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RESEARCH ARTICLE

Comparative study of aspartame and neotame stability in Ice cream and Cake

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Abstract: The suitability of artificial sweeteners aspartame and neotame for the incorporation in products like ice cream and cake was studied. These sweeteners were compared for their stability during processing and storage and the effect of addition on different physico-chemical properties were also considered. The recovery of the HPLC method used for the estimation of aspartame and neotame from ice cream and cake was 96.22-98.57% and 96.08-98.62%, respectively. During pasteurization of ice cream mix (68°C/30 min), 25% of aspartame was degraded, however, no loss of neotame was observed. Aspartame and neotame content decreased significantly from 74.55 to 62.97% and 99.42 to 89.93%, respectively during storage (-18°C/90 days) of ice cream. During baking (180°C/20 min), the degradation of aspartame and neotame was about 50 and 13%, respectively. However, aspartame and neotame content decreased significantly from 49.90 to 24.61% and 87.20 to 62.40%, respectively during storage (25°C/20 days) of cake.

Introduction

Neotame (Dimethyl butyl-aspartyl phenylalanine-methyl ester) (NTM), a derivative of aspartame (Aspartyl phenylalanine-methyl ester) (APM) is gaining popularity in the food industry due to its greater sweetness potency (30-60 time sweeter than APM), higher stability at neutral pH with no adverse health effects as compared to APM (Kumari et al. 2016b). Besides the approval from FDA as food additives, APM consumption always remains controversial among the consumers, as some of the published reports suggested APM's ill health effects and regenerated controversy among consumers (Landrigan and Straif, 2021; Soffritti et al. 2007, Soffritti et al. 2010; Belpoggi et al. 2006; Andreatta et al. 2008; Tibaldi et al. 2020). However, more than 100 studies claimed its safety and negate any association with adverse health effects (EFSA, 2006; EFSA, 2013; Butchko et al. 2002). In 1996, FDA declared APM to be safe at a daily acceptable level of 50 mg/kg/day, except for phenylketonuric person. Phenylalanine (Phe), a degradation product of APM is toxic for phenylketonuric persons as it results in mental retardation (Harper, 1984). However, in NTM, the presence of dimethyl butyl (DMB) group restricts the production of Phe and makes it safer for consumption. Additionally, DMB also provides remarkable sweetness potency, flavour enhancing property, and stability during the baking process. Since 2002, NTM is permitted in the food industry as a general-purpose sweetener but still not extensively used in food products. The stability of the sweetener is greatly affected by pH, temperature, duration and water activity (Tsoubeli and Labuza, 1991). These sweeteners are most stable between pH 4 to 5 (Kumari et al. 2016b). At alkaline or neutral pH, APM can be degraded into different degradation products like aspartyl-phenylalanine (ASP-Phe), which may be further hydrolyzed into aspartic acid or Phe or may cyclize into diketopiperazine (DKP) etc. At acidic pH, α-APM can be isomerized into β-APM, which can be further degraded into aspartyl-phenylalanine (Prodolliet and Bruuelhart, 1993). NTM when used at a much higher concentration (200 ppm) in a beverage (pH 3.2, stored at 20°C/8 weeks) only de-esterified NTM was the principal degradation product (7% of original NTM), however, three minor degradation products less than 1% concentration was obtained by cyclization, beta arrangement or hydrolysis. At a lower initial concentration (15 ppm) these products were not detected (JECFA 52, 2004). Very few published reports are available regarding the stability of NTM in the food system. APM when used in products like yoghurt (heating temperature 85°C/30 min and pH 4.5) and flavoured milk (temperature 90°C/20 min, pH 6.6) about 40-50% of APM was degraded into ASP-Phe, however only 3 to 7% of NTM was degraded at same condition (Kumari et al. 2016b, 2018).

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APM was completely degraded into ASP-Phe and Phe during the preparation of in-bottle sterilized milk, however, 50% NTM was degraded (Kumari et al. 2016b). The stability of APM was also studied in chocolate milk (Keller et al. 1991b), *burfi* (Arora et al. 2010), whey lemon beverage (Arora et al. 2013), orange flavoured soft drinks by HPLC (Yakici and Arici, 2013). APM sweetened cake was evaluated by several workers for its sensory properties (Wetzel et al. 1997; Baeva 2000; Nourmohammadi et al. 2011) and this sweetener was found to be a successful sweetener in frozen desserts also (Keller et al. 1991a; Guzeler et al. 2011, Rathod et al. 2013). Cake and ice cream are the most widely consumed products by all age groups and have potential industrial applications for sweeteners. But before using any artificial sweeteners in these products, their stability and breakdown products should be properly analyzed. The acceptability of APM in ice cream and cake in terms of sensory properties has been reported in the literature, however, the relevant information is still lacking on its stability in ice cream and cake during processing and storage. Data also lacks on comparative stability study of APM and NTM as affected by different processing parameters in these products.

