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Biogenic amines in vacuum-packed and non-vacuum-packed flesh of carp (*Cyprinus carpio*) stored at different temperatures

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Abstract

Samples of carp flesh (*Cyprinus carpio*), vacuum-and non-vacuum-packed were stored at 3 and 15 °C. Chemical, sensory and microbial qualities were measured throughout the storage time to determine the changes that took place and to evaluate the effects of both storage temperature and the means of packaging. Seven biogenic amines (putrescine, cadaverine, spermidine, spermine, histamine, tyramine and tryptamine) were determined. Putrescine and cadaverine showed the best correspondence with the sensory and microbial states of the samples. Low storage temperature had a dominant effect, resulting in low biogenic amine content and better quality of samples. The effect of vacuum-packaging was less obvious, especially in samples kept at 15 °C. Application of vacuum-packaging at 3 °C prolonged the shelf life by about 4–5 days.

Keywords: Biogenic amines; Putrescine; Freshwater fish; Carp; Quality changes

1. Introduction

Fish are a rich source of high-quality protein, essential vitamins and healthful polyunsaturated fatty acids. The high content of proteins, on the other hand, represents a risk in the decomposition processes. The disintegration of proteins yields peptides and amino acids, which are susceptible to further decay, resulting in biogenic amines, that can be widely distributed in proteinaceous foods.

Amines, such as putrescine (PUT), cadaverine (CAD), spermidine (SPD), spermine (SPM), histamine (HI), tyramine (TY) and tryptamine (TR), being low molecular weight compounds, are likely to arise via several biochemical pathways (Miyazaki & Yang, 1987). Small amounts are biosynthesised in animal cells but, in larger quantities, they are also produced by microbial decarboxylation of amino acids. The amount and type of amine formed is therefore influenced by the food composition, microbial flora and by several parameters, which promote bacterial growth during storage – such as temperature, ripening and packaging (Halasz, Barath, Sarkadi, & Holzapfel, 1994). Food quality has been linked to these amines in fermented but, primarily in non-fermented foodstuffs. In stored flesh, amines are generated by the action of spoilage bacterial decarboxylases.

The reasons for amine determination in foods are twofold. The first is their potential toxicity; the second is the possibility of using them as food quality markers. Though biogenic amines in low concentrations are essential for many physiological functions (Bardocz et al., 1995; Eliassen, Reistad, Risoen, & Ronning, 2002), ingestion of large amounts may result in health problems (Joosten, 1988).

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Toxicity of amines strongly depends on the individual efficiency of detoxication (Shalaby, 1996). During the digestion process in the human gut, low amounts of biogenic amines are metabolized to physiologically less-active degradation products. This detoxification system includes specific enzymes (e.g. diamine oxidase, DAO). However, upon intake of high loads of biogenic amines with foods, this detoxification system is unable to eliminate biogenic amines sufficiently. Moreover, in the case of insufficient DAO activity, caused by e.g. generic predisposition, gastrointestinal diseases or inhibition of DAO activity due to secondary effects of medicines or alcohol, low amounts of biogenic amines cannot be metabolized efficiently (Bodmer, Imark, & Kneubühl, 1999). Biogenic amines, such as histamine and tyramine, are considered as antinutritional compounds. For sensitive individuals they represent a health risk, especially when their effect is potentiated by other substances. Poisoning by histamine with its allergy-like symptoms is usually related to the consumption of scombroid fish, such as tuna or mackerel (Veciana Nogués, Marine Font, & Vidal Carou, 1997; Wu, Yang, Yang, Ger, & Deng, 1997), and it is considered to be one of the commonest forms of food intoxication reported (Slorach, 1991). Histamine in fish samples can be rapidly determined by gas chromatography (Hwang, Wang, & Choong, 2003).

Biogenic amines in fish can serve as indices of decomposition. Mietz and Karmas (1978) found that the HI content varied extensively with the fish species and they did not recommend using histamine determination for fish flesh quality assessment. These authors proposed the complex BAI criterion, which is given by the formula: $BAI = (PUT + CAD + HI)/(1 + SPD + SPM)$. Recently this opinion was confirmed by Mendes (1999), examining histamine formation in sardines and mackerel. Correlation of HI formation with a decline in organoleptic quality in sardines was not found. Yamanaka, Shiomi, and Kikuchi (1989) evaluated the decomposition of chum salmon (*Oncorhynchus keta*) and proposed cadaverine as the best indicator of spoilage. A good correlation was found between organoleptic evaluation and levels of PUT and CAD in skipjack tuna (Sims, Fran, & York, 1992). Veciana Nogués et al. (1997) proposed, for tuna assessment, the use of an index calculated from the sum of the contents of HI, TY, PUT and CAD. It can be concluded, that amines formed in the matrix depend on the fish species.

