± SD. Means with the different superscript letters in the same row are significantly different at $p \leq 0.05$.

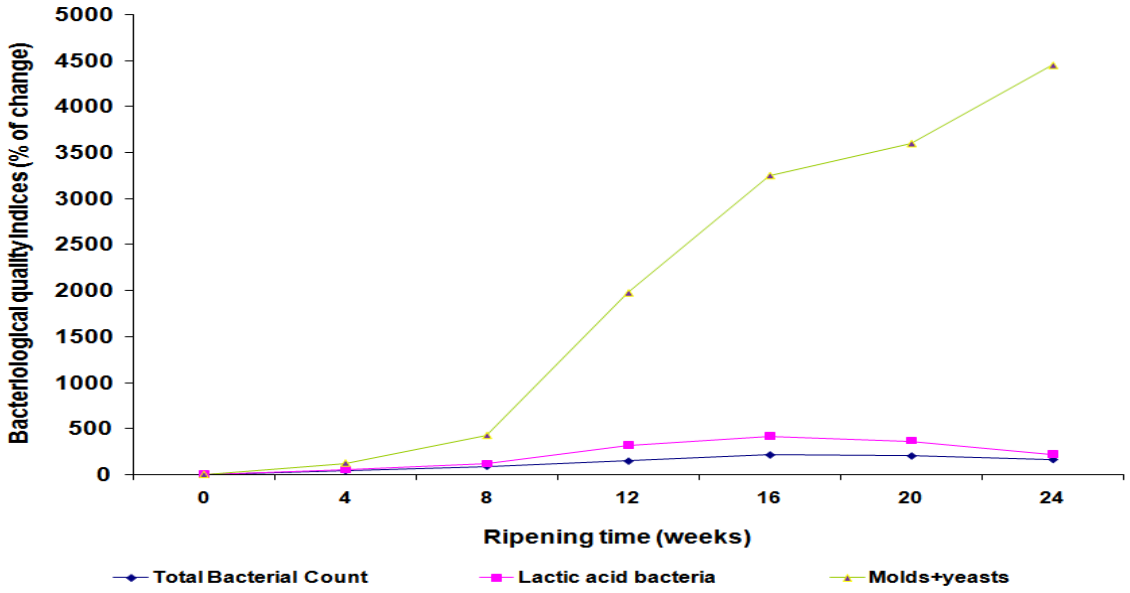


Fig. (3): Bacteriological quality indices of Egyptian-type hard cheese during the ripening period.

The amount and type of amine formed in foods including cheese depends on the nature of the commodity and on the microorganisms present. Many *enteobacteriaceae*, and certain *lactobacilli* e.g. *Lactobacillus buchneri*, *pediococci* and *enterococci* are particularly active in the formation of biogenic amines. Histamine represents one of the highest biological activities of all the amines, and formed by the enzyme, histidine decarboxylase. Numerous bacterial genera have been reported to possess histidine decarboxylase activity, *Escherichia*, *Salmonella*, *Clostridium*, *Bacillus* and *Lactobacillus*. **Sumner *et al.* (1990)** reported that *L. buchneri* strains in culture produce histamine at levels of <1 - 4070 nmol/ml, while *L. brevis* strains produced < 1-6 nmol/ml. Also, **Engesser *et al.* (1989)** investigated 26 authentic and isolated strains of lactic acid bacteria for histamine and tyramine production. Under the test conditions only one strain (*L. buchneri*) was found to be a strong producer of histamine, while only *Enterococcus faecalis* formed significant concentrations of tyramine.

In general, amine formation by bacteria is influenced by many factors include temperature, pH, oxygen supply and fermentable carbohydrate. Regarding the temperature, **Halasz *et al.* (1994)** mentioned that *Enterobacter cloacae* produced 2 mg/ml putrescine after 24 hours of incubation at 20^0C but was unable to synthesize amine at 10^0C. Also, *Klebsiella pneumoniae* was not as sensitive to temperature but did show less extensive cadaverine production at 10^0C than at 20^0C. In related to pH, **Hudson and Duane (1978)** reported that the optimum pH for the amino acid decarboxylases activities was in the range 2.5 to 6.5. Also, the oxygen supply also appears to have a significant effect on the biosynthesis of amines. In this connection, **Halasz *et al.* (1994)** mentioned that *Enterobacter cloacae* only produced about half as much putrescine in an anerobic situation than under aerobic conditions, and *K. pneumoniae* synthesized significantly less cadaverine but gained the ability to produce putrescine under anaerobic conditions. Furthermore, the presence of fermentable carbohydrate, such as glucose, enhance the amino acid decarboxylase activity (**Hudson and Duane, 1978**).