Hence, the present study was therefore designed to analyze the stability of APM and NTM by HPLC method in ice cream and cake during processing and storage. The effect of different processing parameters such as pH, temperature, water activity, and storage period was also studied.

Materials and Methods

Water and acetonitrile (HPLC grade), aspartyl-phenylalanine methyl ester (aspartame), L-phenylalanine were procured from Sigma-Aldrich (Layoffs, Missouri, USA). HPLC grade potassium dihydrogen phosphate and dipotassium hydrogen phosphate were purchased from Qualigens fine chemicals (Mumbai, India). The degradation product L-Aspartyl-L-phenylalanine was procured from Sigma-Aldrich (Steinheim, Germany). Zinc sulphate and potassium ferrocyanide were procured from S.D. Fine Chem. Ltd. (Mumbai, India). SPE cartridge DSC-18LT Discovery (6 ml tube of 1 g capacity) and Vacuum assembly Visiprep™ DL were procured from Supelco (Bellefonte, PA, USA).

Milk sample was obtained from the dairy plant of National Dairy Research Institute (Karnal, India). APM and NTM were procured from NutraSweet Sweetener Company (Georgia, USA). Polydextrose was procured from Danisco India Private Limited (Gurgaon, India) and maltodextrin from M/S Sukhjit Starch and Chemicals limited (Punjab, India). The wheat flour was obtained from Victoria foods Private Limited (New Delhi, India) and shortening from Bunge India Private Limited (Mumbai, India). The cornflour and baking powder were obtained from Weikfield foods Private Limited (Maharastra, India). Whey protein concentrate-70 (WPC-70) was procured from Modern Dairies Limited (Haryana, India) and cake gel from AB Mauri India Private Limited (Maharashtra, India). The vanilla flavour was obtained from Ajanta Enterprises (Himachal Pradesh, India) and the mixture of stabilizer and emulsifier from Danisco India Private Limited (Gurgaon, India).

Preparation of Ice cream

Ice cream was prepared according to the method described by Kumari et al. (2016a). The sugar was completely replaced by APM and NTM in ice cream at an acceptable level of APM (1200 mg/kg ice cream mix) and NTM (30 mg/kg ice cream mix). As a bulking agent, WPC-70 (1.5%) and maltodextrin (9.9%) were used.

Preparation of Cake

The cake was prepared according to the method of Kumari et al. (2016a). For the preparation of APM and NTM sweetened cake, the sugar was completely replaced by the best acceptable level of APM (7000 mg/kg cake mix) and NTM (60 mg/kg cake mix), respectively.

Extraction of APM and NTM from Ice cream

For the extraction of APM, the APM sweetened ice cream sample was first degassed using on an ultrasonication bath for 20 min at 30°C. Ten grams of the degassed sample were taken in a 100 ml beaker. 50 ml aqueous methanol solution (20%) was added into it and sonicated for 20 min at 40°C. It was cooled to ambient temperature (30°C) and transferred to 100 ml volumetric flask. Three milliliters of each carrez I (3.6 g potassium ferrocyanide in 100 ml water) and carrez II (7.2 g zinc sulphate in 100 ml water) were added into it and kept undisturbed for 10 min. Diluted up to the mark by water (HPLC grade) and filtered through filter paper (Whatman no.1). The NTM extraction from ice cream was done as per the method of kumari et al. (2016a). The filtrate is further purified by passing through an SPE cartridge.

Extraction of APM and NTM from cake

For the extraction of APM, five grams of APM sweetened cake sample was added into 50 ml of aqueous methanol solution (20%). After vortex for 2 min, it was sonicated for 20 min at 40°C. It was cooled to ambient temperature (30°C) and centrifuged (Kubota Corporation 6800, Tokyo, Japan) for 10 min at 2000×g. The supernatant was transferred into a 50 ml volumetric flask and added into 2 ml carrez 1 and II each and shaken vigorously. It was kept undisturbed for 10 min at ambient temperature (30°C), diluted up to the mark with water (HPLC grade) and filtered through filter paper (Whatman no.1). Further purification was done by passing through the SPE cartridge. The extraction of NTM from cake was done as per the method described by Kumari et al. (2016a).