In contrast to the number of studies of changes in biogenic amine contents in sea fish (Mackie, Pirie, Ritchie, & Yamanaka, 1997; Sato, Okuzumi, & Fujii, 1995; Veciana Nogués, Albala Hurtado, Marine Font, & Vidal Carou, 1996; Yamanaka et al., 1989), there is little information on fresh water fish (Křížek, Pavlíček, & Vácha, 2002). The objective of the present work was to determine how the temperature of storage and manner of the flesh packaging can affect biogenic amine formation in carp muscle.

2. Materials and methods

2.1. Fish samples

2.1.1. Carp production

The carp samples (*Cyprinus carpio*) were obtained from a fish farm in Pohorelice near Brno. In order to ensure the best uniformity of the natural microbial load, all fish were caught in the same fish pond and were treated in the same way. The fish were gutted and, after decapitation and trimming away the fishtail, the body was cut into two halves (fillets with backbones). Portions of about 100 g of muscles from the chest area served as samples.

2.1.2. Sample packaging

2.1.2.1. Non-vacuum-packaging. Samples were placed in PE bags. Their wrapping was careful, but purposely not airtight, simulating normal conditions in households, preventing samples mainly from desiccation.

2.1.2.2. Vacuum-packaging. Samples were wrapped in PE PP foil (thickness 90 μm) and sealed under vacuum, level 8 (98%). This process was realized in the fish farm manufacture on a professional wrapping machine, Turbovac 700-ST-FLL (Leybold Vakuum GmbH, Köln, Germany).

2.1.3. Sampling

Experiments were designed as dynamic. Samples were analyzed in duplicate every 24 h. They were stored up to the apparent stage of decomposition. The times of storage at temperature 3 °C were 14 and 16 days, for samples NV and V, respectively; at temperature 15 °C those were 6 (NV) and 14 (V) days.

Within the period of sampling, complementary sensory tests were done (see Section 2.4).

2.2. Analytical method

2.2.1. Sample extraction and derivatization

Samples were homogenised in either a commercial food handblender (Philips, Vienna, Austria) or an Ultra-Turrax T25 homogeniser (Ika Labortechnik, Staufen, Germany).

Biogenic amines (PUT, CAD, SPD, SPM, HI, TY and TR) were extracted from 40 g of homogenised flesh samples with diluted perchloric acid, p.a. (0.6 M). After filtration, the volume was made up to 150 ml with perchloric acid. Centrifugation was not necessary as the carp flesh had a relatively low content of fat. The amines

were determined as the *N*-substituted benzamides, after derivatization with benzoylchloride by micellar electrokinetic capillary chromatography, using capillary zone electrophoresis machine. Two parallel analyses were carried out. The procedure has been described in detail by Křížek and Pelikánová (1998).

2.2.2. Apparatus

Analyses were carried out using a Spectraphoresis 2000, a fully automated system for capillary zone electrophoresis equipped with a multi-wavelength UV–VIS scanning detector (Thermo Separation Products, Fremont, CA, USA). Separations were achieved in a plain fused silica capillary column of 43 cm total length (36 cm effective length to the detector) and 75 μm inner diameter (CElect FS75 CE column, Supelco, Bellefonte, PA, USA).

Data processing was performed using Spectacle and PC 1000 CE software v. 3.0 (Thermo Separation Products).

2.3. Microbiological analysis

Mesophilic viable count (MVC), psychrotrophic viable count (PVC), coliform bacteria and enterococci were investigated. MVC were performed in pure plates with Plate-Count Agar (HiMedia, Mumbai, India) according to CSN ISO 4833. The same plates were used for the determination of PVC. The plates were incubated for 7 days at 4 °C. Coliform bacteria count was done according to the CSN ISO 4832. The violet Red Bill agar (HiMedia, Mumbai, India) was used. Enterococci were counted on Slanetz–Bartley agar (Hi-Media, Mumbai, India). The plates were incubated at 37° C for 24–48 h.

