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Determination of Cadaverine and Putrescine in Different Types of Cheese

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Abstract: Cheese can accumulate Biogenic amines, with concentrations varying from just traces to more than 1000 mg Kg^{-1}. Their toxicity had led to the general agreement that they should not be allowed to accumulate in food. A total of eighty randomly collected samples of four varieties of cheese Mish, Ras, Cheddar and Edam (20 each).The samples were collected from Kafr El-Sheikh and EL- Gharbia Governorates, for determination of pH values, Total Volatile Nitrogen (TVN), Thiobarbituric acid values (TBA) and quantify its contents of cadaverine and Putrescine using HPLC. Mean values ranged from 4.30 to 6.08 for pH, from 4.17 to 11.81 mg/100g for TVN, from 0.09 to 0.23 mg/Kg for TBA, Cadaverine was found in 80, 70, 65 and 55%, while Putrescine was found in 80, 75, 65 and 60% respectively in Mish, Ras, Cheddar and Edam cheese with mean value from 7.21 to11.67 for Cadaverine and from 8.57 to 16.38 for Putrescine. 85, 90, 100 and 100% Cadaverine also 70, 90, 90 and %95 Putrescine in Mish, Ras, Cheddar and Edam cheese respectively were accepted according toMaximum Residual Limit of EOS 20mg /100g while 60, 65, 75 and 85% cadaverine also 40, 85, 60 and 70% Putrescine in Mish, Ras, Cheddar and Edam cheese respectively were accepted according to FDA which is10mg/100g. Finally Correlation coefficient (r) between pH, Total Volatile Nitrogen (TVN), Thiobarbituric acid values (TBA) and levels of Biogenic amines in examined cheese samples declared that pH had significant negative correlation with Putrescine in Mish cheese only (p<0.05). Strict hygienic measures must be adopted from farm to fork to insure good quality end product safe for human specially at risk group.

Key words: Biogenic Amines · Total Volatile Nitrogen · Thiobarbituric Acid · Quality characteristics · Cheese

INTRODUCTION

Cheese is the most popular fermented dairy product and some cheese varieties can be preserved for several months or even years. In addition, the fermentation of milk, drying and salting of the curd are common practices to extend the shelf-life of dairy products as much as possible [1].Recent trends in food quality and safety promote an increasing search for trace compounds that can affect human health. Biogenic amines (BAs) belong to this group of substances [2].

As cheese is one of the fermented foods most commonly associated with Biogenic amines poisoning; mainly Tyramine, Histamine, Putrescine and Cadaverine. BAs formation in cheeses is caused by decarboxylase-positive microorganisms.Recently, the lactic acid bacteria (LAB) were identified as BAs producer. BAs are organic bases of low molecular weight that possess biological activity, which formed in high protein containing food like: cheese, meat and, fish [3].

BAs can be formed and degraded as a result of normal metabolic activity and are usually produced by the decarboxylation of free amino acids. BAs production by bacteria considers one of the defense mechanisms used to withstand an acidic environment. Decarboxylase enzymes have an optimum pH around 5.0 [4].

Consumption of food containing high levels of BAs specially cadaverine and Putrescine which called di-amines is considered undesirable since it can be associated with several toxicological problems such as respiratory distress, headache, bradycardia, lockjaw, paresis of the extremities, hypotension and allergies. These problems are especially severe in consumers with low levels of the enzymes involved in the detoxification

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system (Mono and di-amine oxidases), either by genetic disorders or medical treatments. An additional problem of di-aminesis potentiating histamine toxicity, these amines play a role as di-amine oxidase inhibitors and so their presence may explain why the intake of aged cheese is more toxic [5, 6].

BAs and decarboxylase enzyme are thermostable once formed in the food will remain. Moreover, recently the market has reacted to customer requirements (Ready to eat products), cheese grating was reported to facilitate microbial contamination leading to the presence of BA-producing bacteria and consequently accumulation of BAs [7].

The importance of observing BAs content lies in potential toxicity to human, mainly when the concentration is up to 100 mg/kg. Thus, the presence of BAs significantly influences the food quality and safety [8].

The aim of the present study was to quantify Cadaverine and Putrescine in different kinds of cheese (Mish, Ras, Cheddar and Edam) and correlate this to factors that may affect biogenic amine content, such as pH, Total Volatile Nitrogen (TVN) and Thiobarbituric acid number (TBA).

