ANALYSIS OF ACESULFAME-K AND ITS STABILITY IN INDIGENOUS DAIRY PRODUCTS

A THESIS SUBMITTED TO THE
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IN PARTIAL FULFILMENT OF THE REQUIREMENTS
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MASTER OF SCIENCE
IN
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(DAIRY CHEMISTRY)

BY

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Date: JUNE 12th, 2006

(**VIVEK SHARMA**)
MAJOR ADVISOR

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LIST OF ABBREVIATIONS

APCC	= Asian Pacific Coconut Community
FDM	= Fat in Dry Matter
FID	= Flame Thermionic Detector
GLC	= Gas Liquid Chromatography
HDL	= High Density Lipoprotein
LDL	= Low Density Lipoprotein
MFFS	= Moisture in Fat Free Solids
MSS	= Mean sums of square
OD	= Optical Density
PAGE	= Polyacrylamide Gel Electrophoresis
RP-HPLC	= Reverse Phase High Performance Liquid Chromatography
SEM	= Scanning Electron Microscopy
SNF	= Solid Non Fat
TFFA	= Total Free Fatty Acids
TVFA	= Total volatile fatty acids
UNIDO	= United Nations Industrial Development Organization
UV	= Ultraviolet
VLDL	= Very Low Density Lipoprotein
WSF	= Water Soluble Fraction

ABSTRACT

Gouda cheese is a quick ripening variety and can be used for the manufacture of processed cheese/cheese spread. Coconut cream filled Gouda cheese was prepared from skim milk and coconut cream using the method of Scott (1987) with certain modifications. The product was evaluated for compositional, biochemical, structural, textural, microbial and sensory attributes during ripening along with control.

Yield of filled cheese was found slightly higher than that of control. There was a significant increase of moisture in filled cheese as compared to control. Moisture in fat free solids (MFFS) in control was significantly higher than in the filled cheese. The fat in filled cheese was significantly lower than that of control, but the fat in dry matter (FDM) in filled cheese was significantly higher as compared to control. There was no significant difference in protein and lactose. Salt and salt in moisture in filled cheese was significantly higher than that of control. Both the cheeses in present study had FDM of more than 50%.

pH, titratable acidity and lactate content of both the cheeses increased progressively throughout the ripening. pH of filled cheese was significantly higher than that of control throughout the ripening. Control had significantly higher values for titratable acidity and total lactate as compared to filled cheese at all stages of ripening.

Filled cheese had significantly higher values for total free fatty acids (TFFA) and total volatile fatty acids (TVFA) as compared to control during whole ripening period. Peroxides did not develop during ripening in both the cheeses. Control had higher values for total carbonyls as compared to filled cheese at all stages of ripening. TFFA, TVFA and total carbonyls showed an increasing trend for both the cheeses throughout the ripening. Further, there was a significant difference in short, medium, long and unsaturated fatty acids between the two cheeses. The contents of short chain fatty acids, medium chain fatty acids and long chain fatty acids significantly increased and unsaturated fatty acids decreased during ripening in both the cheeses.

The soluble protein and ripening index in both the cheeses increased during ripening. There was no significant difference in these parameters between two cheeses.

The electrophoretic pattern of cheese revealed that α_{s1} casein degraded preferentially over β casein in both the cheeses. At any sampling age, the PAGE pattern of the control and filled cheese were similar.

Peptide profiles obtained by RP-HPLC of both the cheeses were almost similar throughout the ripening. Similar pattern of peaks were observed in both the cheeses at any sampling age. There was no significant difference in hydrophobic to hydrophilic ratio (HO/HI) between control and filled cheese. However, effect of ripening on HO/HI is highly significant.

The hardness, cohesiveness, gumminess and chewiness of both the cheeses increased to a maximum at 1 month of ripening and decreased thereafter. Springiness reduced consistently with the progress of ripening in both the cheeses. Hardness, gumminess springiness and chewiness were higher in filled cheese than that of control throughout the ripening. Cohesiveness was lower in filled cheese as compared to control throughout the ripening.

Total viable bacteria and lactic acid bacterial counts were slightly higher in control as compared to filled cheese during ripening. These counts of both cheeses increased during early stages of ripening followed by a rapid decline as the ripening progressed.

The score for flavour was in normal to good range, while colour ranged between good to very good range for both cheeses after 4 months of ripening. Filled cheese was found to have coconut flavour in early stages of ripening which was masked as ripening progressed. The body and texture scores were slightly higher in filled cheese than in control when fully ripened.

Filled Gouda cheese was characterized by a 3–dimensional, stable microstructure in comparison to control as exhibited by scanning electron microscopy (SEM).

Filled Gouda cheese with an acceptable quality could be prepared from skim milk and coconut cream. Apart from providing a successful outlet for utilization of surplus milk and coconuts, this will present an alternate variety to the cheese loving clientele.

सारांश

बर्फी, कलाकन्द तथा सुगन्धित दूध बनाने में शर्करा (स्यूकरोस) के स्थान पर एस्लफेम-के का प्रयोग किया गया । संवेदी मूल्यांकन से प्रकट होता है कि 0.045 प्रतिशत बर्फी और कलाकन्द तथा 0.04 प्रतिशत सुगन्धित दूध पर इन डेरी उत्पादों को तैयार करने में जब एस्लफेम-के का प्रयोग किया गया तो यह मिठास बोध में उच्चतम तथा नियंत्रण से समरूप था । एस्लफेम-के से मीठे बनाए गए डेरी उत्पादों में वही वांछनीय मिठास, रंग, बनाव एवं संरचना/गाढ़ापन एवं मुहंस्वांद संग्रहण के सात दिनों के बाद भी कायम पाई गई। नियंत्रण की अम्लता में तथा एस्लफेम-के द्वारा मीठे उत्पादों में संग्रहण के दौरान महत्वपूर्ण वृद्धि पाई गई । इसी प्रकार संग्रहण करने पर सभी नमूनों में अम्लता का स्तर कम पाया गया ।अनुरूप नियंत्रित नमूनों की अपेक्षा एस्लफेम-के द्वारा मीठे उत्पादों में अनुमाप्य अम्लता अधिकतम थी । सग्रंहण के दौरान स्यानता में परिवर्तन एस्लफेम-के द्वारा मीठे सुगन्धित दूध तथा नियंत्रित नमूनों दोनों में, महत्वपूर्ण नही थी । स्यूकरोस (शर्करा) के साथ नियंत्रित दूध की तुलना में एस्लफेम-के द्वारा मीठे सुगन्धित दूध में स्यानता की मात्रा कम थी । संग्रहण की पूरी अवधि के दौरान विभिन्न एस्लफेम-के द्वारा मीठी बरफी तथा कलाकन्द के विभिन्न संरचनात्मक गुणों का स्तर कम था। बरफी तथा कलाकन्द में एस्लफेम-के के प्रयोग से तन्त्र की सघनता कम हो गई ।

संग्रहण के दौरान एस्लफेम-के द्वारा मीठे बरफी तथा कलाकन्द की अपेक्षा शर्करा से नियंत्रित नमूनों में उज्ज्वलता कम थी । तथापि, सुगन्धित दूध में नियंत्रित नमूनों की तुलना में एस्लफेम-के युक्त नमूनों मे उज्ज्वलता अधिक पाई गई । संग्रहण की सम्पूर्ण अवधि के दौरान उनके अनुरूप नियंत्रित नमूनों की अपेक्षा एस्लफेम-के युक्त मीठे उत्पादों में कुल प्लेट काऊन्ट वंशक्रम से बढ़े ।

डेरी उत्पादों में एस्लफेम-के के वियोजन के लिए एक ठोस प्रावस्था निष्कर्षण विधि मानकित की गई । एस्लफेम-के एवं इसके अवक्रमित उत्पाद एस्टोऐस्टामाइड के वियोजन के लिए एच पी एल सी विश्लेषणात्मक अवस्थाएं मानकिंत की गई । उच्च दबाव वर्ण रेखिए विश्लेषण यह प्रमाणित करते है कि एस्लफेम-के अवक्रमित नही होते । बरफी, कलाकन्द तथा सुगन्धित दूध मे मीठास काफी स्थायी था क्योंकि इन उत्पादों में इसकी स्थिरता प्रमाणित करने में संग्रहण के दौरान एच पी एल सी विश्लेषण पर कोई विघटन नही पाया गया।

CHAPTER - 1

Introduction

1. INTRODUCTION

In the last couple of decades, the growing concern about health and quality has encouraged people to exercise, eat healthy food, decrease the consumption of food rich in sugar, salt and fat. Previously, food science was concerned with the development of food for human survival, now it was substituted for the concept of production of quality food. Recently, the idea has become to use food as a means of promoting welfare and health, while reducing the risk of diseases. The food industry has responded to this demand and, as a consequence, there has been a fast increase in diet foods and beverages available to consumers in many markets of the world. With increased consumer interest in reducing sugar intake, food products made with sweeteners other than sugar have become more popular. The discovery of a great number of sweeteners during the last decade has triggered the development of new sugar-free products, particularly for diabetics, people on special diets. (Spinheiro *et al*, 2005). Sweetness of taste plays a preponderant role in food preference. Several food ingredients stimulate the sensation of sweetness by interacting with taste receptor cells on the tongue. The power of these ingredients varies with the properties of the food system, temperature, physical state, the presence of other flavours, and with characteristics of the person, such as genetics, health status, and age. There are many different sweeteners, which can be grouped into three main types viz; **i)** sugars (refined sugars, sucrose, fructose, glucose, dextrose, maltose, etc), **ii)** sugar replacements, polyols, or sugar alcohols (sorbitol, manitol, xylitol, isomalt, etc), and **iii)** intense sweeteners (saccharin, aspartame, acesulfeme-K, sucralose etc). The first two groups are nutritive sweeteners. These include sweeteners with sweetening power near or inferior to sucrose, and add functional properties to foods through their effects on sensory, physical, microbial and chemical characteristics (Vallvey, 2004).

Sweeteners are alternative substances to sugars, which give food a sweet taste and are used to replace sucrose partially or totally. During the past few decades, low-calorie artificial sweeteners, such as aspartame, saccharin, acesulfame and sucralose, have become sugar alternatives to replace sucrose and have been widely used in dairy products in Europe and USA. There has been an increasing demand for “light” milk products. Sugar free dairy products, which may be calorie reduced, non cariogenic and suitable for diabetics, can be manufactured by using intense sweeteners. Sweeteners are now used in variety dairy products like cocoa beverages, flavored milk drinks or milk based desserts. Recently in our country the ***Prevention of Food Adulteration Act*** (PFA, 2004) has also allowed the usage of artificial sweeteners in dairy products and there is a paucity of data with regard to their behaviour / stability in these indigenous dairy products. So, keeping in view the above factors, the current study has been undertaken with the following objectives:

1. Optimization of levels of acesulfame-K in flavored milk, burfi and kalakand.
2. Isolation of acesulfame-K from dairy products by their clean up.
3. Standardization of analytical conditions for HPLC analysis.
4. Storage studies with regard to stability of acesulfame-K in indigenous dairy products.

CHAPTER - 2

Review of Literature

2. REVIEW OF LITERATURE

It is proposed to review literature on intense sweetener acesulfame-K in foods including dairy products under the following sub- heads:

2.1 GENERAL ASPECTS OF SWEETENERS

2.1.1 Sweetener and sweetness

Sweet means "pleasing" to the taste and sweetness is the pleasure or enjoyment that comes from food that tastes sweet. Man’s preference for sugar and sweeteners is firmly anchored back through history. Archeological findings of primitive cavern paintings depict caveman stealing honey from a bees nest, show that the desire for sweetness was alive and well even then. Today, sucrose or table sugar is the taste standard by which all other sweeteners are measured. An "ideal" sweetener tastes like sucrose, is colourless, odourless, readily soluble, stable and economical. Some sweeteners like sugar contain calories and some are low-calorie or calories free.

Sweet foods provide pleasure and they also help to improve food acceptance and palatability. This is why sweetness can play a powerful role in determining what and how much people eat. With the abundance of affordable food in many parts of the world today, people often eat too much food and consume too many calories. So, an important health goal is to include the pleasure of sweet foods without taking in too many calories. Fortunately, there are several ways that sweet foods can fit into a calorie-balanced and healthy lifestyle. Using low-calorie sweeteners is one way that people can have the pleasure of sweetness without too many calories (www.sweeteners.org/faq.html, 2005).

2.1.2 Benefits of low calorie sweeteners

The first low-calorie sweetener, saccharin was discovered in 1878. Since then, a number of other low-calorie sweeteners including cyclamate, aspartame, acesulfame-K, aspartame-acesulfame salt, neohesperidine DC, thaumatin, and sucralose have been produced and used around the world. The consumption of low-calorie sweeteners continues to increase. Consumer demand for low-calorie foods and beverages has been the major force behind this growth. The increasing interest in a health-conscious lifestyle and advances in food technology are pushing the development of more and better tasting low-calorie foods and beverages. In 2002, in terms of sugar equivalence, the global intense sweeteners market grew by 4.3%; global consumption of sugar, in comparison, grew by 3.1% (LMC International 2002). Low calorie sweeteners in 2002 accounted for almost 11% of the overall sweetener market compared to about 8% in 1990 (LMC International, 2002).

Low-calorie sweeteners have been shown to play a useful role in helping people lose and maintain weight. Preventing obesity is an important factor in reducing the risk of Type II diabetes or non-insulin dependent diabetes (NIDDM). Low-calorie sweeteners make it possible to include a greater variety of sweet foods without adding too many calories.

Low-calorie sweeteners are also beneficial in the management of and the reduction of dental caries (tooth decay). Since oral bacteria that cause tooth decay cannot use low-calorie sweeteners, foods and beverages sweetened with low-calorie sweeteners and aid in the promotion of dental health. Low-calorie sweeteners are also used in oral hygiene products for the same reason. (PMC specialities group, 2004)

2.1.3 Classification of sweeteners

The classification of the main sweeteners has been depicted in fig 2.1. Bulk sweeteners conferring body and texture to foods, are completely metabolized by the body and provide an important part to our energy.