2.4. Sensory tests

Sensory tests were only complementary to the main objective of this study – the determination of chemical changes in the flesh matrix – and were simplified to three levels: good (1), acceptable (2) and poor (3). The organoleptic properties were based mainly on odour and general appearance. Table 1 better specifies the three levels.

2.5. Statistical evaluation

Samples were prepared in duplicate and each sample was analysed twice. Fitting of curves, describing the kinetics of amine formation, was done by the mathematical software Maple V v 5.0 (Waterloo Maple Inc, Waterloo, Canada). Correlation coefficients and other statistics were calculated using Statistica v. 5.1 (StatSoft, Inc., Tulsa, OK, USA).

3. Results and discussion

Fifty two samples were analyzed in duplicate. The mean amine contents in all samples (regardless of storage time) are given in Table 2. Putrescine and cadaverine are predominant amines in all samples. Their contents are about 5 or 10 times higher than other amines. Experiments were designed as dynamic. Mean contents of all amine concentrations, for all sampling days and sensory scores, are shown in Table 3–6.

Former experiments with carp flesh (Křížek et al., 2002) revealed that the content of PUT, or alternatively the total content of PUT + CAD shows the best correspondence (of all biogenic amines) between the concentration and the sensory stage of the flesh (Table 7). It was not surprising that samples stored at lower temperature and samples vacuum-packed showed better storage ability. The influence of temperature on the storage ability was essential, but the manner of packaging was secondary.

At 15 °C, samples of both methods of wrapping were fully spoiled (sensory level 3) after 3 days of storage. At this temperature the rates of increase of negative sensory properties and the PUT (or PUT + CAD) contents were similar for both kinds of wrapping. Similar results were obtained by Mendes (1999), testing bluefish stored at 20 °C. Cadaverine, at this temperature, reached the highest concentration after 2 days of storage.

Table 2
Mean contents of biogenic amines and concentration ranges of all samples

Amine	Mean contents (mg/kg)	Range (mg/kg)
PUT	44.9	0.3–203
CAD	92.5	ND–517
SPD	8.8	4.2–18.0
SPM	10.7	1.8–16.0
HI	10.9	ND–79.8
TY	14.7	ND–86.0
TR	1.4	ND–31.1

Table 1
Sensory scheme applied by five trained panellists

	Quality (sensory score)		
	Good (1)	Acceptable (2)	Poor (3)
Odour	Meaty, neutral	Neutral, slightly spicy	Fishy, repulsive
Appearance (colour/texture)	White/tightly elastic	Greyish/solid	Grey/muddy

Table 3
Mean contents of biogenic amines in vacuum-packed samples (V) kept at 3 °C

Amine	Time (days)																
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Putrescine	1.1	0.53	0.30	0.74	1.6	2.0	0.83	2.6	1.4	4.0	6.8	5.2	4.2	7.1	2.1	6.3	21.6
Cadaverine	0.37	0.10	0.10	0.10	3.0	2.8	0.18	4.6	5.0	15.3	16.3	13.0	2.4	13.2	6.1	14.8	58.0
Spermidine	10.7	10.3	4.2	6.9	6.6	6.8	7.8	6.8	8.1	7.8	8.8	10.1	4.4	9.6	4.5	13.2	11.0
Spermine	10.2	11.7	13.6	10.7	8.3	9.6	10.2	10.9	11.9	11.8	11.9	11.6	1.8	12.6	3.3	9.2	6.5
Histamine	0.07	ND	0.02	ND	0.33	0.18	ND	ND	ND	ND	ND	ND	0.23	ND	ND	ND	4.1
Tyramine	ND	ND	ND	0.23	0.41	0.29	0.48	0.96	0.19	ND	1.3	0.91	1.7	0.80	2.2	1.2	6.9
Tryptamine	0.20	ND	0.11	ND	0.16	0.08	ND	0.24	ND	0.12	ND	0.09	0.09	ND	ND	0.61	ND
Sensory	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.5	1.5	2.0	2.0	2.0	2.0	2.0	2.0	2.5	2.5

Table 4
Mean contents of biogenic amines in non-vacuum-packed samples (NV) kept at 3 °C