MATERIALS AND METHODS

Collection of Samples: A total of eighty randomly collected samples of four varieties of cheese Mish, Ras, Cheddar and Edam (20 each). The samples were collected from street-vendors, groceries and supermarkets of different sanitary levels from Kafr El-Sheikh and EL-Gharbia Governorates, during the period from March to July 2016. Each sample was separately packed in polyethylene bag and immediately transferred to the laboratory in an insulated ice box for determination of pH values, Total Volatile Nitrogen (TVN), Thiobarbituric acid values (TBA) and quantify its contents of cadaverine and Putrescine.

Determination of pH: pH value was measured by using an electrical pH meter (Bye model 6020, USA) according to Pearson [9].

Determination of Total Volatile Nitrogen (TVN)
TVN was determination according to FAO [10].

Determination of Thiobarbituric acid number (TBA) according to Pikul *et al.* [11].

Determination of Biogenic Amines: Cadaverine and Putrescine levels were determined in examined samples according to the protocol recommended by Krause *et al.* [12] and Pinho *et al.* [13].

Reagents Preparation: Dansyl chloride solution was prepared by dissolving 500mg of dansyl chloride in 100 ml acetone. Stock standard solutions of the tested amines were prepared by dissolving 25 mg of each standard pure amine (Cadaverine -2HCl & putrescine 2HCl) in 25 ml distilled water individually.

Extraction of Samples: Twenty five grams of each cheese sample were blended with 125 ml of 5% Trichloroacetic acid (TCA) for 3 min using a blender then filtration was achieved using filter paper Whatman No1. Thus, 10 ml of the filtrate were transferred into a suitable glass tube with 4g NaCl and 1 ml of 50 % NaOH. The filtrate was extracted three times (2min each) by using 5 ml n-butanol: chloroform (1:1 v/v) and the upper clear layer was transferred to 100 ml separating funnel by using disposable Pasteur pipette. To combine the organic extracts (Upper layer), 15 ml of n-heptane were added in separating funnel and extracted three times with 1.0 ml portions of 0.2 N HCl, the HCl layer was collected in a glass Stoppard tube. Solution was evaporated just to dryness using water bath at 95°C with aid of a gentle current of air.

Formation of Dansylamines: One hundred µl of each stock standard solution (or sample extract) were transferred to 50 ml vial and dried under vacuum. About 0.5 ml of saturated $NaHCO_3$ 5% solution was added to the residue of the sample extract (or the standard).Vial was stoppered and carefully mixed. Carefully, 1.0 ml dansyl chloride solution was added to the vial and mixed thoroughly using vortex mixer. The reaction mixture was incubated at 55°C for 45 min. Accurately, 10 ml of distilled water were added to the reaction mixture, then vial was stoppered and shacked vigorously using vortex mixer, the extraction of dansylated biogenic amines was carried out using 5ml of diethyl ether for 3 times again vial was stoppered, shacked for 11.0 min and the ether layers were collected in a culture tube using disposable Pasteur pipette. The combined ether extracts were carefully evaporated at 35°C in a bath with aid of current air. The obtained dry material was dissolved in 1ml methanol and 10µl were injected in HPLC.

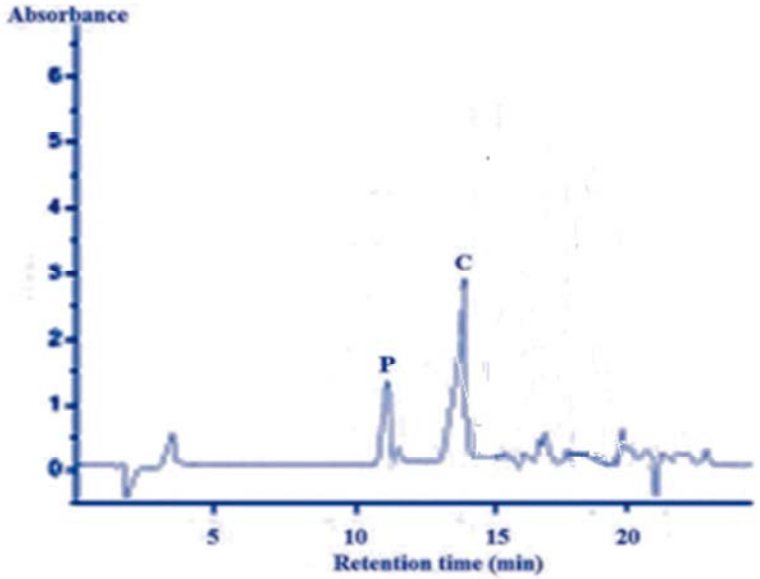

Fig. 1: Chromatograms of the areas of biogenic amines standard solutions derivatized by HPLC
P: Putrescine [Retention time (RT) = 11.2 min]
C:Cadaverine [Retention time (RT) = 13.5 min]