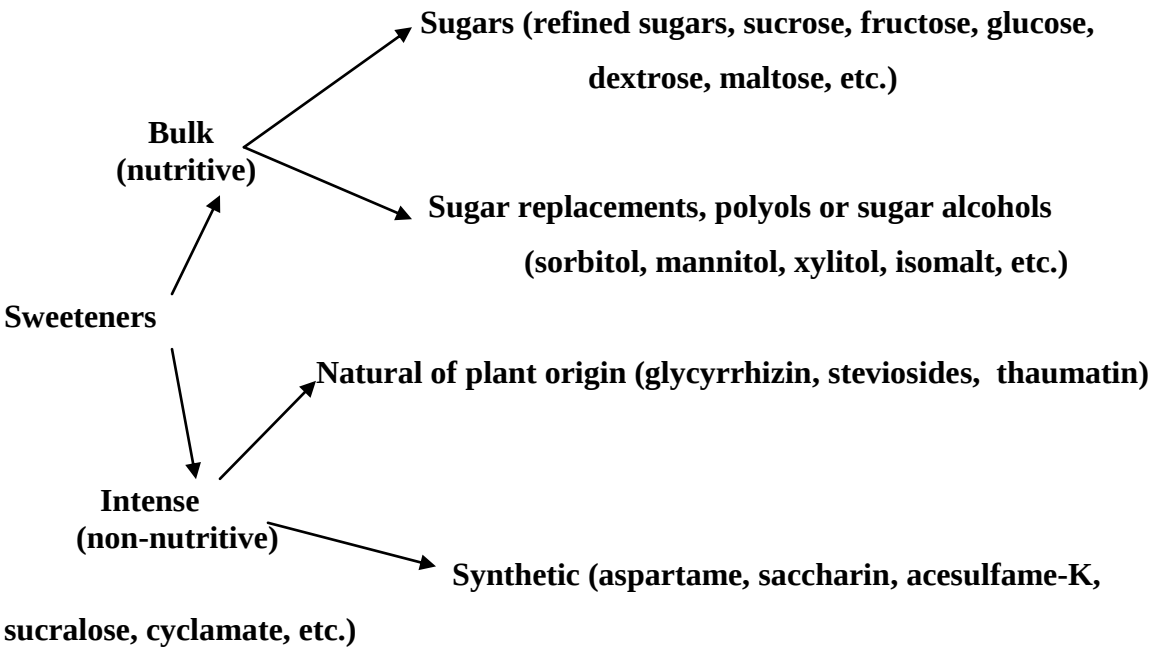

Figure 2.1. Classification of sweeteners

They are also referred to as nutritive or calorie sweeteners. On the other hand, intense sweeteners are generally not metabolized by the body and are excreted unchanged. These are used at very low levels in foods, hence are referred to as non- nutritive or non-calorie sweeteners (Prodolliet, 1996).

2.1.4 An Ideal sweetener

An analysis of the organoleptic and functional properties of each single sweetener has clearly showed that none of the currently known sugar substitutes comes close to the taste and functional properties of sucrose. Most exhibit one or more differences like taste properties, viz. sweetness lag, lingering aftertaste or bitterness, lack of bulking properties, stability problems during storage and competitive prices.

The characteristics of an ideal sweetener are:

- Non toxic
- Taste like sugar
- As sweet as or sweeter than sucrose
- Pleasant taste with no aftertaste
- Colorless and odorless
- Readily soluble
- Low / no calories
- Doesn't promote tooth decay
- Diabetic management
- Stable in all processing environments
- Easily available
- Cost competitive
- Weight maintenance

(Nabors, 2001)

2.1.5 Acesulfame-K

Acesulfame-K was accidentally discovered in 1967 by Karl Clauss and Harold Jensen, while working in the laboratories of Hoechst AG (Germany), the reactions of fluorosulfonylisocyanate with acetylenes, resulted into a product 5, 6-dimethyl-1, 2, 3-oxathiazin-4 (3H)-one-2, 2-dioxide), which was found to be sweet. As a consequence of the 1969 ban on cyclamates in the USA, a systematic program was begun at Hoechst to optimize the properties found in this compound. In the end, it was determined that the preferred product was 6-methyl-1, 2, 3-oxathiazine-4 (3H) –one –2, 2 –dioxide. The potassium salt of acesulfame is known as acesulfame-k (DuBois, 1992).

2.1.6 Structure of Acesulfame-K

This sweetener is composed of carbon, nitrogen, oxygen, hydrogen, sulphur and potassium atoms. The chemical formula of acesulfame-K is $C_4H_4NO_4SK$. The structure of acesulfame-K has been depicted in **(**Ophardt, 2003) Fig.2.2.

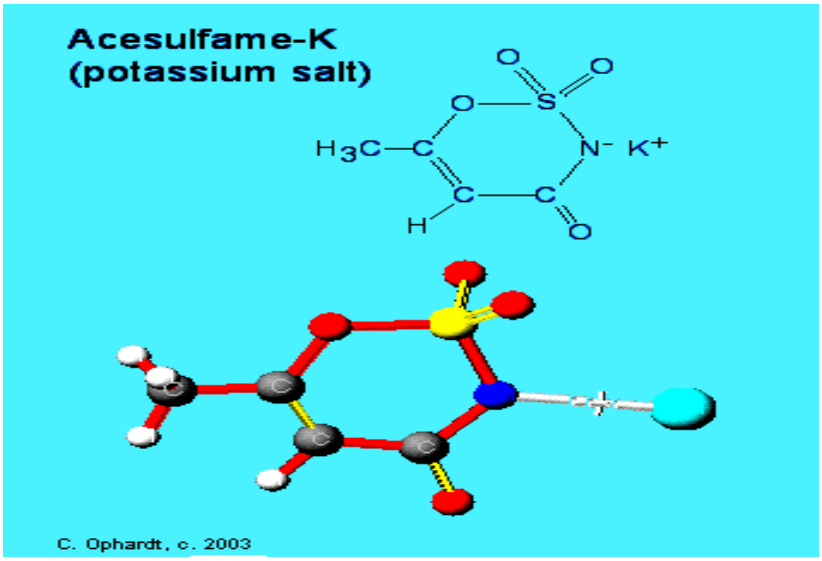

Fig 2.2. Acesulfame-K (K salt of 6-methyl-1, 2, 3-oxathiazine-4 (3H)-one-2, 2- dioxide)

2.2 PHYSICO-CHEMICAL PROPERTIES OF ACESULFAME-K

The physical and chemical properties of acesulfame-K and its different forms are depicted in table 2.1.

2.2.1. Sweetness

Acesulfame-K is about 200 times sweeter than a 3% sucrose solution. Although it has a rapid onset of sweetness, acesufame-K at medium concentrations has little aftertaste and a higher quality of sweetness than sodium saccharin. At higher concentrations, a lingering, bitter, chemical, synthetic taste can be detected (Franta and Beck., 1986)

2.2.2. Synergistic Effects

Mixtures of acesulfame with aspartame or sodium cyclamate has a synergistic effect. Whereas, slight synergistic effects can be noted with saccharin (Franta and Beck, 1986)

2.2.3. Solubility

Acesulfame-K is readily soluble in water. Solubility rises sharply with increased temperature. At 20^0C, approximately 270g dissolve in 1 litre water, at 100^0C far more than 1000g. Stock solutions of higher concentrations can therefore be produced even at room temperature. In most organic solvents, e.g. alcohols, the solubility is low. In anhydrous ethanol the solubility is only about 1 g / litre. However, with increased water content, the solubility increases. (Von Rymon Lipinski, 1985).

2.2.4. Decomposition / Melting

Acesulfame-K does not have a definite melting point. Instead of melting, acesulfame-K starts to decompose at about 225^oC under the conditions of melting point determination. A higher decomposition point might be observed with a fast temperature rise, and prolonged exposure to slightly lower temperatures might also result in decomposition. (Von Rymon Lipinski, 1985)

2.2.5. Caloric value / Metabolism

As acesulfame-K is not metabolized, it is noncaloric. It is excreted unchanged and does not accumulate in the body (Fanta and Beck, 1986).

Table 2.1: Properties of Acesulfame-K

Mol.wt (Da)	201.24
M.P (^{0}c)	200[a]
Solubility (g/L.water.20^0c)	270
Relative sweetness	130-200
Shape	White, odorless, crystalline powder
Specific gravity	1.83
Caloric value (kJ/ g)	0

[a]Decompose before melting (Vallvey, 2004)

2.3 NATIONAL AND INTERNATIONAL REGULATORY GROUPS

The maximum limit of artificial sweetener acesulfame-K (ppm) as approved by different national and international regulatory bodies has been depicted in table 2.2 (PFA) and table 2.3 (CODEX).

Table 2.2: PFA standards for acesulfame-K

This is proposed by PFA in products like soft drinks, biscuits, breads, cakes, sweets (burfi, peda, khoya and similar products), chewing gums, chocolates and sugar based confectionaries.

Product	*Maximum limit of acesulfame-K
Carbonated water	300 PPM
Soft drink concentrate	300 PPM
Biscuits, bread, cakes and pasteries	1000 PPM
Sweets, (carbohydrates based and milk product based): Halwa, Mysore pak, Boondi ladoo, jalabi, Khoya Burfi, Peda, Gulab Jamun, rasogolla and similar milk product based sweets sold by any name.	500 PPM
Chocolate (white, milk, plain, composite and filled)	500 PPM
Sugar based/ sugar free confectionery	3500PPM
Chewing gum/ Bubble gum	5000PPM
Synthetic syrup for dispenser	1500PPM

(PFA, 2004)

Table.2.3: CODEX Limits for acesulfame-K in dairy products

This is proposed by CODEX in products like dairy based drinks, dairy based desserts and dairy chocolates.

Dairy product	Maximum level used * (mg/kg)
Dairy based drink: flavored, fermented and chocolate drinks	500
Dairy based desserts: ice cream, ice milk, pudding and fruit / flavored yoghurt	1000
Dairy chocolates: milk chocolate bar, chocolate flakes and white chocolate	500

(CODEX, 2005)

2.4 MECHANISM OF SWEETNESS

Sweetness plays an important role in food preference. Prodolliet (1996) reviewed that sweetness is elicited by compounds of different chemical structures having a common structural feature, called glycophore, which is responsible for the sweet sensation through receptor-sweetener interaction. The primary mechanism for sweet taste response is the intermolecular hydrogen bonding between glycol unit and taste bud receptor site. The sweet glycophore confers the quality of sweeteners resides in an AH, B couple. A and B are electronegative atoms separated by distance of greater than 2.5 A° but less than 4 A^{o}. Hydrogen (H) attached to one of the electronegative atom by covalent bond in sugar AH and B are the glycol groups. The so-called tripartite, AH, B, γ-glycophore (where γ is lipophilic site) is acknowledged to be necessary for artificial sweetener. (Shallenberger,1967 and Birch, 1991).

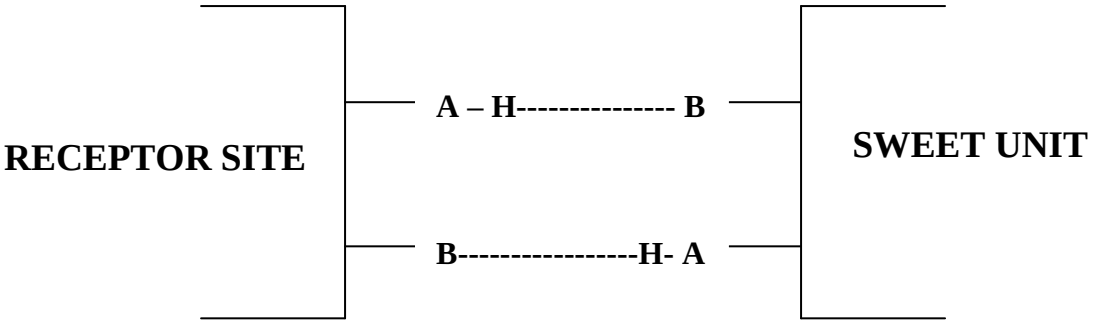

Fig.2.3. Mechanism for sweet taste response

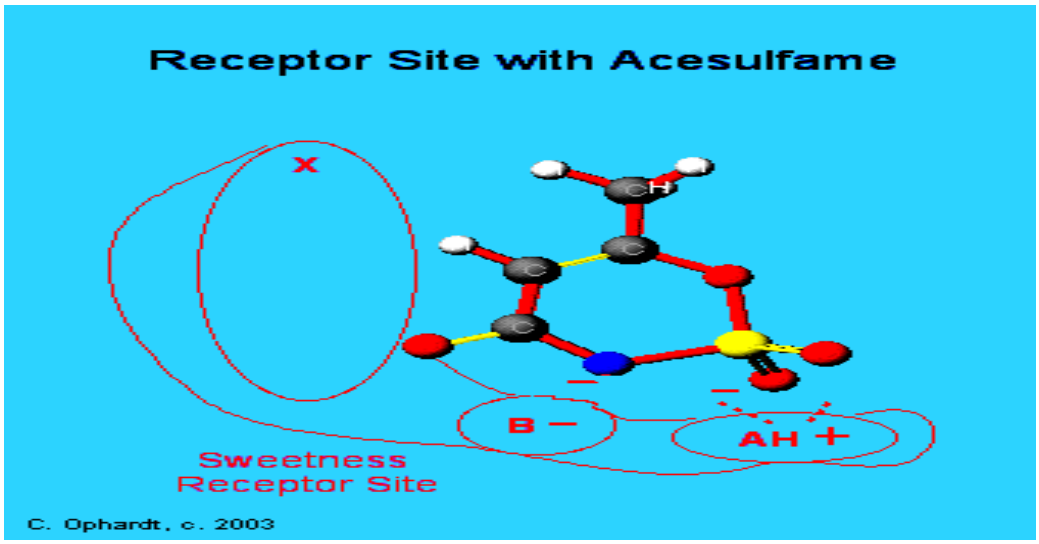

Fig.2.4. Receptor site with acesulfame-K

2.4.1 Sweetness Receptor Site:

Area (AH+): This area has hydrogen available to hydrogen bond to oxygen that is part of the sulfur group.