The solid-phase extraction of APM and NTM from sample filtrate was done as per the method described by Kumari et al. (2016b). The 20µl of extracts were filtered through a Hamilton microlitre

syringe filter (Hamilton Company, Nevada, USA) before being injected into the HPLC system.

HPLC conditions

Waters HPLC apparatus equipped with PDA detector (Waters 2998, Massachusetts, USA), Empower 2 software and manual injector with 20 µl sample loop were used for the analysis of sweeteners. Phenomenex C_{18} column (4.6×250 mm, pore size 100 Å, particle size 5 µm) maintained at 40°C was used for analysis of sweeteners. APM, NTM and its degradation products were quantified according to the method described by Kumari et al. (2016a and 2016b). APM and its degradation products (Phe and ASP-Phe) were analyzed under gradient conditions as described by Kumari et al. (2016b). The mobile phase A consisted of 0.02 M phosphate buffer, pH 5.0: acetonitrile (97:3) and mobile phase B consisted of 0.02 M phosphate buffer, pH 3.5: acetonitrile (80:20). The gradient condition is depicted in Table 1. The flow rate of the mobile phase was 1 ml/min and wavelength for analysis was 200 nm. NTM was analyzed under the isocratic condition as followed by Kumari et al. (2016a). The mobile phase consisted of a 60:40 mixture of acetonitrile and water (0.09%TFA) was used at 0.6 ml/min flow rate and 210 nm wavelength.

Method validation

Individual solutions of APM, ASP-Phe, Phe (5-100 mg/l) were prepared in mobile phase A and B (1:1) and NTM solution (5-100 mg/l) was prepared in methanol: water (20:100). These prepared solutions were used for the preparation of the standard curve, which was plotted by taking concentration against peak area.

The LOD and LOQ were determined by using the formula LOD= 3 (standard deviation of curve's response)/calibration curve slope and LOQ= 10 (standard deviation of curve's response)/calibration curve slope.

The precision and accuracy of the method were calculated according to the method mentioned by Kumari et al. (2016a).

The percentage recovery of the method was calculated by using the following formula:

$$\% \text{ Recovery} = \frac{X}{Y} \times 100$$

Where,

X = amount of sweetener recovered

Y = amount of sweetener added to the product

pH

The pH of ice cream and cake was determined by the method as described in IS: SP: 18, part XI (1981) and AOAC method (2005), respectively.

Water activity

The water activity was measured by water activity meter "Aqual Lab' (Model Series 3 TE) supplied by Decagon Devices, W. A., USA. (Plate-6).

Stability monitoring

Effect of pasteurization and baking

The ice cream mix sample was cooled to 4-7°C just after heating the ice cream mix (68°C/30 min) and was analyzed in triplicate for the sweeteners in ice cream mix

The cake was cooled to ambient temperature (30°C) after baking (180°C/20 min) and was analyzed in triplicate for the sweeteners in cake

Storage stability

Ice cream samples were stored at -18°C for 90 days and samples were analyzed weekly during storage for the sweetener content.

Cake samples were stored at 25°C for 20 days and samples were analyzed at 0, 4th, 8th, 12th, 16th and 20th days of storage the sweetener content.

Statistical analysis

Means, relative standard deviation (RSD), standard error mean (SEM), 95% confidence intervals and linear regression analysis were evaluated by Microsoft Excel (2007). A significant difference between mean values was tested using ANOVA.

Results and Discussion

Method validation

Table 2 represented the correlation coefficients and regression equation of the standard curve. The correlation coefficient for

Table 1 Gradient programming for detection of Aspartame and its degradation products

Time (min)	A concentration (%)	B concentration (%)
0.1	100	0
8	100	0
13	0	100
25	0	100
27	100	0
30	100	0