Apparatus HPLC Conditions: High performance liquid chromatography (HPLC) was an Agilent 1100 HPLC system, Agilen Technologies, Waldbronn, Germany, equipped with quaternary pump model G 1311A, UV detector (Model G 1314A) set at 254nm wavelength, auto sampler (Model G1329A VP-ODS) and Shim pack (150× 4.6 mm) column (Shimadzu, Kyoto, Japan) was used for biogenic amines separation according to the following Gradient solvent program: Samples were eluted with a step gradient of 0.02N acetic acid Solvent A, Methanol Solvent B and Acetonitrile Solvent C for 25 min .The flow rate 1ml / min. The Program was set a linear gradient starting from 60% solvent A, 20% solvent B and 20% solvent C, respectively changed to 15%, 35% and 50% at15 min. then at 20 min. return to 60%, 20% and 20% respectively till 25min. of elution.

Identification and Quantification of Compounds: Retention times and absorbance ratios against those of standards were used to identify the separated biogenic amines and to check their purity. Quantitative determination of each biogenic amines were determined from its respective peak areas and their corresponding response factors. Data were integrated and recorded using Chemstation Software program.

Statistical Analysis: The obtained results were statistically evaluated according to Feldmen *et al*. [14].

RESULTS AND DISCUSSION

Cheese Spoilage is largely due to the microbial invasion, which brings about several physico-chemical and biochemical changes leading to the development of off-flavour subsequently, increase in pH might be attributed to the facts that microbial activity causes break down of citrate and hydrolysis of protein [5].

Results presented in Table (1) declared that the mean pH values were 4.30 , 6.08, 5.10 and 5.51, Total Volatile Nitrogen (TVN) "mg / 100 gm" were 11.81, 7.93, 5.57 and 4.17 and Thiobarbituric acid values (TBA) "mg/Kg" were 0.23^{a}, 0.15^{c}, 0.12 and 0.09 in Mish, Ras, Cheddar and Edam cheese respectively. Mean values in the same column with different letters is significantly different ($P \leq 0.05$).

El-Aswad [7] found that pH values ranged from 6.21 to 4.07 also malonaldehyde (MA) content ranged from 0.20 to 0.50 in soft cheese samples as affected by storage for 24 months, also Ragab [24] reported that pH values and malonaldehyde content ranged from 4.60 to 5.78 and 0.21 to 0.72 , respectively in Mish cheese samples as affected by elongation of storage period.

Undesirable changes occur in cheese due to action of proteolytic and lipolytic bacteria. Lipolysis results directly in the formation of flavor compounds by liberation of free fatty acids (FFA). A major product produced from the degradation of lipid hydroperoxides in foods, is *maloaldehyde*, which is often used as an indicator for

Table 1: pH values, Total Volatile Nitrogen (TVN) mg / 100 gm and Thiobarbituric acid values (TBA) mg/Kg in the examined Cheese samples (n=20)

	pH value			T.V.N.			T.B.A.		
Cheese type	Min.	Max.	Mean±SE*	Min.	Max.	Mean±SE*	Min.	Max.	Mean±SE*
Mish	4.13	4.59	4.30 ± 0.032^{d}	3.87	16.90	11.81 ± 0.95^{a}	0.07	0.35	0.23 ± 0.02^{a}
Ras	5.64	6.42	6.08 ± 0.052^{a}	2.45	13.08	7.93± 0.91^{b}	0.04	0.25	0.15 ±0.012^{b}
Cheddar	4.91	5.29	5.10 ± 0.03^{c}	1.83	9.43	5.57 ± 063^{c}	0.02	0.19	0.12 ± .013^{bc}
Edam	5.32	5.74	5.51 ± 0.028^{b}	1.58	7.92	4.17 ± 0.44^{c}	0.02	0.14	0.09 ± .01^{c}

* SE =Standard error of mean

Table 2: Cadaverine and Putrescine levels (mg / 100 gm) in the examined cheese samples (n=20)