Area (B-): This area has partially negative oxygen available to hydrogen bond to the partially positive hydrogen of the amine group.

Area (X): This area is more or less perpendicular to the other two areas interacts through hydrophobic or non-polar properties to the non-polar the benzene on the acesulfame-K molecule (www.elmhurst.edu/~chm/vchembook/549acesulfame-K.html, 2005).

2.5 APPLICATIONS

2.5.1 Diabetic food formulations

Acesulfame-K in combination with sugar alcohols, especially sorbitol, tends to round off the sweeteness and provides full and uniform sweetness. This property can be especially useful in providing sweetness to diabetic food formulations where caloric reduction is a goal. (McCormick, 1984)

2.5.2 Beverages

Acesulfame-K in combination with lactose, maltose, glucose, fructose or glucose-fructose syrups remains stable and caloric sugars adds viscosity to beverages (McCormick, 1984). Acesulfame-K was used in whey based fruit beverages at a level of 0.275%. (Beukema and Jelen, 1990)

2.5.3 Brownies and cookies

Acesulfame-K was also used in brownies (Almeida, 1998). Acesulfame-K in combination with malitol, lactitol and sorbitol was used in sugar free cookies. (Zoulias *et al*., 2000)

2.5.4 Chewing gums

Acesulfame-K combination with aspartame was used in chewing gums. (Muhammad *et al*., 1997)

2.5.5 Dairy products

Acesulfame-K was also used in strawberry yoghurt at the level of 0.02%. The consumer panelists described acesulfame-K yoghurt as having a bitter aftertaste, which resulted in its low score. (Keating and White, 1990)

2.6 STABILITY OF ACESULFAME-K

2.6.1 Crystalline state

Exceptional stability in the crystalline state is reported for samples stored for about 10 years at ambient temperature (in summer 27^0C and in winter 20^0C). (Franta and Beck., 1986)

2.6.2 Aqueous media

In aqueous media, acesulfame-K is distinguished by good stability. After several months of storage at room temperature, virtually no change in acesulfame-K concentration was found in the pH range common for beverages. Prolonged continuous exposure to 30^0C, does not cause losses exceeding 10%, the threshold for recognition of sweetness differences. (Von Rymon Lipinski and Hanger, 2004).

2.6.3 Beverages

Acesulfame-K containing beverages can be pasteurized under normal pasteurization conditions without loss of sweetness. Pasteurizing for longer periods at lower temperatures is possible, as is short-term pasteurization for a few seconds at high temperatures. Sterilization is possible without losses under the normal conditions (i.e., temperatures around 200^0C for products having lower pH levels and 121^0C for products having pH around and higher than 4). In a solution of pH 4.0 which was heated to 120^0C for 1hour, no loss of acesulfame-K could be measured. (Von Rymon Lipinski and Hanger, 2004)

2.6.4 Baking

In baking studies, no indication of decomposition of acesulfame-K was found. This corresponds to the observation that acesulfame-K decomposes at temperatures well above 200^0C (Von Rymon Lipinski and Hanger, 2004).

2.6.5 Buffered solutions

Extensive studies were performed with buffered aqueous solutions. Results for pH levels and storage conditions for commonly found soft drinks are given in table2.3. After 10 years storage of a solution buffered to pH 7.5 at room temperature, no significant loss of acesulfame-K was detected.

Table.2.4: Stability of acesulfame-k in Buffered aqueous solutions

20^0C Storage time (Weeks)	pH 3.0 Recovery (%)	pH 3.5 Recovery (%)
16	98	98
30	98	99
50	98	99
100	95	98
30^0C storage (Weeks)	**pH 3.0 recovery (%)**	**pH 3.5 recovery (%)**
16	97	100
30	95	97
50	91	96

(Von Rymon Lipinski and Hanger, 2004)

2.7 ACCEPTABLE DAILY INTAKE AND SAFETY

This sweetener was evaluated for safety by JECFA in 1983. The FDA first approved acesulfame-K in 1988, and it is currently approved as general purpose sweetener, not including meat and poultry. Both FDA JECFA have set an ADI of upto 15 mg/kg bw/day. The European commission's SCF reevaluated this sweetener and supported its safety but recommended an ADI at 9 mg/kg of bw/day. The amount of acesulfame-K added to food products is very small because of its intense sweeteners power and because it is often used in combination with other sweeteners. The estimated daily intake is estimated at 20% of the ADI because of its intense sweetening power. Estimated intakes in children are below the ADI (ranges from 3 to 9 mg/kg bw/day) (ADA, 2004).

2.8 TOXICOLOGICAL STUDIES

The study on the kinetics and transformation of this substance showed that sweetener was quickly absorbed. In humans, rats and dogs, over 99% was excreted in 24 hrs and was excreted unchanged. Acute toxicity studies of acesulfame-K showed it to have an oral LD^{50} of 7,431 mg/kg of body weight. This toxicity was suggested to be due to the potassium, which comprises about 20% of the acesulfame-K molecule and has oral LD^{50} of 2,430 mg/kg of body weight. The symptoms in animals with high doses resembled the paralysis and cardiovascular effects seen with potassium excess. No toxic effect was observed at 3% level of acesulfame-K in the diet, which would be 1.5mg/kg/day for the rat. Pharmacological studies of mice fed acesulfame-K showed no adverse effects. Finally Authors concluded that doses in food and beverages at up to 10mg/kg/day would not be anticipated to cause an observable effect. Other possible adverse effects were tested and found to have no data to indicate any problems. (Jones, 1992)

2.9 DEGRADATION AND DECOMPOSITION OF ACESULFAME-K

Potential decomposition products of acesulfame-K could be found under extreme conditions. Under such conditions, compounds of hydrolytic decomposition are mainly acetone, carbon dioxide (co_2), ammonium salts, sulfate, and amidosulfate. In the hydrolytic decomposition, the ring system is initially opened. This quickly yields the end products of hydrolysis (Von Rymon Lipinski and Hanger, 2004). The decomposition products of acesulfame-K were depicted in fig.2.5.

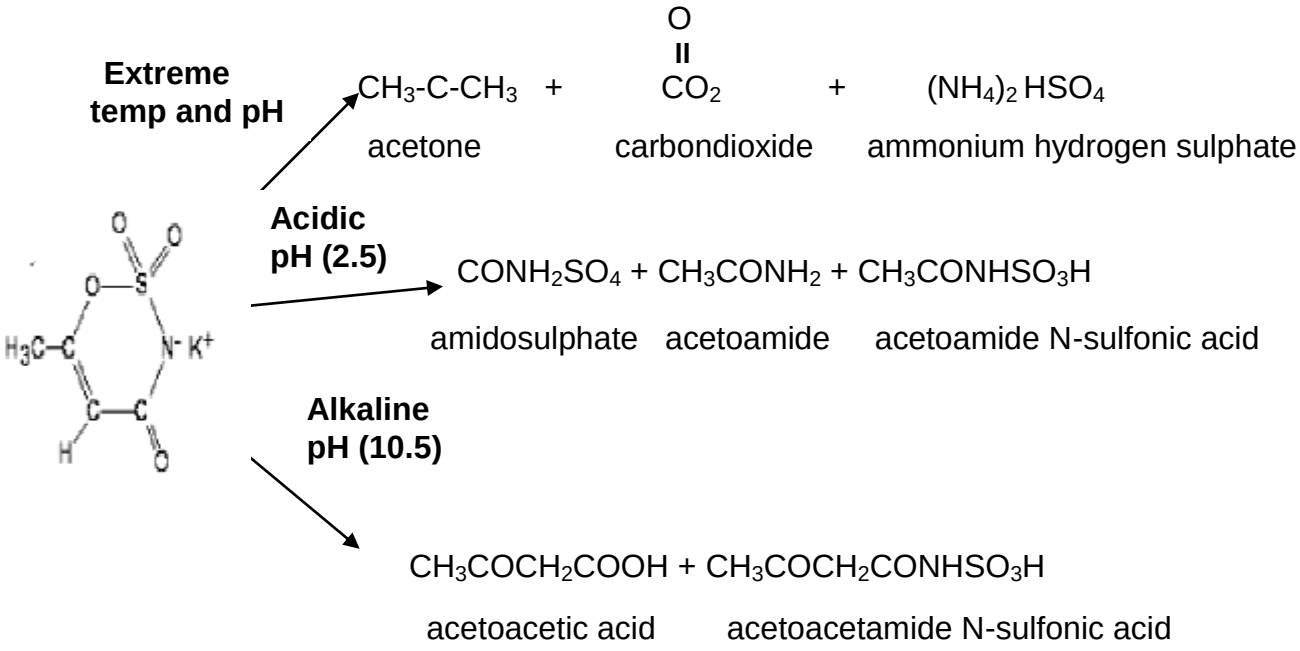

Figure.2.5. Decomposition products of acesulfame-K
(Arpe, 1978)

2.10 ANALYSIS METHODS

2.10.1 Sample preparation

Preparation of the samples from the products containing acesulfame-K as sweetener involves the homogenization, dilution with water, ultrasonification, clarification and extraction by centrifugation and solid phase extraction etc. These sample preparation techniques are depicted as flowchart in figure 2.6

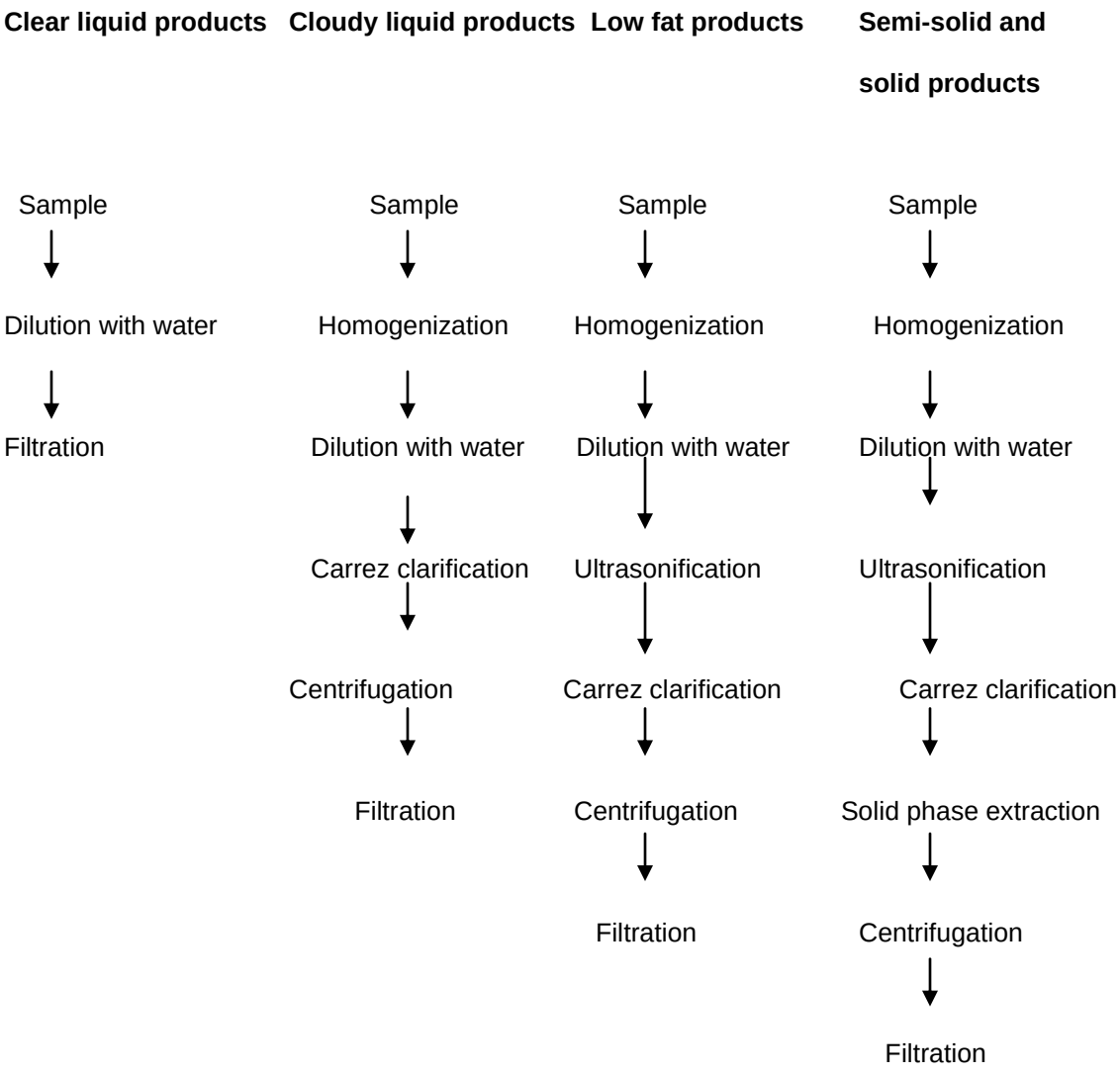

Figure 2.6. Sample preparation techniques

(BSEN.1999, as cited by Wood *et al.*, 2004)

2.10.2 High-performance liquid chromatography (HPLC) method for analysis of acesulfame-K in foods

HPLC is the most popular system for separating and analyzing acesulfame-K. Most of the reported systems for the separation of acesulfame-K and other additives in foods were conducted by reversed-phase HPLC on silica based C-18 bonded phase columns. The analysis of acesulfame-K in different types of foods and beverages using HPLC are given in Table 2.5. Most of the mobile phases used in HPLC procedures include isocratic elution and binary gradient elution. The composition of this solvent phase includes water, aqueous phosphoric, acetic or citric acid or its salts with acetonitrile, methanol, or 2-propanol as organic modifier. The nature and proportion of an organic modifier and the pH of the mobile phase is usually the key to separations in terms of retention time, resolution and analysis time. The pH control must be adjusted by use of buffers, especially acetate, phosphate and citrate.