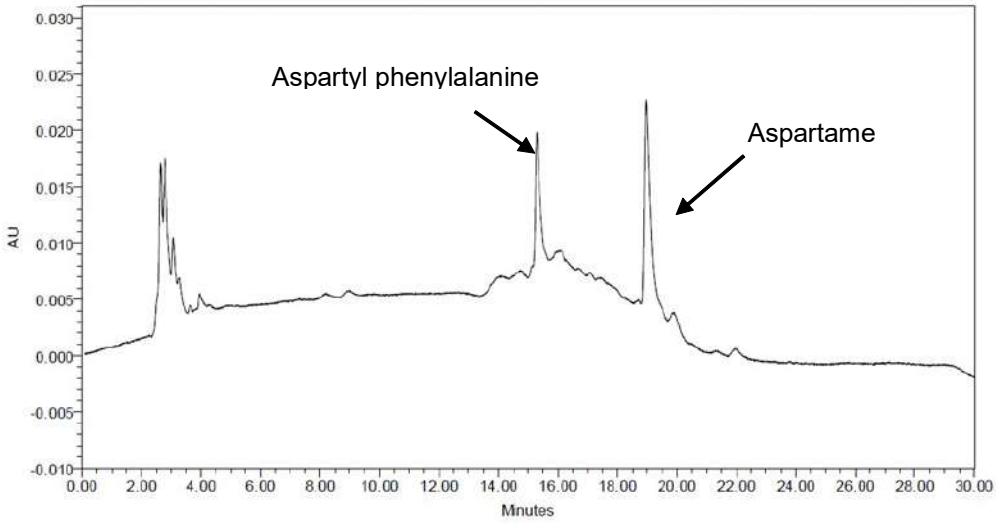

Fig. 1a Chromatogram of aspartame and its degradation product in ice cream

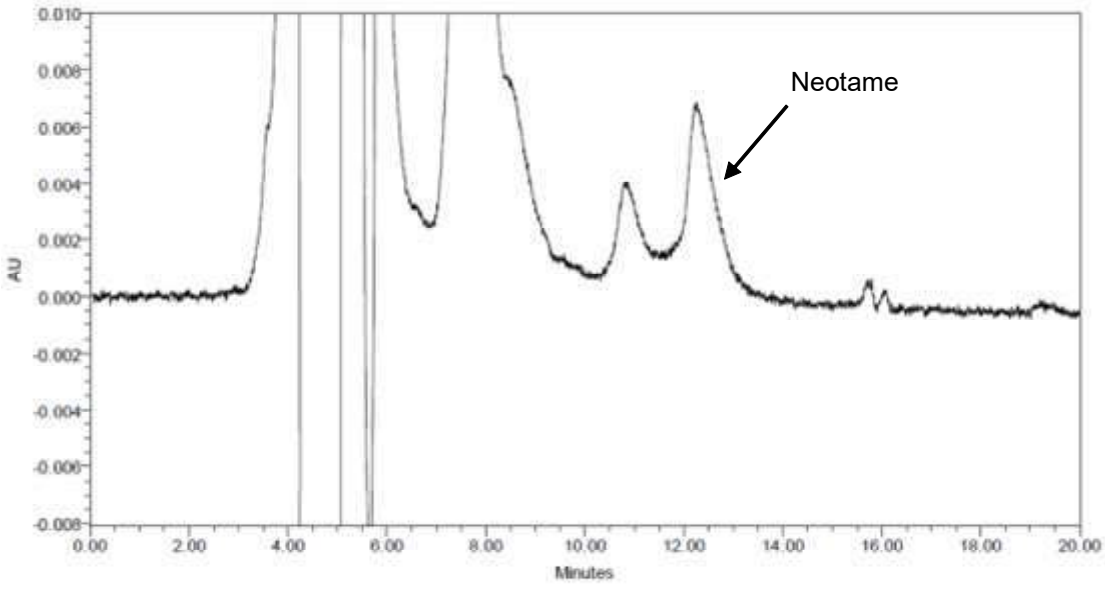

Fig. 1b Chromatogram of neotame in ice cream

Table 2 Sensitivity and Linearity of the method

Sweetener/Degradation product	Regression equation	R^2 Correlation Coefficient	LOD (mg/l)	LOQ (mg/l)
Aspartame	y = 45137x-4036.5	1.0	1.5	4.5
Aspartyl-phenylalanine	y = 8683.7x-21040	0.998	1.0	3.0
Phenylalanine	y = 30095x-14721	0.9989	1.0	3.0
Neotame	y = 54004x-32859	1.0	0.25	0.50

APM, NTM and its degradation product was greater than 0.99. The calibration curve was found to be linear from 5 to 100 mg/l concentration.

Table 2 represented the values of LOD and LOQ. A similar value of LOD for APM was reported by George et al. (2010), Lawrence and Charbonneau (1988) and Arora et al. (2008; 2010; 2013) by using the C_{18} column at wavelength 200 nm. Our results for NTM were in accordance with Yang and Chen (2009 and 2010). The chromatogram for APM, NTM and its degradation products in ice cream and cake samples were represented in Figures 1a, 1b, 2a and 2b.