Biogenic amine distribution within the Roumy cheese, an Egyptian-type hard cheese :

The differences between the inner and the outer part were tested using the Roumy cheese repining samples (Table 5 and Figure 4). Higher tyramine content in the outer-part in comparison with the core was found in the samples after 24 weeks of ripening (723.20 vs. 369.50 mg/kg, $P<0.01$). Similar results were found regarding sum of total biogenic amines (1003.36 vs. 500.78 mg/kg $P<0.01$). Also, higher phenylethylamine, histamine and putrescine were recorded in the outer-part in comparison with the core in the same samples. Such behavior was recorded by **Komprda *et al.* (2007)** in Dutch-type hard cheese but with lower content of tyramine and BA than in the present study. Also, **Petridis and Steinhart (1996-a)** reported an increase in histamine and tyramine content in the blocks of a Swiss-type cheese from the core outwards; the outermost segment differed significantly ($P<0.05$) in this aspect from all other inner segments. Such data could be explained by the effect of many factors including water activity (aW) and growth conditions of microorganisms on the formation of BA. For example, **Petridis and Steinhart (1996-a)** used the cheese ripened under the rind

and considered their results rather surprising due to the supposed lower water activity (aW) in the edge in comparison with the inner parts. Also, **Komprda *et al.* (2007)** explained such results by the good growth conditions for the aerobic and aerotolerant microorganisms (*enterococci and coliforms*) in the outer part of the cheese. They also reported that the cheese was ripened in the foil but relatively high aW values under the foil could be reasonably supposed together with favourable conditions for the growth of anaerobes. Anaerobic counts, but also TBC and LAB were higher ($P<0.01$) in the outer-part samples as compared to the core. On the other side, **Novella-Rodrigues *et al.* (2003)** found higher tyramine content and lower tryptamine content inside the hard-ripened raw-milk goat cheese as compared to the edge. They mentioned that unspecified different external and internal micro-environmental conditions, and possible differences of tyramine and tryptamine producers regarding O2 requirements. Finally, **Komprda *et al.* (2007)** reviewed that starter culture and time of ripening could be affected on the content of quantitatively important biogenic amines and counts of microorganisms on different parts of hard cheese.

Table (5): Biogenic amine distribution (mg/kg) within the Roumy cheese, an Egyptian-type hard cheese.

Biogenic amine	Distribution zone (layer)									
	1		2		3		4		5	
Tryptamine (Try)	4.19±	1.24 ^{a}	3.12±	0.67 ^{b}	1.87±	0.57 ^{c}	0.98±	0.23 ^{d}	5.02±	0.81 ^{a}
Phenylethylamine (Phe)	82.25±	8.90 ^{a}	74.20±	9.43 ^{b}	67.41±	12.40 ^{b}	54.42±	15.85 ^{c}	49.11±	6.34 ^{d}
Histamine (His)	8.04±	0.98 ^{a}	8.04±	1.08 ^{a}	8.04±	1.77 ^{a}	4.80±	0.76 ^{b}	4.64±	0.79 ^{b}
Tyramine (Tyr)	723.20±	43.67 ^{a}	651.00±	32.67 ^{a}	498.20±	29.56 ^{b}	398.54±	33.77 ^{c}	369.50±	38.17 ^{c}
Putrescine (Put)	142.00±	7.98 ^{a}	113.50±	8.76 ^{b}	76.20±	7.93 ^{c}	60.80±	8.39 ^{d}	58.78±	7.23 ^{d}
Cadaverine (Cad)	31.20±	4.56 ^{a}	24.18±	2.90 ^{b}	17.60±	2.36 ^{c}	12.12±	3.05 ^{d}	10.67±	1.08 ^{d}
Spermidine (Sper)	9.28±	2.98 ^{a}	7.30±	1.09 ^{a}	3.21±	0.85 ^{b}	2.32±	0.61 ^{bc}	2.02±	0.58 ^{bc}
Spermine (Spr)	3.20±	0.59 ^{a}	2.90±	0.63 ^{b}	1.88±	0.54 ^{b}	1.88±	0.19 ^{b}	1.04±	0.11 ^{c}
Sum of BA	1003.36±	104.56 ^{a}	884.24±	29.50 ^{b}	674.41±	23.76 ^{d}	535.86±	40.87 ^{d}	500.78±	56.45 ^{d}

Each value represents the mean of three replicates ± SD. Means with the different superscript letters in the same row are significantly different at $p\leq0.05$.