Table 2.5: HPLC methods for the determination of acesulfame-K in foods

	FOOD	SAMPLE PREPARATION	COLUMN	MOBILE PHASE	DETECTION (nm)	REFERENCES
1	Orange drink Diabetic chocolate	Solid phase extraction (SPE) Defatting, SPE	Partisil 10 ODS3, 10 μm	Methanol - tetrabutyl ammonium hydrogen sulfate in phosphate buffer (32.5: 67.5), pH 4.0	227	Slack (1989)
2	Beverage	None	μ Bondapak C18, 10 μm	Methanol - acetic acid - water (20:5:75)	254	Veerabhadrarao *et al.,* (1987)
3	Beverage, Jam	Dilution with water	Spherisorbis ODS-1, 5 μm	Methanol - 0.02 M KH_2PO_4 (8:92), pH 6.7	227	Hannisdal (1992)
4	Diet soft drinks, tabletop sweeteners Diet puddings & dessert toppings	Dilution with water, filtration Ethanol extraction, filtration, dilution with mobile phase	μ Bondapak C18, 10 μm	Acetonitrile - 0.02 M KH_2PO_4 (3:97), pH 5.0 + Acetonitrile - 0.02 M KH_2PO_4 (20:80), pH 3.5	200	Lawrence and Charbonneau (1988)

Contd..

5	Tabletop sweetener, candy Liquid beverage Other foods	Dilution with water Dilution with water, degassing, clean up on C18 cartridge Water extraction, carrez clarification **Recoveries (%): 95.2 – 106.8 (AK)**	µ Bondapak C18	Acetonitrile - 0.0125 M KH_2PO_4 (10:90), pH 3.5	220	Prodolliet and Bruelhart (1993)
6	Cherry, nectar, brine Yoghurt Chocolate Mayonnaise	None Carrez clarification Defatting, Carrez clarification Defatting, Water extraction	Lichrospher N – select B, 5µm	Acetonitrile - 0.02 M KH_2PO_4 (10:90)	220	Lehr and Schmid (1993)
7	Candy Gum	Dissolution in water Extraction with glacial acetic acid – $CHCl_3$ (50:25)	AS4, anion exchange	2.8 mM Na_2CO_3	228	Biemer (1989)
8	Food stuffs	Dilution with water, SPE, Carrez clarification	µ Bondapak C18	Phosphate buffer-Acetonitrile (90:10, 80:20, 85:15, 95:5, 98:2)	220	(BSEN 12856: 1999) cited by Wood *et al.,* (2004)

Contd..

10	Soy sauce, pickles, miso, powdered instant puddings	Dialyze with 1% H3PO4, cleanup on Bond Elut SCX for AS and on Bond Elut C18 for AK and SA	Fine pak C18 S	Tetrapropyl-ammonium hydroxide in methanol – water (20:80), pH 4.0	210	Morriyasu *et al.,* (1991)
11	Beverages	None	μ Bondapak C18, 10 μm	Acetonitrile - 0.02 M KH_2PO_4 (3:97), pH 5.0	546	Lawrence (1987)
13	Vanilla yoghurt with cherries, multivitamin juice	Methanol extraction, filtration Recoveries (%): 63.0 – 81.1	Li Chrospher 60 RP – Select B, 5 μm	20 mM tetra-n-butyl ammonium hydrogen sulfate (75:15 to 5:95), 20 mM phosphate (pH 4.3) – acetonitrile (90:10 to 5:95)	217, 285	Haush (1996)

Contd..

14	Fishery products, creams, mayonnaise, ice cream	Water extraction, SPE Recoveries (%): 84 - 102	Nucleosil 100–5 C18, 5 μm	20 mM KH_2PO_4–acetonitrile–water, 20 mM phosphate buffer (pH 6.7)-acetonitrile	232	Ostermeyer (1995)
15	Soy sauce, sugared fruit, dried roast beef	Acetone extraction, cleanuo on silica cartridge Recoveries (%): 76 - 95	Shoko monomeric / Shoko polymeric; C18, 5 μm	Acetonitrile – 50 mM α-hydroxy isobutyric acid (pH 4.5; 2.2:3.4), 2.5 mM hexadecyltrimethyl ammonium bromide	233	Chen and Fu (1996)
17	RTS beverages, sports beverages, ice candy Dry beverage mixes, tomato sauce	Dilute with water, filtration Extraction with ethyl acetate or $CHCl_3$ – acetic acid (4:1), evaporate, dissolve with mobile phase	μ Bondapak C18, 10 μm	Methanol – acetic acid – water (20:5:75), Methanol – acetic acid – water (35:5:60), Acetate buffer (pH 3.0) – methanol (95:5)	254	Veerabhadrarao *et al.,* (1987)

2.10.3 Other Methods for analysis of acesulfame-K in foods

The miscellaneous methods used for the determination of acesulfame-K in foods. Thin layer chromatography (TLC) has been used for the identification and/or determination of acesulfame-K in combination with other sweetners, because the equipment needed is simple, inexpensive and flexible (Das *et al*., 1970; Von Rymon Lipinski and Brixius, 1979). The method like Capillary electrophoresis has become a viable alternative to HPLC for the determination of acesulfame-K, because of its highly efficient separation, low solvent consumption, and ease of automation. It has also been used for the determination of sweetener mixtures in foods and beverages (Thompson *et al*., 1995; Schnierie *et al*., 1998; Boyce, 1999; Frazier *et al*., 2000). Sastry *et al.* (1995) detected acesulfame-K and saccharin in beverages, ice candy, syrups and ice cream using UV-visible spectrophotometry. Nikolelis and Pantoulias (2001) detected acesulfame-K in tabletop sweeteners, dried soft drinks, wines and yoghurts using flow-through sensor.

Table 2.6: Other methods used for the determination of acesulfame-K in foods

	TECHNIQUE	FOOD	SAMPLE PREPARATION	STATIONARY PHASE/ COLUMN/ FILM	MOBILE PHASE/ BUFFER/ CONDITIONS	DETECTION (UV) (nm)	REFERENCES
1	Thin layer chromatography	Food, Cosmetics Tabletop sweeteners	None None	Polyamide Kieselgel 60 F254	Xylene – propanol – formic acid (5:5:1) Ethanol acetate-methanol-formic acid-water (70:15:5:0.5)	0.2 % Dichlorofluorescein in methanol (UV) UV	Von Rymon Lipinski and Brixius (1979) Annon, (1990)
2	Flow-through sensor/ Electrochemical biosensor/ Bilayer lipid membranes	Tabletop sweeteners, diet soft drinks, wines, yoghurts	None	Filter-supported bilayer lipid membranes of egg phosphatidylcholine	0.1 M KCl, Temperature: 25 ± 1°C	-	Nikolelis and Pantoulias., (2001)

Contd..

3	Capillary electrophoresis	Standards	None	Capillary zone electophoresis (CZE) electro kinetic injection at 5 KV, 7 s	1.5 mM tetra borate	Potentiometric coated wire ion selective electrode (ISE)	Schnierle *et al.*, (1998)
4	UV – Visible Spectrophotometry	Ice cream	HCl addition, extraction as above, re-extract with 1% Na_2CO_3, adjust to pH 7.0	Sevron Blue 5G reagent	$CHCl_3$/Phosphate buffer, pH 7.0	655	Sastry *et al.,* (1995)

2.11 FLAVOURED MILK

Flavoured milks are milks to which some flavours have been added. Renner and Zwermann (1988) optimized the sugar content for flavoured milk drinks. On the basis of scores received, optimum sugar contents for the 4 flavours cocoa, banana, strawberry and vanilla were estimated to be 3.9, 3.7, 3.6 and 3.9%, respectively. Yau *et. al.* (1989) studied the effect of carbonation and sweetener type (sucrose, high-fructose maize syrup, aspartame and pear concentrate) on flavour properties of uncarbonated or carbonated blueberry flavoured milks. Carbonation increased the sensory rating of overall flavour intensity, sweetness and blueberry flavour. Sweetener type found to have a significant effect on the sensory rating of viscosity, but no effect on that of overall flavour intensity, sweetness or blueberry flavour. Sucrose and high-fructose maize syrup were found more acceptable sweeteners than aspartame or pear concentrate.

Endres (1991) optimized the sugar content of milk and yoghurt drinks in the nutrition of children. In sensory tests with 222 school children aged 8-14, the optimum sugar content was found to be 3.8% for chocolate milk, 3.7% for strawberry milk, 3.4% for vanilla milk and banana milk, and 8.3% for drinking yoghurt. Age and sex had little effect on these results. Hanif et. al. (1996) studied the acceptability of cow and buffalo flavoured milk drinks. Milk flavoured with vanilla, chocolate and sweet orange flavours containing 1 ml/l flavour scored most acceptable, whereas milk flavoured with mango flavour at 2 ml/l scored most acceptable. Agrawal *et. al.* (1991) prepared coconut milk by blending skim milk powder with the coconut milk of freshly grated coconut along with added buffer salts and sugar. It contained 6% skim milk powder and 9.65% toatal solids. On sterilization and can be utilized as coconut flavoured milk for the food industry.

Rodriguez *et. al*. (2001) developed flavoured milk with a mixture of milks from the cow and the buffalo and contained flavour of orange, strawberry and lemon. A taste panel preferred milk made from 6.25% cow milk and 83.75%

buffalo milk. Carpenter (2006) patented chocolate and caramel milk drink by mixing together specific proportions of chocolate syrup and caramel syrup until blended; adding the blend to milk; preferably 2% milk; and continuing the stirring and mixing until the chocolate syrup and caramel syrup are thoroughly diluted and dispersed throughout the milk. Viajayalakshmi and Tamilarasi (2001) studied changes in sensory properties and microbiological quality of various dairy products (obtained from a retail outlet in India) including flavoured milk during storage at 6-8°C for up to 6 week. Flavoured milk deteriorated in appearance after just 1 day. Most samples showed higher than expected levels of microorganisms; microbial populations increased in all samples during storage.

2.12 BURFI

Burfi is one of the most popular khoa based sweets all over India. The generic nomenclature " burfi" covers a wide range of product variations that include plain, danedar, dudh, chocolate, fruit and coconut burfi. It has a mildly caramelized and pleasant flavour. Its colour may range from off-white to creamy or light caramel. Multilayered and multi-coloured varieties are also produced.

The important steps in the preparation of burfi are desiccation of milk into khoa of different consistencies, incorporation of sugar either in crystalline forum or as syrup, admixture of other ingredients and subsequent desiccation to get the desired body and texture (soft and semi-hard or hard) characteristics of the variety. The colouring and flavouring materials, if any, are added in the initial and final stages of preparations, respectively. The product, while still hot and possessing a semisolid consistency, is poured onto previously prepared moulds and cooled either rapidly or over a period of time to get the desired body (coarse or fine grained). After cooling, the mass is cut into pieces of required size and shape and packed. Sachdeva (1980) standardized the process for manufacture of burfi from cow and buffalo milk standardized to 4.5% and 6% fat respectively. Burfi prepared from cow milk with 4.5% fat was found sticky and gummy due to insufficient release of free fat. Buffalo milk burfi was liked most by panel of judges.

Bhatele (1983) had shown that burfi of an acceptable quality on the basis of sensory evaluation could be prepared by keeping 30% sugar in the final product using khoa of middle stage 64-67% TS and heating the mixture for 3-5 minute in an open jacketed, manually stirred kettle with steam pressure maintained at 0.5Kg/cm^2 and proper kneading and whipping simultaneously. Reddy (1985) studied the suitability of using vacuum concentrated milk and skim milk powder for the manufacture of burfi. Gothwal and Shukla (1995) studied the effect of refined wheat flour (Maida) and sugar on the browning of milk, khoa and khoa-based sweets. Refined wheat flour (maida) at a concentration of 8% increased browning index (20 to 22%) during heat treatment in both cow and buffalo milk. At a concentration of 10%, maida caused an increase in browning index in burfi (13%), kalakand (13%), milk Peda and milk cake (18%).

Ramakrishna *et. al*., (2005) studied moisture sorption characteristics of milk burfi, and traditional Indian sweet, using sugar substitutes. The product was prepared replacing sugar with sorbitol, maltodextrin (MD)+polydextrose (PD), and PD alone, along with aspartame to give an equi-sweetness level compared to sugar. The isotherms followed typical sigmoidal shape, characteristic of sugar-rich products. The curve for burfi with sorbitol shifted to the left compared to that of sugar, whereas for those prepared with MD+PD or PD, the curves are similar to that of burfi made with sugar.

2.13 KALAKAND

Kalakand is an Indian milk product where milk is desiccated with small quantities of citric acid and sugar. It is a sweet dairy product with a granular texture, caramel flavour, and paste-like consistency and generally made by evaporating buffaloes' acidified milk. It is typified by pleasant caramel flavour and granular texture. The granular mass is fused and held together in a loosely compacted body. The colour of kalakand varies from off-white to light caramel. Magdum (1979) prepared kalakand by boiling milk in an iron karahi placed over a brisk non smoky fire with continuous circular motion using ladle. Citric acid and sugar was added at 0.05% and 7% of level of milk respectively. Gill and De

(1974) also used 0.05% citric acid after 10-15 minutes of boiling milk. Mathur (1991) reported the manufacture of kalkand from danedar khoa by working various ingredients such as sugar, aromatic spices etc. over fire in a shallow pan. Arora *et. al*. (1991) studied the chemical and microbiological quality of kalakand sold in the market. Mean chemical composition found was 17.32% (4.75-26.46%) fat, 77.60% (63.59-84.75%) TS, 13.40% (9.40-17.97%) protein, 27.96% (15.38-42.12%) sugar, 16.64% (11.86-21.90%) lactose, 2.53% (1.78-3.48%) ash and 0.39% (0.26-0.70%) acidity. While, mean microbial counts/g found were 84.93 x 105 total bacteria, 290 x 104 staphylococci, 4000 coliforms and 9250 yeasts/moulds.