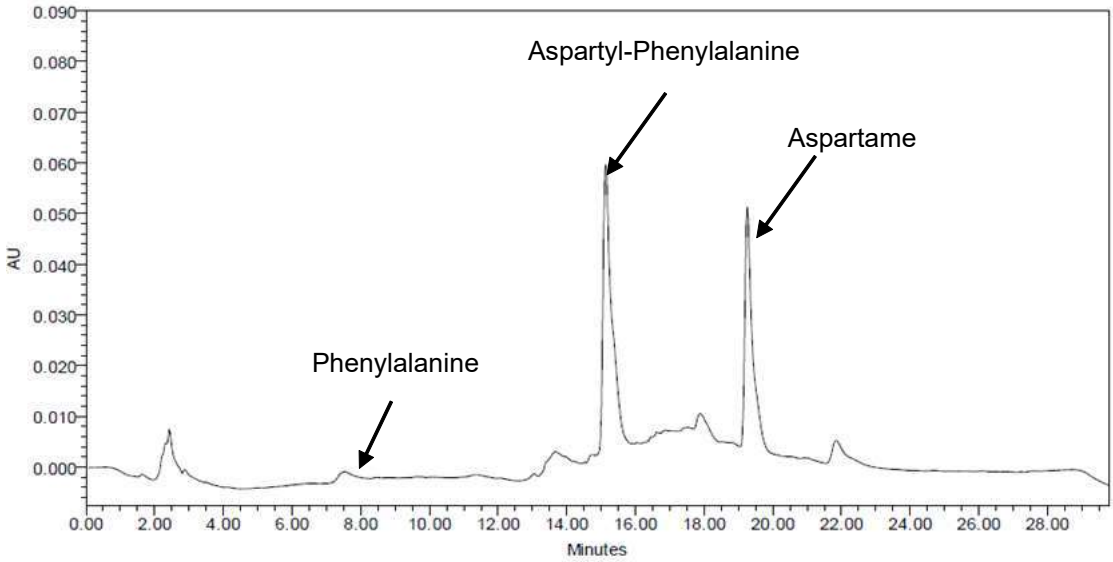

Fig. 2a Chromatogram of aspartame and its degradation products in cake

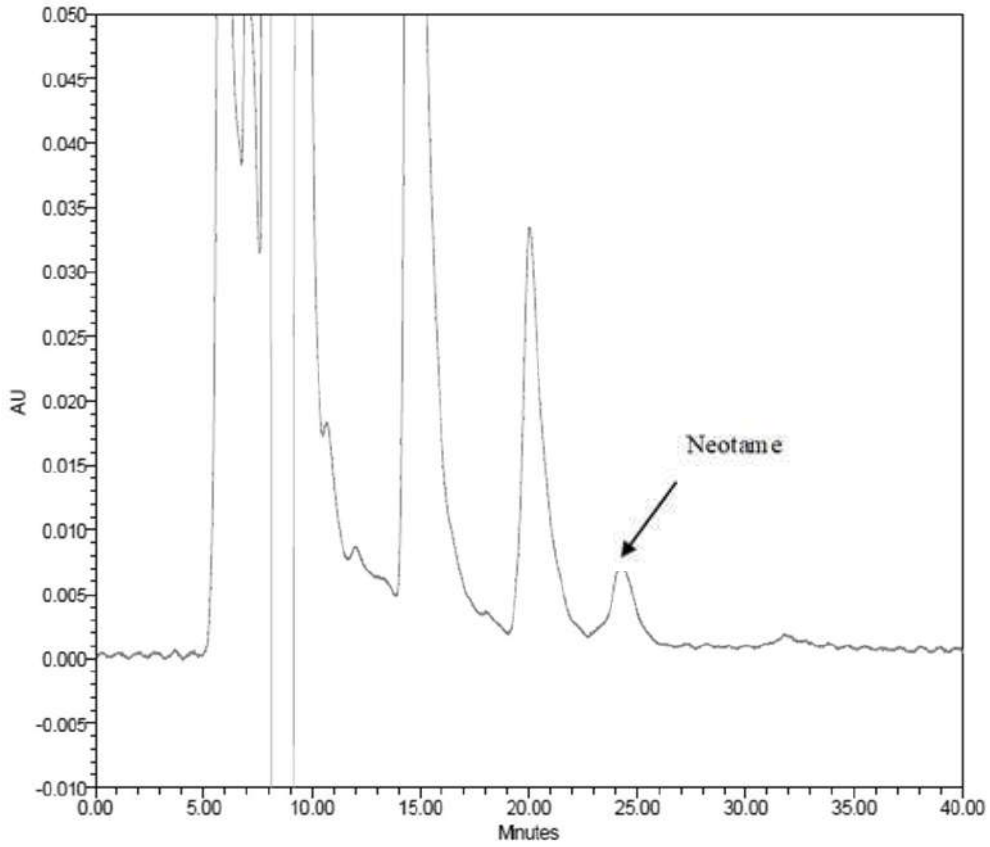

Fig. 2b Chromatogram of neotame in cake

The accuracy was represented as per cent recovery of a specific component in the sample, the recovery (mean±%RSD) of APM and NTM from ice cream and cake is depicted in Table 3 and 4, which shows the high accuracy of the method.