	Cadaverine					Putrescine				
	+ve samples					+ve samples				
Cheese type	No.	%	Min.	Max.	Mean±SE	No.	%	Min.	Max.	Mean±SE
Mish	16	80	1.7	28.4	11.67 ± 2.17^{a}	16	80	1.9	30.7	16.38 ± 2.10^{a}
Ras	14	70	1.4	22.9	10.56 ± 1.87^{a}	15	75	1.6	27.3	8.57 ± 2.00^{b}
Cheddar	13	65	1.0	19.5	9.85 ± 1.67^{a}	13	65	1.2	24.1	11.25 ± 2.04^{ab}
Edam	11	55	1.0	14.3	7.21 ± 1.31^{a}	12	60	1.0	21.9	11.02 ± 1.84^{ab}

Table 3: Acceptability of the examined cheese samples based on their levels of cadaverine and Putrescine (n=20)

	* Max. R.L. at 20mg/100g				** Max. R.L. at 10mg/100g			
	Accepted samples***				Accepted samples***			
	Cadaverine		Putrescine		Cadaverine		Putrescine	
Cheese type	No.	%	No.	%	No.	%	No.	%
Mish	17	85	14	70	12	60	8	40
Ras	18	90	18	90	13	65	17	85
Cheddar	20	100	18	90	15	75	12	60
Edam	20	100	19	95	17	85	14	70

*Maximum Residual Limit according to Egyptian Organization for Standardization "EOS"[15].

** Maximum Residual Limit according to FDA [16].

*** Including negative samples.

Table 4: Correlation coefficient (r) between pH, Total Volatile Nitrogen (TVN), Thiobarbituric acid values (TBA) and levels of Biogenic amines in examined cheese samples

Cheese type	Biogenic amine	pH value	T.V.N.	T.B.A.
Mish	Cadaverine	.087 (.749)	.348 (.187)	.310 (.243)
	Putrescine	-.542* (.030)	-.272 (.308)	-.304 (.252)
Ras	Cadaverine	.378 (.182)	.227 (.434)	.376 (.186)
	Putrescine	.013 (.964)	.089 (.752)	-.019 (.947)
Cheddar	Cadaverine	-.237 (.435)	-.256 (.399)	-.246 (.418)
	Putrescine	.252 (.406)	.232 (.445)	.215 (.481)
Edam	Cadaverine	-.200 (.555)	-.252 (.454)	-.054 (.874)
	Putrescine	-.135 (.676)	-.042 (.896)	.046 (.887)

* Correlation is significant at the 0.05 level (2-tailed)

detecting the level of lipid peroxidation in food and is considered harmful for human health, carcinogenic and mutagenic [17].

Proteolysis of the caseins resulted in formation of FAAs which are important precursors for numerous catabolic reactions, which contribute to the production of fundamental volatile compounds for cheese flavor. Protein degradation is the first factor which can be used to determine the extent of spoilage. Volatile amines are produced from bacterial action on proteins and contribute to the “Off flavours” and odours of cheese [18].

TVBN (the total volatile bases) include ammonia, trimethylamine. Formation of trimethylamine (TMA) in cheese resulted from decomposition of proteins by amino acid decarboxylase that are produced by bacteria, which is used as an indicator of the spoilage in foods, (cheese)[19]. In addition, TMA is a good detector of biogenic amines, because of its volatility, such as fishy odour, which is clear in presence of low levels of TMA and odour like ammonia, in presence of high levels of TMA in a food sample. Cheese is one of fermented food that could be exposed to contamination during the manufacture or storage, therefore, it is likely to contain amines [18].

Results achieved in Table (2) indicated that mean values of cadaverine and Putrescine levels "mg / 100 gm" in the examined cheese samples were 11.67, 10.56 , 9.85 and 7.21 for cadaverine and 16.38, 8.57 , 11.25 and11.02 for Putrescine, in Mish, Ras, Cheddar and Edam cheese respectively. Mean values in the same column with the different letters is significantly different ($P<0.05$).

Cadaverine and Putrescine are diamines which can be formed either as natural polyamines during de novo polyamine biosynthesis or as biogenic amines by decarboxylation. Cadaverine and putrescine has a role in food toxicity as they potentiate the toxicity of other amines, especially histamine, also these diamines can react with nitrite to form carcinogenic nitrosamines. BAs content of cheese depends on the type of cheese, the ripening time, the manufacturing process and the microorganisms present [3, 6].

El-Zahar [20] reported that cadaverin concentration in Mish cheese samples was ranged from3±0.3 to22±1.97 and in Ras cheese from not detected to 20±2.1 which is nearly similar to our result, while Putrescine ranged in Mish cheese from 4 to 20and in Ras cheese from 6 to16, which differ from our present results.

Cadaverine and Putrescine were detected in different cheese samples with different levels and by different authors concluded that the variation in Cadaverine and Putrescine concentration depending on variation in sanitary condition, storage period and storage temperature as increasing temperature leading to increase Cadaverine concentration, these finding were in agreement with our result [21].