Suresh and Jha (1994a) studied the sensory, biochemical and microbiological qualities of kalakand Chemical analysis of market samples showed variations in carbohydrates, fat, protein, and ash contents. Levels of titratable acidity, lactic acid, tyrosine value, peroxide value, and free fatty acids were higher in market samples than in laboratory samples. The presence of sulphydryl and hydroxymethylfurfural groups indicated a greater extent of browning in market kalakand samples. Total plate counts, and counts of coliforms and yeasts + fungi were 29.5 x 103, 6.6 x 102-15 x 102, and 4.4 x 102 cfu/g, respectively, in market samples, vs. 1 x 103, 40, and 10 cfu/g, respectively, in laboratory samples. These workers also optimized the process for kalakand manufacture with an extended shelf-life. Buffalo milk standardized to 6.0% fat with added sugar (7%) and citric acid (0.02%) resulted in a highly acceptable product with significant improvements in sensory scores.

2.14 MICROSTRUCTURE OF INDIGENOUS DAIRY PRODUCTS

Difference between dairy products cannot only be tested but also seen, because manufacturing processes impart special features in product microstructure. Microstructural differences between these products can be observed using Scanning electron microscopy (SEM). Studies of food microstructure help to understand some of very important physical properties of foods such as elasticity or firmness and sensory attributes such as grittiness.

Very limited studies have been carried out through SEM for the Indian indigenous products. Kalab *et al*, 1988, while studiing the microstructure of paneer found an uniform, dense protein matrix resembling cottage cheese structure. Verma, 1989 observed thread like folded structures in both cow and buffalo milk rasogolla. Adhikari *et al*, 1993 reported coalesced, compact casein micelles with associated fat globules in chhana and numerous small voids interspersed throughout the matrix. As a result of cooking of chhana, the fat globules ruptured and finally coalesced to large masses, and the void spaces increased markedly, producing a highly ragged and uneven matrix with cotton-like structure in rasogolla. No work has been done for other indigenous dairy products i.e. burfi and kalakand.

CHAPTER - 3

Materials and Methods

3. MATERIALS AND METHODS

The materials used and the methodology employed in the present investigation is described in this chapter.

3.1 CHEMICALS AND REAGENTS

3.1.1 Acesulfame-k standards: sigma-Aldrich, corporation, Box-14508, ST, Lovfs, Missouri-63178, USA

3.1.2 Acesulfame-k degradation products standards: sigma-Aldrich, corporation, Box-14508, ST, Lovfs, Missouri-63178, USA

3.1.3 Standard solutions of Acesulfame-k and degradation products

Ten mg each of Acesulfame-k and acetoacetamide were dissolved in 10 ml of mobile phase 0.02 M Phosphate buffer (pH 5.0): Acetonitrile (97:3), to get stock standard solutions of concentration 1 mg/ml. 100 µl from each of the stock standard solutions were pipetted into separate 10 ml volumetric flasks and volume was made up to the mark with mobile phase, to get concentrations of 10 ng / µl . Also 100 µl from each of the standard solutions of Acesulfame-k & acetoacetamide (1mg/ml) were pipetted into a 10 ml volumetric flask and volume was made up to the mark with mobile phase, to get concentration of each component as 10 ng / µl in the mix.

3.1.4 Plate count agar (Titan Biotech, Bhiwadi, Rajsthan)

3.1.5 Sterilization agent: Lysol soap solution with cresol procured from Titan Biotech, Bhiwadi, Rajsthan.

3.1.6 Solvents: Acetonitrile and water (HPLC grade, Qualigens Fine Chemicals, Mumbai),

3.1.7 Phosphoric acid, sodium hydroxide, oxalic acid, phenolphthalein, citric acid (AR grade, Sd-fine Chemicals, Mumbai). **di-potassium**

hydrogen phosphate and potassium dihydrogen phosphate (AR grade, Qualigens Fine Chemicals, Mumbai).

3.1.8 0.02M Phosphate buffer, pH 5.0 – Di-potassium hydrogen phosphate (K2HPO4) 21.9 mg of and 2.705 g of potassium dihydrogen phosphate (KH_2PO_4) were dissolved in 1000 ml HPLC water.

3.1.9 Mobile phase – 0.02 M Phosphate buffer (pH 5.0): Acetonitrile (97:3), filtered and degassed.

3.1.10 Carrez solution No. 1 – 3.6 g of potassium ferrocyanide dissolved in 100 ml water.

3.1.11 Carrez solution No. 2 – 7.2 g of zinc sulphate dissolved in 100 ml water

3.1.12 Filter papers Whatman No.1.

3.2 EQUIPMENTS

3.2.1 Stainless steel double jacketed kettle: This kettle is provided with steam line, steam control valve, steam pressure gauge and cold water line in the jacket with steam trap and condensate outlet and arrangement for tilting and keeping it fixed in normal position. The diameter of pan at top was 650 cm and depth at centre 60 cm; capacity 80 kg.

3.2.2 Homogenizer (Goma Engeeniering Pvt. Ltd, H-102, Pune)

3.2.3 Vacuum filtration assembly (Millipore Corporation, Bedford,MA,USA)

3.3.4 Solid phase extraction C_{18} cartridge (Supelco, Bellfonte, PA,USA)

3.2.5 Ultra-sonifier (SONICS, Vibra Cell, Model VCx750, Newtown, CT,USA)

3.2.6 Solid phase extraction vaccum manifold (Visiprep™ DL, Supelco, Bellfonte, PA,USA)

3.2.7 BOD Incubator (NSW 152, Narang Scientific Works, New Delhi)

3.2.8 pH meter (PHAN, Labindia, Mumbai, India)

3.2.9 Ostwald Viscometer (Labco Glassware, Ambala, India**)**

3.2.10 Lyophilizer Martin Christ Rotational freeze dryer, alpha -1-4, Osterode, Germany.

3.2.11 Texture Analyzer (TA-xT2i Stable Micro System, Godalming, Surrey, UK)

3.2.12 Scanning Electron Microscope (Hitachi-S-405 A, Tokyo, Japan)

3.2.13 Colourflex (Hunterlab, Reston, Virginia, USA)

3.2.14 Storage Material – Flavoured milk stored in Nayasa Pears plastic jars and Burfi and Kalakand stored in Avion plastic jars procured from local market, Karnal.

3.2.15 HPLC System & Accessories

Model	:	Shimadzu LC10A
Detector	:	UV, SPD-M10AV
Loop Injector	:	20 µl
Printer / Integrator	:	HP Laser
Computer	:	EPC Professional
Solvent filter	:	0.45 µm, 47 mm diameter- durapore (Millipore)
Syringe	:	Blunt type (Hamilton)

3.3 RAW MATERIALS

3.3.1 Milk: Pooled buffalo milk and skim milk samples were collected from Experimental Dairy, NDRI, Karnal.

3.3.2 Colour: Raspberry red powder IH 7804 procured from Bush Boaken Allen India Ltd. Chennai.

3.3.3 Sugar: Sugar for control preparation was procured from local market.

3.4 EXPERIMENTAL METHODS

3.4.1 Preparation of flavoured milk

The flavoured milk was prepared according to method of Ramasamy (1999) as shown below:

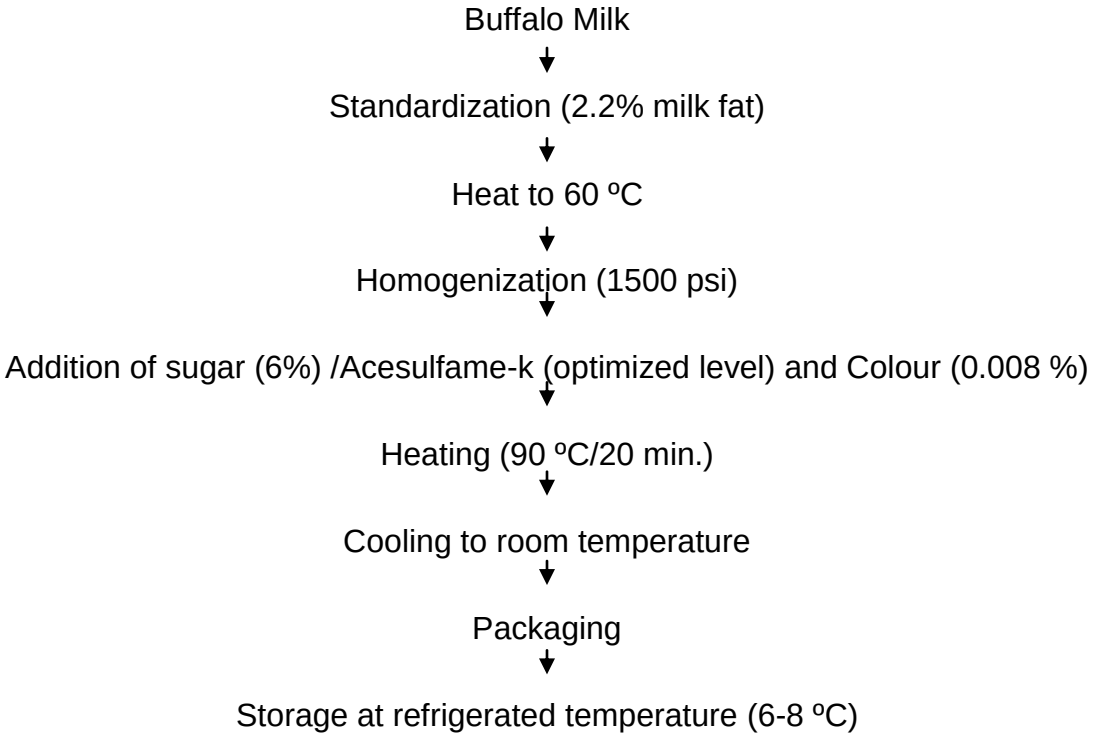

Figure 3.1: Flow chart for manufacture of flavoured milk

3.4.2 Preparation of burfi

Burfi was prepared according to method of Bhatele (1983) as shown below with slight modification for sweetener containing product.

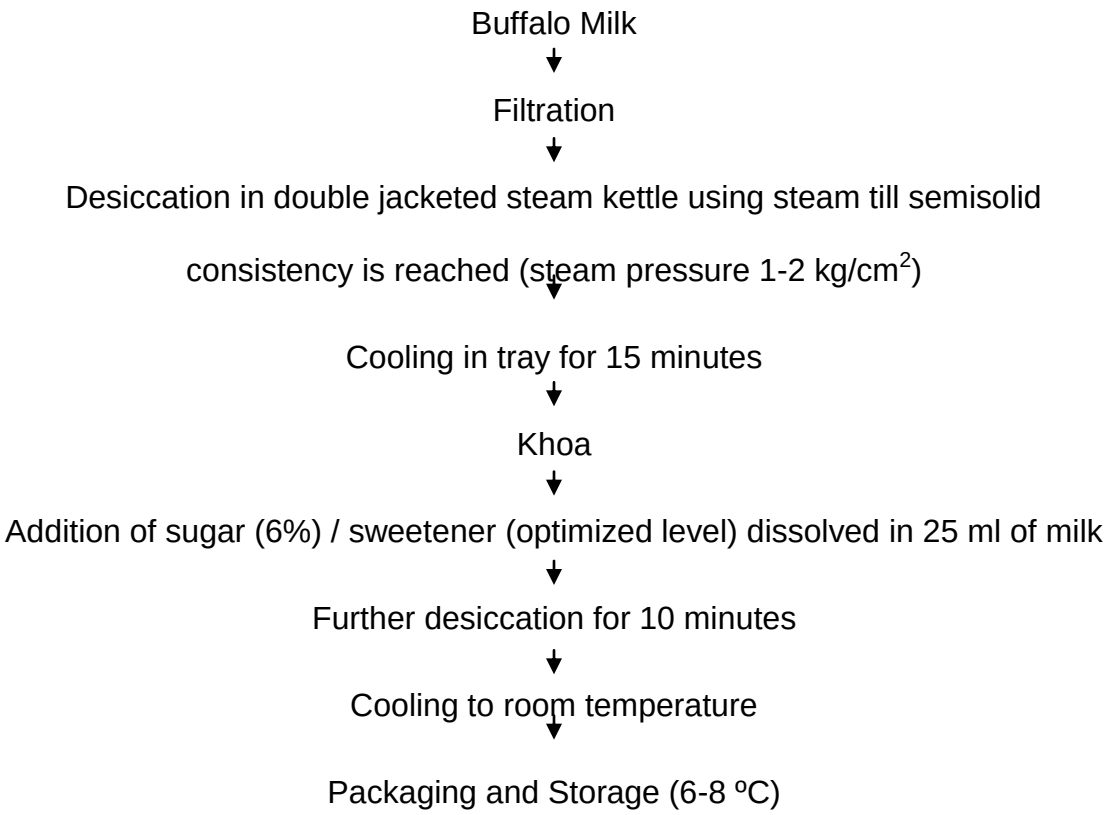

Figure 3.2: Flow chart for manufacture of burfi

Sugar and sweetener levels were added on the basis of milk. For Acesulfame-k sweetened burfi, sweetener was added to khoa after dissolution in 25 ml of milk in order to have uniform incorporation.

3.4.3 Preparation of kalakand

Kalakand was prepared according to method of Suresh and Jha (1994a) as shown below with slight modification for sweetener containing product.