The values for interday and intraday precisions (% RSD) are depicted in Table 3 and 4. All these values were within the 5% limit, indicating repeatability of the method.

pH

The stability of sweeteners is greatly affected by the pH of the food (Kumari et al. 2016b). The pH of ice cream (6.5) and cake (6.9) is close to neutral pH. The activation energy of the sweetener degradation is closely related to the pH condition and it is lower at neutral pH (Ozol, 1986). The stability with respect to pH needs to be monitored during the storage period in the present study.

In the case of ice cream, no significant ($p>0.05$) difference among the sample of control, APM and NTM sweetened ice cream was observed. A significant decrease in the pH of control (6.57-6.52), APM sweetened (6.55-6.52) and NTM sweetened (6.56-6.52) ice cream was observed during the 0^{th} to 90^{th} day of storage at -18°C.

There is a slight decrease in the pH of control (7.05-6.97), APM (6.91-6.85) and NTM (6.93-6.86) sweetened cake during the storage period (25°C/25 days), however, the pH of control, APM and NTM cake did not differ significantly ($p>0.05$) both from each other and throughout the storage period.

Water activity

There is a combined effect of pH and water activity on the activation energy of sweeteners degradation (Bell and Labuza, 1991a). At pH 7 the activation energy of sweetener degradation declines on increasing water activity from 0.56 to 0.99 (Bell and Labuza 1991b). Due to these reasons, the present study monitors the water activity along with the sweetener stability during the storage period.

The water activity of the cake containing APM and NTM was significantly higher ($p<0.05$) than the control (cake with sucrose) (Table 5). The lower water activity of the control cake is due to the presence of sugar, which could not be compensated by the bulking agents (maltodextrin and polydextrose) used for APM and NTM sweetened cake (Wetzel et al.., 1997). The water activity of control, APM and NTM sweetened cake significantly ($p<0.05$) decreases during the storage (25°C/25 days).

Stability of APM and NTM during processing of ice cream and cake

In APM sweetened ice cream, pasteurization (68°C/30 min) of the ice cream mix resulted in partial degradation of APM into ASP-Phe. Results revealed that 74.55±0.08% of APM remained intact and the degradation of APM resulted in the formation of ASP-

Table 3 Precision and Accuracy of the method for analysis of APM (n=6)

Sample	Concentration added (mg/l)	Observed concentration	% Recovery	Intraday Precision (%RSD)	Interday Precision (%RSD)
Ice cream	Low level (1000)	985.7	98.57	0.61	0.86
	Medium level (1200)	1178.64	98.22	0.57	0.92
	High level (1400)	1378.44	98.46	0.85	0.99
Cake	Low level (1000)	962.2	96.22	1.25	1.25
	Medium level (5000)	4815.5	96.31	0.85	1.17
	High level (7000)	6759.9	96.57	0.89	1.21

Table 4 Precision and Accuracy of the method for analysis of NTM (n=6)

Sample	Concentration added (mg/l)	Observed concentration	% Recovery	Intraday Precision (%RSD)	Interday Precision (%RSD)
Ice cream	Low level (20)	19.71	98.54	1.43	1.17
	Medium level (40)	39.43	98.57	0.85	1.86
	High level (60)	59.17	98.62	0.92	1.52
Cake	Low level (25)	24.04	97.98	1.86	2.85
	Medium level (60)	58.78	96.17	1.34	2.70
	High level (100)	96.08	96.08	1.86	2.15

Table 5 Water activity of cake during storage

Storage period (days)	Control	APM Sweetened cake	NTM Sweetened cake
0	0.83 ± 0.01^{aD}	0.91 ± 0.01^{bD}	0.92 ± 0.01^{bD}
4	0.79 ± 0.01^{aC}	0.87 ± 0.01^{bC}	0.87 ± 0.01^{bC}
8	0.76 ± 0.02^{aB}	0.83 ± 0.01^{bB}	0.84 ± 0.01^{bB}
12	0.76 ± 0.01^{aB}	0.82 ± 0.03^{bB}	0.83 ± 0.01^{bB}
16	0.76 ± 0.01^{aB}	0.83 ± 0.01^{bB}	0.84 ± 0.01^{bB}
20	0.74 ± 0.01^{aA}	0.80 ± 0.01^{bA}	0.79 ± 0.01^{bA}

Values are expressed as means±SEM (n=3)