Biogenic amine accumulation is minimized at low temperatures through inhibition of microbial growth and the reduction of enzyme activity. The optimum temperature for the formation of BA by mesophilic bacteria has been reported to be between 20 to 37°C, while production of BA decreases below 5°C or above 40°C. Prolonged storage periods, in particular at abuse temperatures make the food more susceptible to amine formation. Some BA such as putrescine and cadaverine can be formed during the storage of food [2].

Results stated in Table (3) showed the acceptability of the examined cheese samples based on their levels of cadaverine and Putrescine, firstly according to Maximum Residual Limit according toEgyptian Organization for Standardization "EOS" [15] which is 20mg/100g found to be 85%, 90%, 100% and 100% of the examined Mish, Ras, Cheddar and Edam cheese were accepted for Cadaverine level, while 70%, 90%, 90 and 95% of the examined Mish, Ras, Cheddar and Edam cheese respectively were accepted for Putrescine. Concerning to Maximum Residual Limit according to FDA [16] which is10mg/100g, 60%, 65%, 75% and 85% of the examined Mish, Ras, Cheddar and Edam cheese were accepted for Cadaverine levels, while 40%, 85%, 60% and 70% of the examined Mish, Ras, Cheddar and Edam cheese were accepted for Putrescine levels.

Nearly similar results were obtained by Ekbal and Amer [22] while lower results were obtained by El-Kassas and El-Bahy [23]

From these results the indication will be increase level of Cadaverine and Putrescine in Mish and Ras cheese more than Cheddar cheese and this is may be attributed to low quality raw material or post pasteurization contamination leading to contamination with micro-organisms able to produce BAs. So adoption of strict hygienic measures from farm to fork is necessary.

EL-Zahar [20] stated that the presence of high contents of BAs in Mish and Ras cheeses could be related to the enzymatic activity of proteases derived from microorganisms.

Cadaverine and Putrescine production has mainly been related to Gram-negative bacteria, especially in the families *Enterobacteriaceae* and *Pseudomonadaceae*, generally associated with spoilage. Enterobacteria genera *Citrobacter*, *Klebsiella*, *Escherichia*, *Proteus*, *Salmonella* and *Shigella* are associated with production of considerable amounts of cadaverine and putrescine in food. Putrescine is one of the most common BA found in fermented products. Lactic acid bacteria, mainly *Lactobacilli* and *Staphylococci* have also been reported to be able to produce putrescine and/or cadaverine [2].

Results achieved in Table (4) demonstrated that Correlation coefficient (r) between pH, Total Volatile Nitrogen (TVN), Thiobarbituric acid values (TBA) and levels of Biogenic amines in examined cheese samples declared that pH had significant negative correlation with Putrescine in Mish cheese only ($p<0.05$).

Ragab [24] found that pH had significant positive correlation with Putrescine and other BAs in Mish cheese (p<0.05). Similar results represented by El-Aswad [7] in Domiatti cheese pH had significant negative correlation with BAs (p =0.0001 to 0.001 and r=-0.967 to -0.708).

The pH of cheese is appropriate for amine production, generally between 5.0 and 6.5, depending on age and type of cheese [1]. Jawad [18] reported that the levels of TVB-N in all packaged cheeses were at low levels. The low temperature of storage contributed to slow the microbial activities in cheese and decrease the biogenic amines quantities, which significantly correlated with TVB-N quantities. Also Shi *et al.* [25] showed that the low temperatures contributes to the preservation of the food quality through inhibition of the levelsof biogenic amines, which correlated significantly with the levels of total volatile base nitrogen.

Dairy products are often manufactured under controlled or poor hygiene conditions; in addition they are produced following different protocols, which can vary from one cheese maker to another. Cadaverine, Putrescine have been found in many different kinds of cheeses. Not all amines are equally toxic, but these are of major concern [6]. The BAs content of cheese can be extremely variable and depend on: the type of cheese, type of milk, thermal treatment of cheese milk, section of the cheese (Edge/core), the ripening conditions, post ripening processing, type of packaging, storage time and temperature, microbiota responsible for cheese making, availability of free amino acids, pH, water activity, bacterial density, synergistic effects between microorganisms and, primarily, the presence of microorganisms possessing amino acid decarboxylase activity [21].

In conclusion, strict hygienic measures must be adopted to produce safe food, nearly free from trace elements like BAs.that affect human health badly specially those at risk groups.

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