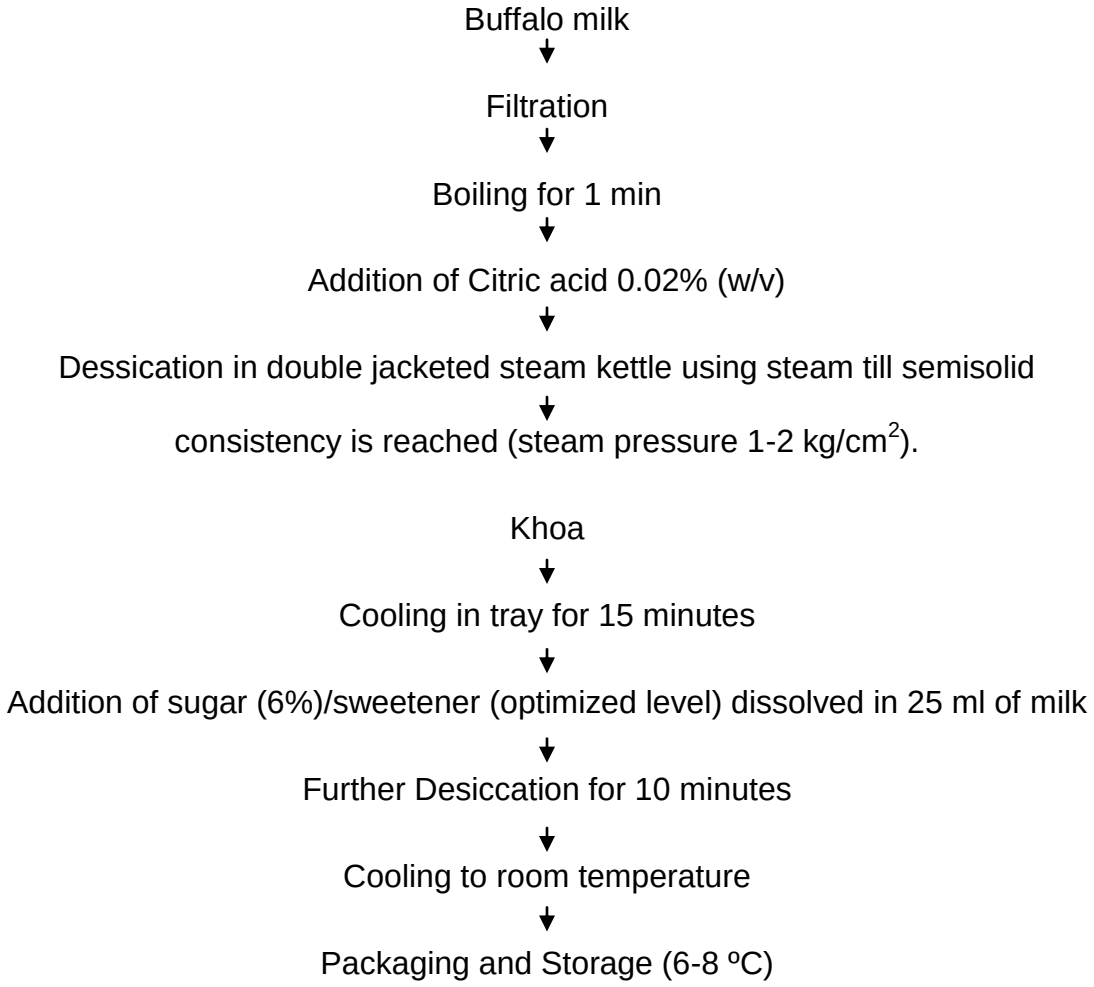

Figure 3.3: Flow Chart for manufacture of Kalakand

The preparation of Kalakand followed the same protocol as that for Burfi. The only difference being that citric acid was added at the rate of 0.02% after boiling of milk for one minute, before desiccation to form khoa.

3.4.4 Optimization of Acesulfame-k levels in flavoured milk, burfi and kalakand

Acesulfame-k levels* were optimized for flavoured milk, burfi and kalakand on the basis of its acceptance by sensory evaluation by a panel of eight judges on 9-point hedonic scale score card shown in annexure I &II.

- Flavoured Milk [0.03-0.05%]
- Burfi [0.035-0.055%]
- Kalakand [0.035-0.055%]

*6% sucrose equivalence (milk basis).

3.4.5 Sample preparation for HPLC analysis using solid phase extraction (SPE) column.

The sample preparation procedure used for isolation of Acesulfame-k from flavoured milk, burfi and kalakand was essentially based upon the method of BS EN 12856: 1999 cited by Wood *et al*. (2004).

3.4.5.1. Activation of solid phase extraction (SPE) C_{18} cartridge

SPE cartridge was activated by passing 3ml methanol and 20 ml water.

3.4.5.2 Sample Preparation

Twenty grams of flavoured milk was taken in a 100 ml beaker. 50 ml of HPLC grade water was added to it and placed the beaker in an ultrasonic sonifier maintained at 40°C for 20 min, after which the solution was cooled to room temperature and transferred to a 100 ml volumetric flask. 2.0 ml of carrez solution No. 1 was added to the flask and mixed, followed by addition of 2.0 ml of carrez solution No. 2. The solution was shaken vigorously and allowed to stand at room temperature for 10 min. After dilution upto the mark with HPLC grade water, filtration was carried out using a Whatman No. 1 filter paper. 2.0 ml of the clarified filtrate was added to the previously activated SPE cartridge and eluted with 10 ml of mobile phase, as colourings, flavourings and fat could not be separated by carrez clarification. The isolate was collected in 25 ml volumetric

flask and finally the volume was made up to the mark with a mobile phase. A blank experiment was also performed simultaneously.

In case of burfi and kalakand 1.75 gm of sample was taken for extraction of Acesulfame-k. The amount of carrez solutions 1 and 2 used for precipitation of proteins was 6 ml each, while the rest of the procedure remained the same as that for flavoured milk. The flow chart of isolation procedure is as given below:

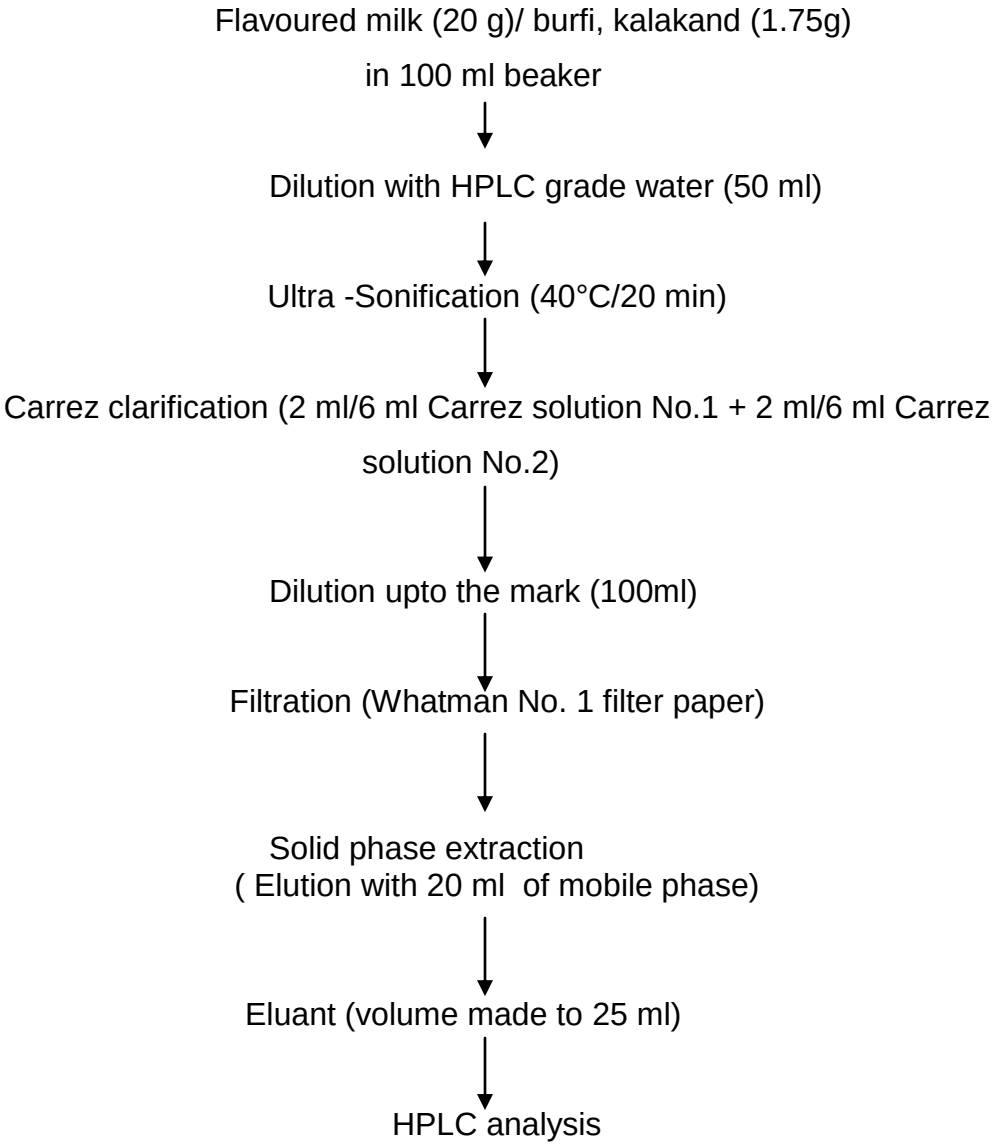

Figure 3.4 Flowchart for isolation of Acesulfame-k

3.4.6 HPLC Analysis

HPLC analysis of reference standards of Acesulfame-k & acetoacetamide and sample isolates from dairy products were performed under the following set of standardized conditions:

Column stationary phase	:	Shimpak C18, S-5µm, 120A, 250x4.6mm ID
Phase	:	Reverse phase
UV detector wavelength	:	200 nm
Mobile Phase	:	0.02 M phosphate buffer, pH 5.0: acetonitrile (97:3)
Flow rate	:	1 ml/min
Run time	:	10 min.
Maximum pressure	:	400 kgmf
Actual pressure	:	80 kgmf

3.4.6.1 Preparation of calibration curve and determination of detection limits

5-point calibration curves were plotted for Acesulfame-k and acetoacetamide. Curves were prepared representing, 50, 100, 150, 200 and 250, ng concentration of Acesulfame-k and acetoacetamide against their corresponding peak areas. Linear regression equations and correlation coefficients (R^2) were determined to see the linearity of the system. The detection limits of the HPLC system for acesulfame-k and acetoacetamide were determined by gradually reducing the concentrations of injections till the peaks disappeared.

3.4.6.2 Peak identification / quantification

Identification of the peak in the unknown sample was made through the comparison of retention times of the reference standards. Quantification was done from the peak areas of the reference standards.

3.4.6.3 Recovery experiments

Spiked samples of flavoured milk, burfi and kalakand with Acesulfame-k at 500 ppm levels on milk basis were isolated and analyzed over HPLC under the standardized set of conditions. The recovery of the method was 84-100%.

$$\text{Percent Recovery} = \frac{\text{Observed concentration} \times 100}{\text{Added concentration}}$$

$$\text{Observed concentration (ppm)} = \frac{C \times A_S \times \textbf{Constant factor}}{A_R}$$

Where, C = Concentration of reference standard (µg)

A_R = Peak area of reference standard

A_S = Peak area of sample

$$\text{Observed concentration (burfi/ kalakand)} = \frac{\text{Observed concentration (ppm) for burfi/ Kalakand On product basis}}{\text{Concentration factor for khoa}}$$

Whereas concentration factor for khoa = $\frac{\text{Amount of milk taken (gm)}}{\text{Amount of khoa prepared (gm)}}$

For flavoured milk, Constant Factor is

$$3125 = \frac{25 \times 100 \times 1000}{20^* \times 2 \times 20}$$

For burfi& kalakand, Constant Factor is

$$35714.3 = \frac{25 \times 100 \times 1000}{1.75^* \times 2 \times 20} \quad \text{(Burfi/Kalakand)}$$

Where, 20* = Weight of sample in gm of flavoured milk
1.75* = Weight of sample in gm of burfi/kalakand
100 = Volume (ml) made up before elution
2 = Volume (ml) of filtrate taken for elution
25 = Volume (ml) made up after elution
20 = Volume of injection (µl) into HPLC system loop
1000 = conversion factor from ml to µl

3.5 Storage and analysis of dairy products

Control and artificially sweetened samples of dairy products (with best selected sweetener level) were stored under refrigerated temperature (6–8ºC) and samples were analyzed on 0, 3rd and 7th day of storage for the following analytical parameters.

3.5.1 Sensory Evaluation

The samples of flavoured milk, burfi and kalakand were evaluated for the sweetness, aroma, colour, consistency, mouthfeel, body & texture and overall acceptability by an expert panel of 8 judges using a 9-point hedonic scale score card as shown in annexure I &II

3.5.2 Physico-chemical analysis

3.5.2.1 Titratable acidity

Titratable acidity of flavoured milk was determined by the method described by American Public Health Association (1995). In case of burfi and kalakand, acidity was determined by the method described in IS: SP 18 (Part XI, 1981).

3.5.2.2 pH

The pH of flavoured milk, burfi and kalakand was determined electrometrically with the pH meter by the method described in IS: SP 18 (Part XI, 1981).

3.5.2.3 Viscosity

Viscosity of milk samples was determined at 27 ± 0.10 ºC using ostwald viscometer. To maintain the temperature exactly at 27 ºC, the experiment was conducted using a thermostatically controlled transparent glass water bath.

3.5.2.4 Colour Measurement

The colour of flavoured milk, burfi and kalakand was measured by using colourflex supplied with universal software 9 version 4.10. The instrument was calibrated with standard black glass and white glass tile as specified by the manufacturer. The light source was xenon flash lamp. Sample was filled upto 2/3rd of the capacity in a cylindrical glass bowl (2.5 cm height and 5 cm diameter) and readings were recorded. Data was received through the software in terms of L^* [lightness, range from zero (black) to 100 (white)], a^* [redness, range from +60 (red) to -60(green)] and b^* [yellowness, range from +60 (yellow) to -60(blue)] values of the international colour system.