Amine	Time (days)														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Putrescine	0.70	0.53	0.91	0.92	3.2	3.9	4.6	4.3	11.8	19.0	13.7	29.6	29.5	40.6	86.0
Cadaverine	ND	ND	ND	0.20	2.3	4.1	5.0	1.7	8.1	11.4	7.2	20.7	21.0	24.9	51.4
Spermidine	8.2	8.8	9.6	7.5	6.8	8.5	7.6	8.5	8.7	6.7	8.7	8.4	7.0	9.9	8.4
Spermine	9.6	11.6	8.0	9.9	9.7	11.5	12.0	12.2	10.3	9.6	10.3	10.2	8.8	11.4	11.1
Histamine	ND	ND	ND	ND	0.08	0.05	0.04	ND	ND	0.08	0.14	0.12	0.33	1.1	0.30
Tyramine	ND	ND	ND	ND	0.19	0.24	ND	0.48	0.19	0.06	0.32	0.20	0.48	0.84	1.1
Tryptamine	0.11	0.57	0.42	0.20	ND	ND	ND	0.19	0.12	0.10	0.18	0.25	1.1	ND	0.73
Sensory	1.0	1.0	1.0	1.0	1.0	1.5	1.5	1.5	1.5	2.0	2.0	2.0	2.5	3.0	3.0

Table 5
Mean contents of biogenic amines in vacuum-packed samples (V) kept at 15 °C

Amine	Time (days)														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Putrescine	1.1	2.0	10.2	19.8	52.8	80.8	94.6	120	156	154	199	172	180	203	195
Cadaverine	0.37	7.9	22.7	48.4	110	190	220	283	360	418	479	395	385	517	488
Spermidine	10.7	7.6	6.7	9.0	7.8	12.3	18.0	12.2	12.8	11.2	14.6	10.4	11.3	9.9	9.5
Spermine	10.2	13.3	9.3	10.1	11.3	12.1	11.4	12.3	12.7	15.4	16.0	14.5	11.4	11.6	10.6
Histamine	0.07	ND	0.27	8.9	49.1	14.3	22.2	42.2	17.4	42.9	43.2	48.8	38.9	44.6	52.0
Tyramine	ND	ND	0.30	1.6	7.7	44.2	67.0	39.4	65.5	68.2	86.0	51.7	57.7	79.9	81.8
Tryptamine	0.20	0.27	0.07	0.34	1.3	1.2	1.9	2.2	2.1	1.8	9.5	5.1	5.5	31.1	5.5
Sensory	1.0	1.0	1.5	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0

Table 6
Mean contents of biogenic amines in non-vacuum-packed samples (NV) kept at 15 °C

Amine	Time (days)						
	0	1	2	3	4	5	6
Putrescine	0.68	0.91	8.9	36.9	84.4	152	181
Cadaverine	ND	0.34	12.4	43.6	107	212	384
Spermidine	8.2	5.6	6.7	8.3	7.9	7.6	6.9
Spermine	9.6	9.2	9.0	12.0	11.6	11.2	9.1
Histamine	ND	0.02	ND	6.3	16.5	55.2	79.8
Tyramine	ND	0.31	0.42	2.1	32.6	40.7	46.3
Tryptamine	0.11	0.24	0.27	0.81	1.2	0.27	1.3
Sensory	1.0	1.0	2.0	3.0	3.0	3.0	3.0

At 3 °C, the difference between the two methods of wrapping was more pronounced. Within the first 8 days of storage, both kinds of samples remained at sensory level one. After this time a different progress appeared. Vacuum-packed samples (V) showed slight degradation to the sensory level 2 (within 9–16 days). PE-packed samples (NV) reached the same sensory level within 9–12 days; then their properties rapidly deteriorated to the

Table 7
Proposed relations among sensory scores and amine concentrations (mg/kg) in carp flesh (Křížek et al., 2002)

	Sensory score		
	1	2	3
PUT	<10	10–20	>20
PUT + CAD	<20	20–45	>45

degree three. Vacuum-packed samples, at 3 °C, did not reach the sensory level 3 within all observed periods of storage. The time behaviour of sensory properties and average PUT content are summarized in Table 8.

A similar key effect of temperature was observed by Baixas Nogueras, Bover Cid, Veciana Nogués, and Vidal Carou (2002) in their study of Mediterranean hake (*Merluccius merluccius*). Ice storage was able to double the rejection times of hake fish stored at 6–8 °C.