$^{a-b}$Means within rows with dissimilar lower case superscript differ significantly ($p<0.05$)

$^{A-D}$Means within the column with dissimilar uppercase superscript differ significantly ($p<0.05$)

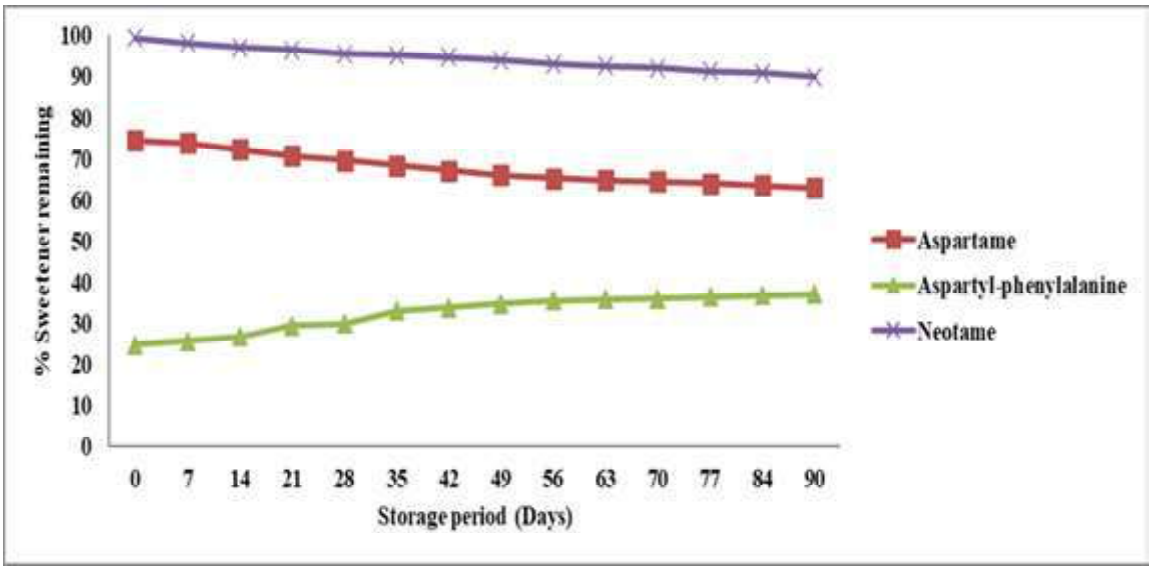

Fig. 3 Levels of sweeteners and degradation products in ice cream during storage (-18°C/90 days)

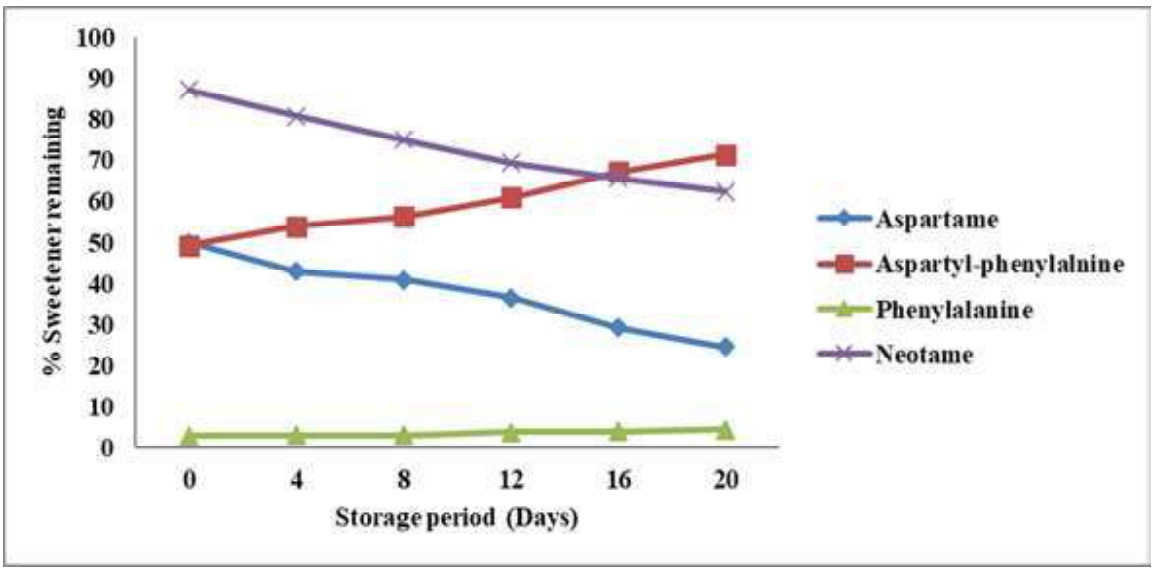

Figure 4. Levels of sweeteners and degradation products in cake during storage (25°C/20 days)

Phe (24.81±0.12%). However, in NTM sweetened ice cream, after pasteurization 99.27 ± 0.21% of the NTM remained intact.