3.5.2.5 Texture Profile Analysis (TPA)

Various textural attributes such as hardness, cohesiveness, springiness, gumminess, and chewiness of burfi and kalakand were studied using TA-xT2i texture analyzer fitted with a 25 kg load cell. The samples of cylindrical shape of height 1 cm were subjected to mono-axial compression of 80% of the initial sample height which were already tempered at 30 ºC. The force distance curve(fig 3.5) was obtained for a dual bite compression cycle employing a cross head speed of 2.5 mm/sec. The test conditions maintained were as under:

Test mode and option	**TPA**
Pre- test speed	5 mm/sec.
Test speed	2.5 mm/sec.
Post test speed	2.5 mm/sec.
Strain	80%
Trigger type	Auto
Probe	P75 Compression Platen

The graphs obtained were analyzed using texture expert exceed software.

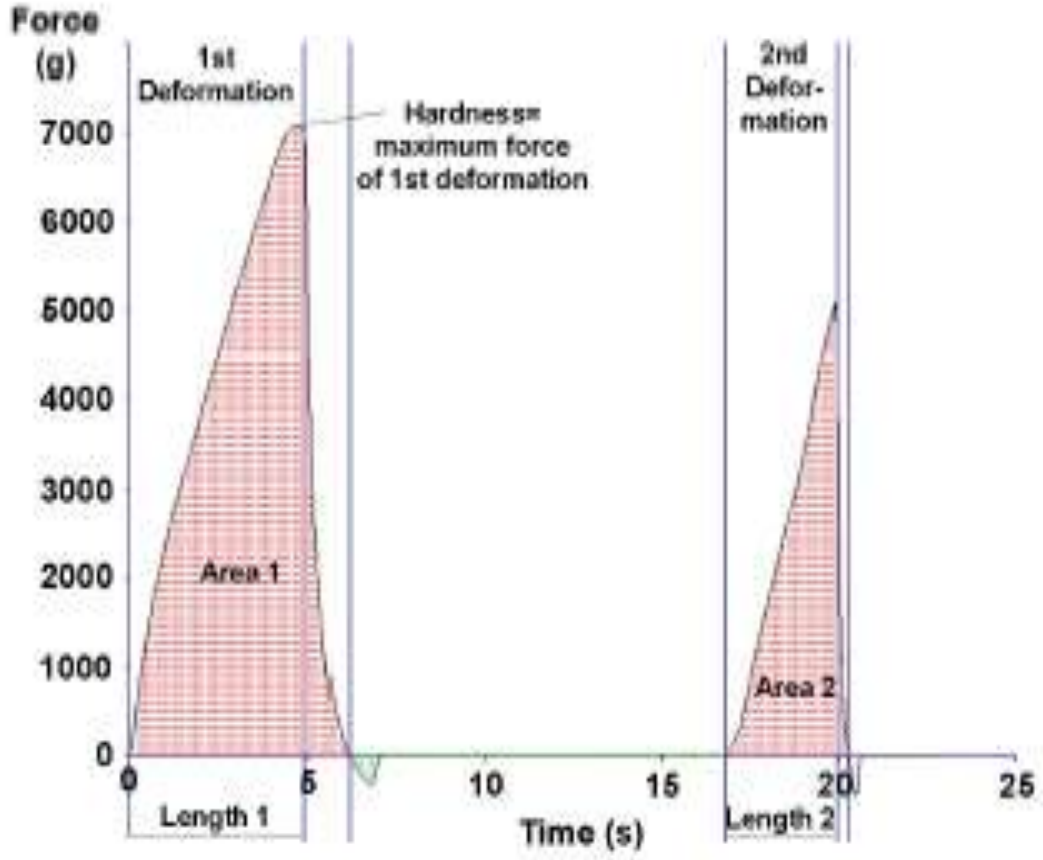

Figure 3.5: A typical 2 bite force distance compression cure for burfi/kalakand.

The following texture profile parameters were obtained.

Hardness: Hardness is the peak force of the first compression of the product.

Adhesiveness: Adhesiveness is indicated by a negative peak following the first peak

Cohesiveness: Cohesiveness is the ratio of area under the second bite curve before reversal of compression to that under the first bite curve

Springiness: Springiness refers to the height that the sample recovers during force relaxation time between first and second compression cycle

Gumminess: Gumminess is related to primary parameter of hardness and cohesiveness and is obtained by multiplication of these 2 parameters.

Chewiness: Chewiness refers to the energy required to masticate food into a state ready for swallowing and is a product of hardness, cohesiveness and springiness.

3.5.3 Microbial analysis

All the dairy products were examined for total plate count according to the method described in IS: SP 18 (Part I, 1980).

3.5.3.1 Preparation of dilution blank

3.5.3.1.1 Dilution blank for flavoured milk

The dilution blank for flavoured milk were made from normal saline (0.80 – 0.85% NaCl) solution. For 1:10, 1:100, 1:1000 dilutions, 9 ml of test solution was poured in test tubes. The mouths of test tubes were closed with cotton plugs. The test tubes containing dilution blanks were then sterilized at 15 psi for 15 min in an autoclave.

3.5.3.1.2 Dilution blank for burfi / kalakand

Dilution blank for burfi and kalakand was made from citrate buffer as well as saline solution. Citrate buffer is prepared by dissolving 10 gm of trisodium citrate in 500 ml of water. 99 ml of this test solution was poured into glass bottles with lid for preparing 1:10 dilutions. For further dilutions i.e. 1:100, 1:1000 dilutions 9 ml of normal saline was poured in test tubes. The mouths of test tubes were closed with cotton plugs. The bottles and test tubes containing dilution blank were then sterilized at 15 psi for 15 min in an autoclave.

3.5.3.2 Preparation of dilution

11.0 gm of burfi/kalakand was weighed in sterile aluminum foil. The contents of the aluminum foil were then transferred to a sterile glass mortar. 20.0 ml of sterile buffer solution at (45 ºC) from 99 ml dilution blank was then added and smooth paste was made using the pestle and transferred to sterile glass bottles. Another 20.0 ml buffer was poured into mortar, rinsed thoroughly and transferred into the bottle. The contents of the glass bottle were mixed well. This gives dilution 1:10. From this initial dilution further dilutions were prepared by transferring 1 ml into 9 ml blanks. In case of flavoured milk serial dilution was done by transferring 1ml of sample initially and then initial dilution into 9 ml blanks.

3.5.3.3 Total Viable Count

Plate Cont Agar was used to enumerate the total viable count in Flavoured milk, Burfi and Kalakand samples.

To rehydrate this medium 23.5 grams of the dry medium suspended in 1000 ml distilled water. The mixture is then boiled to dissolve the medium completely. It is then filled in conical flask and the mouths of the conical flasks were closed with cotton plugs. The conical flasks were then sterilized by autoclaving at 15 psi pressure (121 ºC) for 15 min.

Composition of Plate Count Agar

Ingredients	grams/litre
Tryptone	5.0
Yeast extract	2.5
Dextrose	1.0
Agar	15.0
Final pH (at 25 ºC)	7.0±0.2

3.5.3.4 Plating method

One ml of diluted sample (suitable dilution) was transferred in each of the duplicate petridishes. 10.0-15.0 ml of the melted agar (at 45 ºC) was then poured and the contents were mixed well by rotating in a horizontal position. The contents were allowed to solidify. The plates were then inverted and incubated at 37 ºC for 48 hours and colonies were counted.

3.5.4 Ultrastructure by scanning electron microscopy

3.5.4.1 Preparation of samples and fixation

Burfi / kalkand were cut into 2 x 2 x 2 mm size cubes. The cubes were transferred into micro beakers containing 2.5% buffered gluteraldehyde fixative. The fixation continued in cold for 3 hrs. Cubes were further trimmed to 1 x 1 x 1 mm size to facilitate the diffusion of fixative into the coagulum. The fixed samples

were then buffered with 0.05M cacodylate buffer at 4 to 6 ºC for 6 hrs. The buffered samples were doubly fixed with 2% osmic acid at 4 to 6 ºC for 2 hrs. Each solution was replaced gradually from one to another in the same container during the whole process.

3.5.4.2 Dehydration

After fixation, samples were subjected to dehydration as per the schedule given below.

Concentration of mixture	Duration
30% Ethanol	5 to 10 min in cold
50% Ethanol	5 to 10 min in cold
70% Ethanol	5 to 10 min in cold
90% Ethanol	5 to 10 min in cold
100% Ethanol	30 to 45 min in cold
100% Ethanol	30 to 45 min at room temp.

3.5.4.3 Drying of the samples

Samples were lyophilized for around 1 to 1.5 hrs.

3.5.4.4 Coating of samples

Freeze dried samples were mounted on stabs with adhesive and sputter coated gold at approximately 100 to 200 Aº thickness on Hitachi IB-3 ion coater. The ion current was kept at 6 mA at fine vacuum of 0.05 to 0.07 torr for 2 to 4 min.

3.5.4.5 Scanning electron microscopy

The gold-coated samples mounted on aluminium stabs were placed in specimen holder and inserted into chamber under vacuum. Hitachi –5 –405A scanning electron microscope was operated at 15 KV using second electrode mode and observations were made at different magnifications under accelerating voltage of 25 KV. The images of the selected area were recorded on a black and

white high speed (200 ASA) photographic film with the help of attached camera assembly. The microstructure of cubes was interpreted by comparing the micrograph of samples.

3.5.5 Sample preparation & HPLC analysis

As described in section 3.4.5 & 3.4.6 above

3.6 Statistical analysis

Data reported (For section 3.5.1 to 3.5.4) were expressed as mean values with standard errors. In all experiments, one-way/two-way analysis of variance (ANOVA) with a subsequent least significant difference (LSD) test was applied for multiple sample comparison to test for any significant differences ($P < 0.05$, $P < 0.01$ as the case may be) in the mean values of all the groups as described by Snedecor and Cochran (1994).

CHAPTER - 4

Results and Discussion

4. RESULTS AND DISCUSSION

The present investigation has been carried out to optimize the levels of acesulfame-K in *burfi, kalakand* and flavoured milk. The study was also carried out to check the stability of artificial sweetener (acesulfame-K) in these products along with thier physicochemical, textural, sensory, microbial and microstructural attributes during storage. The results obtained during the course of investigation are presented in this chapter and discussed in detail.

4.1 Optimization of acesulfame-K levels in flavoured milk, *burfi* and *kalakand*

4.1.1 Sensory Evaluation

The requirements of the consumer overshadow all other aspects of food production because without consumer sales, the industry will wither or die. Consumers expect food to be safe and wholesome, to offer value for money, to be of the highest quality and to have an attractive sensory profile. That is, every aspect of appearance, smell, flavour, and mouth feel should match their expectations. Sensory profile is the most important characteristic that contributes to the overall quality of dairy products. It is the property by which consumer first identifies and then judges a specific product.

The sensory evaluation of control with acesulfame-K as well as artificially sweetened products was undertaken to detect any variations produced in the sensory attributes of traditional dairy products by incorporation of acesulfame-K.

On the basis of sucrose equivalence, three different levels of acesulfame-K were selected on milk basis for the preparation of *burfi*, *kalakand* and flavoured milk. Out of these three levels, the best level was selected by comparing the sensory scores. Sensory evaluation of these products was carried out by a team of eight selected panelists from the institute on the basis of 9-point hedonic scale (Annexure- I & II).

Sensory evaluation revealed that acesulfame-K when used at a levels of 0.035% and 0.055% in *burfi* (Table 4.1), *kalakand* (Table 4.2) and 0.03 and 0.05% in *flavoured* milk (Table 4.3) resulted in lower sensory scores and hence was rejected. The level of 0.04% in flavoured milk and 0.045% in *burfi* and *kalakand* scored highest in terms of sweetness perception and almost resembled the control. Colour & appearance and body & texture scores were significantly lower in acesulfame-K sweetened *burfi and kalakand* as compared to control, but no differences were observed in colour & appearance and consistency & mouthfeel of acesulfame-K sweetened *flavoured milk*. However, overall acceptability of acesulfame-K sweetened products was lower ($P<0.05$) than the corresponding controls. Hence, on the basis of sensory evaluation, out of the above three levels, the level of 0.045%, 0.045% and 0.04% was finally selected for the manufacture of *burfi*, *kalakand* and flavoured milk, respectively.