Statistically significant correlations among contents of amines were not numerous. From 168 possible correlations among amines in samples kept under different conditions, only a limited part of the Spearman correlation matrix was statistically significant. Putrescine showed the best correlation with CAD, HI, TY and TR (Table 9). Analogous correlation of CAD with other amines was nearly identical, due to the highly significant correlation of PUT vs. CAD (data not shown).

There were only eight other significant correlations among amines. In vacuum-packed samples at 3 °C: SPM/HI (0.510*); SPM/TY (0.591*); non-vacuum-packed samples at 3 °C: HI/TY (0.681**); vacuum-packed samples at 15 °C: TR/HI (0.736**); TR/TY (0.828**) and in non-vacuum-packed samples at 15 °C: SPD/SPM (0.786*); TR/HI (0.755*); TR/TY (0.847*).

For amines and the sensory score, a similar correlation matrix was observed (Table 10). Both last mentioned (Tables 9 and 10) shows the close mutual relationship among PUT, CAD, TY and in lesser extent HI, TR. Polyamines SPD and SPM that are formed from PUT (Miyazaki & Yang, 1987) were seldom involved in statistically significant correlations. These two amines are not toxicologically important and their contents cannot serve as carp flesh quality indicators.

Table 8
Mean putrescine content (mg/kg) and sensory properties

Packaging/ temperature (°C)	Quality (sensory score)					
	Good (1)		Acceptable (2)		Poor (3)	
	Days	PUT	Days	PUT	Days	PUT
V/3	0–8	1.2	9–16	7.2	–	–
NV/3	0–8	3.4	9–12	22.9	13–14	63.3
V/15	0–2	4.4	–	–	3–14	136
NV/15	0–1	0.8	2–2	8.9	3–6	114

V – vacuum-packaging; NV – non-vacuum-packaging.

Table 9
Spearman correlation coefficients for putrescine content

Packaging/ temperature (°C)	Putrescine correlated with other amines in samples, kept under the same conditions			
	Cadaverine	Histamine	Tyramine	Tryptamine
V/3	0.891***	NS	0.726***	NS
NV/3	0.982***	0.801***	0.791***	NS
V/15	0.971***	0.743**	0.901***	0.964***
NV/15	1.000***	0.937**	1.000***	0.847*

NS – not significant.
* $P \leqslant 0.05$.
** $P \leqslant 0.01$.
*** $P \leqslant 0.001$.

This contrasts with the observations of Mietz and Karmas (1978), probably owing to the fact that those authors studied various kinds of sea species (salmon, rockfish, lobster, shrimp).

In carp flesh samples the kinetic curves, especially of PUT and CAD, as well as those of HI, TY and TR, increased smoothly (Tables 3–6). The exceptions were SPD and SPM, where no statistically important trend was observed (the hypothesis that the slope is zero could not be rejected for all kinds of samples at $P < 0.05$). Baixas Nogueras et al. (2002) studying hake found, that CAD usually starts to increase later than PUT, but its levels at the end of storage are generally higher. The delay of CAD formation can also be seen in our samples stored at 3 °C (Tables 3 and 4), but it was not conspicuous 15 °C samples.

Previous results (Křížek et al., 2002) revealed, that critical concentrations of PUT + CAD in carp flesh are about 20 mg/kg (change of the sensory level 1–2) and 45 mg/kg (change of the sensory level 2–3). As the kinetic curves of amine formation usually show a smooth increase, they can be described by quadratic or cubic regression equations. By solving these equations for the proposed critical concentrations (20 and 45 mg/kg), we obtained estimates of the respective critical times (days), when the given concentration had been reached (Table 11).

Table 11 shows, that vacuum-packaging had a beneficial effect for samples kept at 3 °C. The critical concentration of PUT + CAD in non-vacuum-packaged samples was reached 4–5 days earlier. On the other hand, the manner of packaging had a negligible effect for samples kept at 15 °C.

The microbial quality of fish flesh depends both on aquaculture and on sanitary conditions in the slaughterhouse. When fish are gutted, bacteria from gills, and especially from the gut, can contaminate edible muscles. Study of bacterial contamination of the carp flesh was complementary to biogenic amine determination. Microbiological analysis was aimed at MVC, PVC, coliforms and enterococci.