During the preparation of APM sweetened cake, baking (180°C/ 20 min) resulted in degradation of APM into ASP-Phe and Phe. During baking, 49.30±0.20% of APM remained intact and 49.72±0.09% ASP-Phe and 2.85±0.01% Phe were formed. However, in the case of NTM sweetened cake, during baking 87.29 ± 0.19% of NTM remained intact.

Our results were in accordance with Wetzel and Bell (1998) and Prodolliet and Bruuelhart (1993), who reported that APM is sensitive to degradation when heated for a longer duration at pH >6 and can be hydrolyzed into ASP-Phe or Phe at this pH. Heating (63°C/30 min) of APM containing phosphate buffer (0.1 M, pH 7.0) resulted in 83% loss of APM (Tsoubeli & Labuza, 1991). In pasteurized flavoured milk (90°C/20 min), about 40% of APM was degraded into ASP-Phe (Kumari et al. 2016b). However, in in-bottle sterilized flavoured milk (121°C/15 min), APM was completely degraded into ASP-Phe and Phe. At temperature 136°C/15 sec and pH 6.0, 42% APM loss was observed by O'Donnell (2012). In cake, 33-34% encapsulated APM was recovered after baking, while non-encapsulated APM recovery was 22% (Wetzel and Bell, 1998).

Stability of APM and NTM during storage

In APM sweetened ice cream, the amount of APM significantly decreased ($p<0.05$) in the range of ~75 % to 63% during the storage period (-18°C/90 days). However, the amount of ASP-Phe increased significantly ($p<0.05$) in the range of ~25% to 37% during this period (Figure 3). During storage of NTM sweetened ice cream, the amount of NTM reduced significantly ($p<0.05$) from ~99% to 90% (Figure 3). Although the ice cream

pH (6.5) is not optimal for sweeteners stability, the rate of degradation was slower due to the frozen temperature at storage conditions. Our results were in agreement with Gloria (2003) who reported that in frozen dairy dessert (pH 6.5-7.0), due to frozen state the degradation rate decreased and the maximum stability of sweetener was detected in the lower temperature range used for frozen and refrigerated storage. Besides, due to the lower amount of free moisture, APM stability is more than expected (Abegaz et al. 2012). Our results were in accordance with Nofre and Tinti (2000) who stated that the degradation rate of NTM is lower at a lower temperature. During storage of in-bottle sterilized milk (30°C/60 days) and pasteurized milk (4-7°C/7days), the amount of NTM declined significantly from 50.36 to 8.67% and 91.78 to 87.18%, respectively (Kumari et al. 2016b).

During storage of APM sweetened cake (25°C/20 days), the APM level decreased significantly ($p<0.05$) from ~50% to 25%, however, a significant increase in the level of ASP-Phe (from ~49% to 71%) and Phe (from ~3% to 4%) was observed (Figure 4). The pH of the cake was about 6.9 during storage which is favourable for APM degradation (Homler, 1984; Ozol, 1986; Tsoubeli & Labuza, 1991; Bell & Labuza, 1991b and Gloria, 2003). The amount of NTM declined significantly ($p<0.05$) from ~87% to 62% during the storage of NTM sweetened cake (pH 6.9) (Figure 4). Our results were in accordance with Nofre and Tinti (2000) who found 85% recovery of NTM after baking and on storage at room temperature for 5 days, 81% NTM was retained. Alkaline pH, storage temperature and duration all combinedly affect the sweetener degradation.

Conclusions

In ice cream, pasteurization of ice cream mix resulted in 25.35% loss of APM and the APM amount was decreased to about 12% during storage. However, no loss of NTM was found during pasteurization but NTM amount was decreased to 9% during storage. The extent of decrease of both the sweeteners was comparatively slower due to frozen conditions. In case of cake, 50.7% of APM was lost by baking and about 25% of the loss was observed during storage period. However, only 13% of NTM was lost during baking and about 24% during storage. Heating at a higher temperature for a longer duration resulted in faster degradation of sweeteners. APM was found to be more sensitive towards heat treatment as compared to NTM. Thus NTM was found to be more stable as compared to APM during processing and storage conditions of ice cream and cake. These features allow NTM's application in high heated products.

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