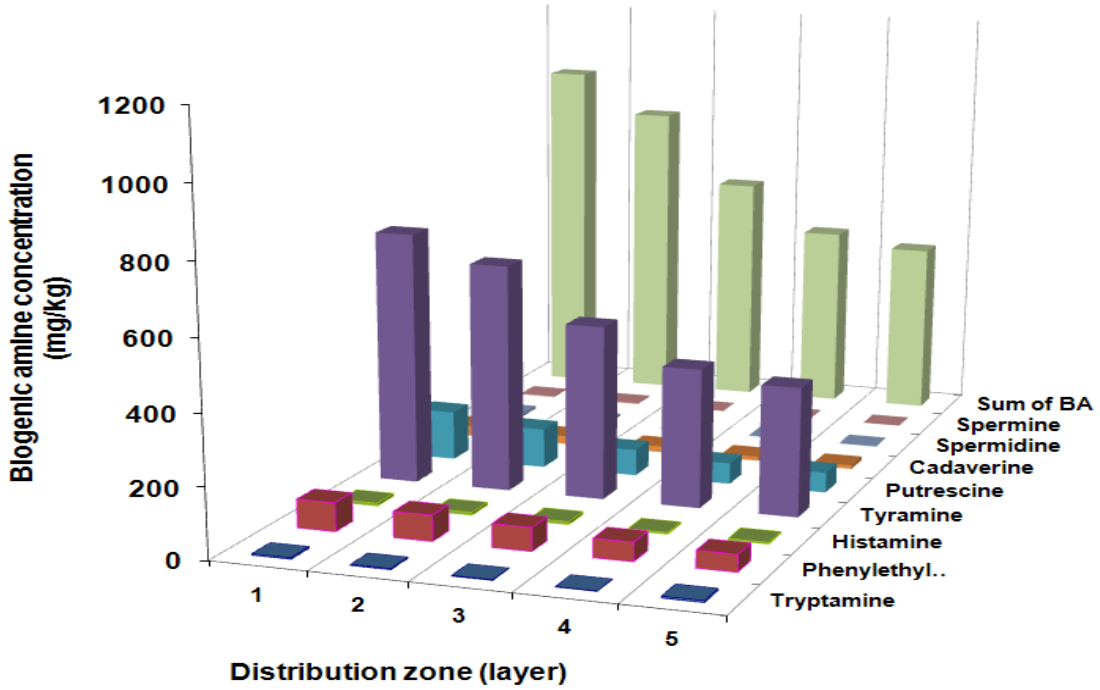


Fig. (4): Distribution of biogenic amines concentration (mg/100 g) in Egyptian-type hard cheese during the ripening period.

Correlation studies:

In the correlation analysis, important differences were found between BA formation in Roumy cheese and many chemical and bacteriological quality indices. Data in Table (6) showed that chemical quality indices have significant positive correlations including AN (r^2=0.803, $P \leq 0.05$), TVFA (r^2=0.785, $P \leq 0.05$), and MDA (r^2=0.849, $P \leq 0.01$) with the BA detected in Roumy cheese affected by repining for 24 weeks. Also, bacteriological quality indices have showed significant positive correlations including TBC (r^2=0.705, $P \leq 0.05$), LAB (r^2=0.738, $P \leq 0.01$), and M&Y (r^2=0.674, $P \leq 0.05$) with the BA detected in Roumy cheese affected by repining for 24 weeks.

Table (6): Correlation between biogenic amines concentration and chemical as well as bacteriological quality indices in Egyptian-type hard cheese during the ripening period.

Parameters	r^2	Parameters	r^2
BA/AN	0.803*	BA/TBC	0.705*
BA/TVFA	0.785*	BA/LAB	0.738**
BA/MDA	0.849**	BA/M&Y	0.674*

*** P ≤ 0.05, ** P ≤ 0.01**

These correlations confirm that if there were no change in the BA in Roumy cheese during repining process, it would be difficult to observe low chemical and bacteriological quality indices. By other meaning, the microbiological analysis based only on basic groups of microorganisms was inconclusive regarding differences in BA content in the cheeses. In similar study, **Komprda *et al.* (2007)** reported that total aerobic counts (cfu g^{-1}) were positively correlated ($P < 0.01$) with histamine ($r = 0.45$), putrescine ($r = 0.40$) and total biogenic amine ($r = 0.60$) content (in mg kg^{-1} of fresh matter) in Dutch hard cheese and with tyramine content ($r = 0.59$; $P < 0.01$) samples. The relationship between content of the biogenic amines (mg kg^{-1}) and lactic acid bacteria (cfu g^{-1}) was similar: $r = 0.53$, 0.62, 0.46 and 0.65 for histamine, tyramine, putrescine and sum of biogenic amines, respectively ($P < 0.01$). Also, Petridis and Steinhart (1996b) found that LAB counts and tyramine content were correlated closely in the Swiss-type cheese.

Conclusion:

Roumy cheese is one of the main types of cheese in Egypt. At the traditional method of Roumy cheese production, the ripening process is prolongated to more than six months to obtain the best desirable color, flavor and consistency of the cheese. Tyramine content in all cheeses increased linearly in the course of ripening, and the concentration of this biogenic amine (more than 300 mg/ kg) in the cheeses samples after 12 weeks of ripening substantially exceeded an established toxicological limit for tyramine (100 mg /kg of the consumed food). Individuals suffering from food intolerances and food allergies, and patients receiving monoaminoxidase (MAO) inhibitors, should avoid consumption of such ripened cheese. In the time interval from the 12^{th} week of ripening, Roumy cheese contained also higher levels of putrescine, a known tyramine toxicity potentiator.