Table 10
Spearman correlation coefficients among amines and sensory score

Packaging/ temperature (°C)	PUT	PUT + CAD	SPD	SPM	HI	TY	TR
V/3	0.887***	0.920***	NS	NS	NS	0.748***	NS
NV/3	0.960***	0.968***	NS	NS	0.843***	0.801***	NS
V/15	0.698**	0.698**	NS	NS	0.698**	0.699**	0.683**
NV/15	0.896**	0.896**	NS	NS	0.814*	0.896**	0.854*

NS – not significant.
* $P \leqslant 0.05$.
** $P \leqslant 0.01$.
*** $P \leqslant 0.001$.

Table 11
Calculated days of storage of carp flesh when given putrescine + cadaverine levels can be expected (r – regression correlation coefficient, Sens. – means sensory score observed at calculated time)

Calculated critical days of storage				
Packaging/ temperature (°C)	PUT + CAD 20 mg/kg	PUT + CAD 45 mg/kg	r	Sens.
V/3	13	16	0.851	2.0–2.5
NV/3	9	11	0.971	2.0–2.0
V/15	1.7	2.4	0.998	1.5–2.0
NV/15	2.0	2.5	0.999	2.0–2.5

Microbial quality of samples was in accordance with the biogenic amines and sensory evaluation. Samples NV, at 3 °C, started with MVC levels of 3.1×10^4 CFU/g, and after 14 days of storage, reached levels 1.8×10^9 CFU/g. In samples V, at 3 °C, the MVC levels were of 10–100 times lower. Samples NV at 15 °C reached 2.0×10^9 CFU/g as early as the 6th day. The counts of the psychrotrophic bacteria were lower than MVC, reaching 5.6×10^8 CFU/g (NV, 3 °C) and 1.3×10^6 CFU/g (V, 3 °C). At 15 °C, their counts were less different: 3.0×10^6 CFU/g (NV) and 1.2×10^6 CFU/g (V). The initial counts of coliforms were 1.5×10^3 CFU/g and these counts rose continuously, reaching 4.5×10^9 CFU/g (15 °C, NV) and 9.0×10^8 CFU/g (15 °C, V) and 4.0×10^7 CFU/g (3 °C, NV) and 1.2×10^5 CFU/g (3 °C, V). Our results are in agreement with Gelman, Glatman, Drabkin, and Harpaz (2001) and Frank, Baranowski, Chongsiriwatan, Brust, and Premaratne (1985), who reported that the biogenic amine formation is more related to the activity of mesophilic than psychrotrophic bacteria. On the other hand, Veciana Nogués et al. (1997), studying tuna fish, pointed out that the formation of PUT, CAD and TY in samples stored at 0 °C cannot be excluded.

The best correlations (linear regression) among bacteria and amines were found for MVC, coliforms ($\log_{10}$ CFU/g) and the $\log_{10}$ of PUT contents or the total of $\log_{10}$ PUT and CAD contents (Table 12).

4. Conclusions

Biogenic amines in stored carp flesh do not represent any health hazard for individuals, because the contents of the most problematic amines – HI and TY – are low compared to selected kinds of sea fish.

Contents of PUT, or the total content of PUT and CAD, seem to be good quality markers, as they were in the best correlations with both sensory levels and total microbial counts within all experiments.

Though the effect of vacuum-packaging for biogenic amine formation was explicit, especially at lower temperature, the effect of temperature was predominant.

Application of vacuum-packaging at 3 °C prolonged the shelf life by about 4–5 days; at 15 °C its effect was negligible.

Table 12
Correlation coefficients (r) for $\log_{10}$ CFU vs. $\log_{10}$ c(amine)

	Mesophilic viable counts				Coliforms				Psychrotrophs			
	NV/3 °C	NV/15 °C	V/3 °C	V/15 °C	NV/3 °C	NV/15 °C	V/3 °C	V/15 °C	NV/3 °C	NV/15 °C	V/3 °C	V/15 °C
PUT	0.751**	0.895**	0.743**	0.789**	0.792**	0.907**	0.673**	0.798**	0.885**	–	NS	NS
PUT + CAD	0.765**	0.914**	0.764**	0.817**	0.812**	0.925**	0.746**	0.831**	0.870**	–	NS	NS
HI	NS	0.753*	NS	0.773**	0.622*	0.763**	NS	0.713**	0.688*	–	NS	NS
TY	0.640**	0.953**	NS	0.834**	0.635**	0.953**	0.497*	0.832**	0.665*	–	NS	NS

NS – not significant.
* $P \leqslant 0.05$.
** $P \leqslant 0.01$.

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