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تكوين وتوزيع الأمينات الحيوية فى الجبنة الرومى كإحدى أشكال الجبن الجافة المصرية أثناء مرحلة التسوية

ناهد شحاته – حنان رضوان

قسم الاقتصاد المنزلى، كلية التربية النوعية، جامعة الزقازيق، الزقازيق، مصر

تمت من خلال هذه الدراسة تقدير محتوى الأمينات الحيوية (التربتامين، الفينايل إيثايل أمين، الهستامين، التيرامين، البيوتراسين– الكادافرين– الإسبرميدين، الاسبرمين) ومقاييس الجودة الكيميائية (النتروجين الأمينى، الأحماض الدهنية الكلية الطيارة، المالونالدهيد) والمسحات الميكروبية (العد البكتيرى الكلى، بكتريا حامض اللآكتيك، عدد الخمائر والفطريات) خلال فترات متقطعة من تسوية الجبن الرومى التى تعد واحدة من أشهر أنواع الجبن الجافة المصرية بلغت صفر إلى ٢٤ أسبوع. ولقد سجلت الأمينات الحيوية التربتامين، الفينايل إيثايل أمين، الهستامين، التيرامين، البيوتراسين– الكادافرين– الإسبرميدين، الاسبرمين متوسطات مقدارها ١.٨٩، ١.٦٩، ٢.٠٢، ١٤.٧٨، ١.٩٢، ٢.٩٠، ٠.٥٤ و ٠.٤٢ ملجرام/كجم على التوالي وباستطالة فترة النضج سجلت جميع هذه الأمينات زيادات تدريجية بنسب مختلفة تختلف من مركب لآخر حيث سجلت تلك الأمينات قيما مقدارها ٠.٩٨، ٨٨.٩٠، ٤.٨٠، ٥١١.٨٩، ٨٦.٥٥، ٢٦.٣٤، ٢.٣٢، ٢.٨٢ ملجرام/كجم على التوالى فى نهاية فترة التسوية التى امتدت لفترة ٢٤ أسبوع. مما يوضح انخفاض التربتامين بنسبة ٤٨.١٥% بينما سجلت باقى الأمينات الحيوية الفينايل إيثايل أمين، الهستامين، التيرامين، البيوتراسين– الكادافرين– الإسبرميدين، الاسبرمين معدلات زيادة بلغت ٥١٥٧.٥٣، ١٣٧.٢٠، ٣٣٦٣.٤٠، ٤٤٠٧.٨١، ٨٠٨.٢٨، ٣٢٩.٦٣، ٥٨٥.٧١% فوق خط البداية على التوالى. ولقد سجلت نفس السلوكية لمؤشرات الجودة الكيميائية الجودة الكيميائية (النتروجين الأمينى، الأحماض الدهنية الكلية الطيارة، المالونالدهيد) والمسحات الميكروبية (العد البكتيرى الكلى، بكتريا حامض اللآكتيك، عدد الخمائر والفطريات). كما أوضحت دراسات التوزيع تواجد نركيزات عالية بدرجة معنوية ($P < 0.01$) من جميع أفراد مجموعة الأمينات الحيوية فى الأجزاء الخارجية من أقراص الجبن الجاف عند المقارنة بالعينات المأخوذة من مركز أقراص الجبن الرومى. كما أظهر تحليل الارتباط أن تحديد مؤشرات الجودة الكيميائية بالإضافة إلى مؤشرات الميكروبيولوجية من جهة ومحتوى الأمينات الحيوية من جهة

أخرى قد أعطى نتائج متسقة. ومن وجهة النظر الغذائية والسمية، زاد محتوى التيرامين خطيا (P <0.01) , وبدرجة معنوية مع زيادة وقت النضج في الجبن الرومي، وبعد ١٢ أسبوعا من النضج حيث بلغ ٣٦٩.٥٠ ملجرام / كجم متجاوزا حد السمية (١٠٠ ملجرام/كجم) من الطعام المأخوذ. لذلك يجب على الأفراد الذين يعانون من عدم تحمل الطعام والحساسية الغذائية، والمرضى الذين يتلقون مثبطات مونوامينوكسيداز monoaminoxidase (MAO) inhibitors، تجنب استهلاك مثل هذا النوع من الجبن خاصة الناضج منه.

الكلمات الاسترشادية: الأمينات الحيوية، النتروجين الأميني، الأحماض الدهنية الكلية الطيارة، المالونالدهيد، المسحات الميكروبية، الطبقة الخارجية للقرص، مركز القرص، حدود السمية.