Table 4.1 Optimization of acesulfame-K level in *burfi*

Characteristics	Level of acesulfame-K added (%)			
	Control	**0.035%**	**0.045%**	**0.055%**
Sweetness	8.3 ± 0.3^{a}	6.4 ± 0.2^{b}	7.8 ± 0.1^{a}	6.7 ± 0.5^{b}
Colour and appearance	8.3 ± 0.3^{a}	7.4 ± 0.2^{b}	7.4 ± 0.0^{b}	7.3 ± 0.5^{b}
Body and Texture	8.8 ± 0.3^{a}	7.8 ± 0.2^{b}	7.8 ± 0.3^{b}	7.0 ± 0.4^{c}
Overall acceptability	8.3 ± 0.3^{a}	6.9 ± 0.3^{b}	7.9 ± 0.1^{c}	4.8 ± 0.6^{b}

Means in each row with different superscripts were significantly different (LSD test, $P < 0.05$) from each other. Data are presented as means ± SEM (n = 8)

Table 4.2 Optimization of acesulfame-K level in *kalakand*

Characteristics	Level of acesulfame-K added (%)			
	Control	**0.035%**	**0.045%**	**0.055%**
Sweetness	8.2 ± 0.2^{a}	5.9 ± 0.3^{b}	7.8 ± 0.2^{a}	5.8 ± 0.4^{c}
Colour and appearance	8.2 ± 0.2^{a}	7.3 ± 0.2^{b}	7.4 ± 0.4^{b}	7.4 ± 0.6^{b}
Body & texture	8.6 ± 0.3^{a}	6.9 ± 0.3^{b}	6.8 ± 0.4^{b}	6.9 ± 0.3^{b}
Overall acceptability	8.2 ± 0.2^{a}	6.5 ± 0.3^{b}	7.9 ± 0.1^{c}	6.0 ± 0.6^{b}

Means in each row with different superscripts were significantly different (LSD test, $P < 0.05$) from each other. Data are presented as means ± SEM (n = 8)

Table 4.3 Optimization of acesulfame-K level in flavoured milk

Characteristics	Level of Acesulfame-K added (%)			
	Control	**0.03%**	**0.04%**	**0.05%**
Sweetness	8.3 ± 0.3^{a}	6.4 ± 0.2^{b}	7.8 ± 0.1^{a}	6.7 ± 0.5^{b}
Colour and appearance	8.3 ± 0.3^{a}	8.2 ± 0.2^{a}	8.2 ± 0.0^{a}	8.3 ± 0.5^{a}
Consistency & mouthfeel	8.8 ± 0.3^{a}	8.7 ± 0.2^{a}	8.7 ± 0.3^{a}	8.7 ± 0.4^{a}
Overall acceptability	8.3 ± 0.3^{a}	6.9 ± 0.3^{b}	7.9 ± 0.1^{c}	6.8 ± 0.6^{b}

Means in each row with different superscripts were significantly different (LSD test, $P < 0.05$) from each other. Data are presented as means ± SEM (n = 8)

4.2 Storage and analysis of dairy products

The dairy products are generally stored at low temperature (<10˚C) to maintain their hygienic quality and enhance the shelf life. During storage certain chemical changes could occur in acesulfame-K sweetened products therefore a detailed study regarding the physico-chemical and sensory attributes of artificially sweetened products was conducted. This may contribute a great deal to enable the manufacturers to produce these products in the organized sector under hygienic environment with better quality and lower cost.

4.2.1 Changes in sensory profile during storage

The sensory evaluation of control as well as artificially sweetened products during storage for seven days (6-8˚C) was undertaken to detect any variations produced in the sensory attributes of *burfi*, *kalakand* and flavoured milk by incorporation of acesulfame-K.

4.2.1.1 Sweeteness

Elucidation of sweetness has been one of the most intractable problems in dairy chemistry. Mean flavour scores of control and artificially sweetened *burfi* and *kalakand* over the entire period of storage are presented in table 4.4. The scores for sweetness were significantly higher ($P<0.05$) in control *burfi* as compared to artificially sweetened *burfi* throughout the storage period. This might be due to the lingering bitter after taste of acesulfame-K in *burfi*.

Analysis of variance also revealed that there was no significant difference ($P>0.05$) for the sweetness scores in both control with sucrose as well as in *burfi* with acesulfame-K for the entire storage period. This indicates that *burfi* still possessed the same desirable sweetness even after 7 days of storage.

A similar trend was observed in both *kalakand* (Table 4.4) and flavoured milk (Table 4.5)

4.2.1.2 Colour

The colour of heat desiccated products like khoa (base material for burfi & kalakand) is influenced by the type of milk used and also on the intensity of heating and sugar used. Whereas, the colour of flavoured milk is influenced by the quantity of colour used in the process. The added colour helps to manufacture flavoured milk of uniform colour irrespective of colour differences occurring in the raw materials from batch to batch.

The colour scores for *burfi* and *kalakand* are shown in table 4.4. The scores revealed that sucrose sweetened *burfi* and *kalakand* controls had a significantly ($P<0.05$) better colour scores than the acesulfame-K sweetened products throughout the storage period.

Analysis of variance also revealed that the period of storage had effect ($P>0.01$) on the colour scores of both control as well as in products with acesulfame-K.

However, for flavoured milk no difference was observed between the colour scores of control and the acesulfame-K sweetened product during the entire period of storage. It was also observed that there was no difference in the colour scores of both control and in the product with acesulfame-K for the entire storage period.

4.2.1.3 Body and texture/ Consistency and mouthfeel

The mean body and texture scores as given by the expert panel for the control and acesulfame-K sweetened *burfi* and *kalakand* for the entire period are shown in table 4.4. The scores revealed that sucrose sweetened *burfi* had a significantly ($P<0.05$) better body and texture than the acesulfame-K sweetened *burfi* throughout the storage period. The higher scores for body and texture in control *burfi* could be attributed to sucrose. Sucrose helps to form a network and create a binding effect in the product. Hence, the inclusion of sugar improved the mouthfeel of the product. On the contrary, acesulfame-K has insufficient water binding capacity and thus resulted in a product with slightly low score. There was a slight decrease in the body and texture scores of both control as well as in acesulfame-K sweetened *burfi* for the entire

storage period. However, analysis of variance revealed that the difference was non significant (P>0.05). A similar trend was also observed in *kalakand*.

The mean consistency and mouthfeel scores as recorded by the panel of judges for the control and acesulfame-K sweetened flavoured milk for the entire period are shown in table 4.5. The scores revealed that sucrose sweetened flavoured milk did not differ in consistency and mouthfeel from the acesulfame-K sweetened product throughout the storage period. Similarly no change was observed in the consistency and mouthfeel scores of both control as well as in the acesulfame-K sweetened flavoured milk for the entire storage period.

4.2.1.4 Overall acceptability.

Table 4.4 and 4.5 revealed that *burfi*, *kalakand* and flavoured milk made with sucrose seemed to be preferred by the expert panel of judges to the products made by utilizing acesulfame-K. These products had an overall acceptance of being “moderately liked” to “like very much”. The lower ranking of acesulfame-K sweetened products may be due to a bitter after taste and loose body and texture of the resulting product. Most importantly, based on the 8 member expert panel evaluations, we conclude that alternatively sweetened *burfi*, *kalakand* and flavoured milk have the potential to be highly accepted by the consumer. The results of sensory evaluation have shown the possibility of using artificial sweeteners in the preparation of indigenous dairy products.

Table 4.4 Sensory parameters of acesulfame-K sweetened *burfi* and *kalakand* during storage

Sensory parameters	*Burfi*			*Kalakand*		
	Storage (Days)			Storage (Days)		
	0	3	7	0	3	7
Sweetness						
Control	8.2±0.20^{a,A}	8.4±0.25^{a,A}	8.3±0.00^{a,A}	8.2±0.20^{a,A}	8.4±0.25^{a,A}	8.5±0.22^{a,A}
Acesulfame-K	7.6±0.40^{a,B}	7.5±0.37^{a,B}	7.6±0.25^{a,B}	7.6±0.32^{a,B}	7.6±0.25^{a,B}	7.4±0.32^{a,B}
Color and appearance						
Control	8.8±0.20^{a,A}	8.6±0.37^{a,A}	8.6±0.20^{a,A}	8.2±0.20^{a,A}	8.3±0.25^{a,A}	8.2±0.25^{a,A}
Acesulfame-K	7.4±0.75^{a,B}	7.6±0.25^{a,B}	7.4±0.25^{a,B}	7.4±0.32^{a,B}	7.4±0.40^{a,B}	7.3±0.37^{a,B}
Body and texture						
Control	8.6±0.40^{a,A}	8.2±0.37^{a,A}	8.2±0.49^{a,A}	8.6±0.25^{a,A}	8.1±0.32^{a,A}	8.2±0.32^{a,A}
Acesulfame-K	6.5±0.74^{a,B}	6.4±0.40^{a,B}	6.6±0.51^{a,B}	6.7±0.20^{a,B}	6.4±0.60^{a,B}	6.5±0.37^{a,B}
Overall acceptability						
Control	8.4±0.25^{a,A}	8.2±0.20^{a,A}	8.2±0.20^{a,A}	8.4±0.25^{a,A}	8.4±0.25^{a,A}	8.1±0.10^{a,A}
Acesulfame-K	7.1±0.59^{a,B}	6.9±0.32^{a,B}	7.1±0.54^{a,B}	7.2±0.37^{a,B}	7.5±0.39^{a,B}	7.1±0.25^{a,B}

Means in each row with different superscripts (a, b, c) were significantly different (LSD test, $P < 0.05$) from each other. Means in a column with different superscripts (A, B) were significantly different (LSD test, $P < 0.05$) from each other. Data are presented as means ± SEM (n = 8)

Table 4.5 Sensory parameters of acesulfame-K sweetened flavoured milk during storage

Sensory parameters	Storage period (days)		
	0	3	7
Sweetness			
Control acesulfame-K	8.2±0.20^{a,A} 7.4±0.25^{a,B}	8.2±0.25^{a,A} 7.4±2.6^{a,B}	8.4±0.22^{a,A} 7.2±0.33^{a,B}
Color and appearance			
Control acesulfame-K	8.2±0.20^{a,A} 8.4±0.19^{a,A}	8.4±0.25^{a,A} 8.2±0.40^{a,A}	8.2±0.25^{a,A} 8.2±0.33^{a,A}
Consistency and mouthfeel			
Control acesulfame-K	8.6±0.25^{a,A} 8.5±0.22^{a,A}	8.6±0.32^{a,A} 8.4±0.60^{a,A}	8.5±0.30^{a,A} 8.6±0.44^{a,A}
Overall acceptability			
Control acesulfame-K	8.4±0.25^{a,A} 7.2±0.38^{a,B}	8.2±0.20^{a,A} 7.4±0.25^{a,B}	8.1±0.25^{a,A} 7.3±0.22^{b,B}

Means in each row with different superscripts (a, b, c) were significantly different (LSD test, $P < 0.05$) from each other. Means in a column with different superscripts (A, B) were significantly different (LSD test, $P < 0.05$) from each other. Data are presented as means ± SEM (n = 8)

4.2.2 Physico-chemical analysis

Table 4.6 – 4.7 shows the physico chemical attributes of *burfi*, *kalakand* and flavoured milk,

4.2.2.1Titratable acidity

Results regarding the titratable acidity of *burfi*, *kalakand* and flavoured milk manufactured using sucrose and acesulfame-K is presented in table 4.6. It is evident from the table that there was a significant ($P<0.05$) increase in acidity of control as well as acesulfame-K sweetened products during storage. Statistical analysis of the data also revealed that there was a significant difference ($P<0.05$) in titrtable acidity between control and products sweetened with acesulfame-K. This difference in titratable acidity may be due to the slight preservative effect of sucrose, which led to the retarded microbial growth (Table 4.12) in control samples resulting in low acidity values as compared to products sweetened with acesulfame-K at all stages of storage.

Table 4.6 Titratable acidity of acesulfame-K sweetened *burfi*, *kalakand* and flavoured milk during storage

Titratable acidity (% LA)	Storage period (days)		
	0	3	7
Burfi			
Control Acesulfame-K	$0.36\pm0.01^{a,A}$ $0.36\pm0.00^{a,A}$	$0.38\pm0.01^{b,A}$ $0.39\pm0.01^{b,B}$	$0.39\pm0.02^{b,A}$ $0.43\pm0.01^{c,B}$
Kalakand			
Control Acesulfame-K	$0.36\pm0.02^{a,A}$ $0.39\pm0.02^{a,A}$	$0.39\pm0.01^{b,A}$ $0.44\pm0.01^{b,B}$	$0.41\pm0.02^{c,A}$ $0.47\pm0.01^{c,B}$
Flavoured Milk			
Control Acesulfame-K	$0.14\pm0.01^{a,A}$ $0.15\pm0.01^{a,A}$	$0.16\pm0.01^{b,A}$ $0.18\pm0.02^{b,B}$	$0.18\pm0.02^{c,A}$ $0.22\pm0.02^{b,B}$

Means in each row with different superscripts (a, b, c) were significantly different (LSD test, $P < 0.05$) from each other. Means in a column with different superscripts (A, B) were significantly different (LSD test, $P < 0.05$) from each other. Data are presented as means ± SEM (n = 5)

4.2.2.2 pH

The pH profile of *burfi* sweetened with acesulfame-K and its corresponding control with sucrose is depicted in table 4.7. It is evident from the table that there was a slight but statistically insignificant ($P>0.05$) decrease in pH of acesulfame-K sweetened *burfi* and control during the entire period of storage. Statistical analysis of the data also revealed that pH of control and *burfi* sweetened with acesulfame-K did not differ significantly ($P>0.05$) during storage.

The pH profile of *kalakand* sweetened with acesulfame-K and its control with sucrose is depicted in table 4.7. It is evident from the table that there was a significant ($P<0.05$) decrease in pH of control only on the seventh day of storage. However, in case of acesulfame-K sweetened *kalakand*, a significant decrease ($P<0.05$) in pH was observed throughout the storage period. Statistical analysis of the data also revealed that there was a significant difference ($P<0.05$) in pH between control and *kalakand* sweetened with acesulfame-K on the seventh day of storage.

It is evident from the table 4.7 that there was a significant ($P<0.05$) decrease in pH of acesulfame-K sweetened flavoured milk during the entire period of storage. However, in case of corresponding control also a similar decrease in pH was observed, but it was significant only after three days of storage. Statistical analysis of the data also revealed that there was a significant difference ($P<0.05$) in pH between control and products sweetened with acesulfame-K on seventh day of storage.

Table 4.7 pH of acesulfame-K sweetened *burfi*, *kalakand* and flavoured milk during storage

pH	Storage period (days)		
	0 day	3 day	7 day
Burfi			
Control	6.46±0.01^{a,A}	6.45±0.01^{a,A}	6.44±0.01^{a,A}
Acesulfame-K	6.45±0.00^{a,A}	6.42±0.01^{a,A}	6.40±0.01^{a,A}
Kalakand			
Control	6.38±0.01^{a,A}	6.35±0.018^{a,A}	6.30±0.01^{b,A}
Acesulfame-K	6.36±0.02^{a,A}	6.32±0.04^{b,A}	6.26±0.01^{c,B}
Flavoured Milk			
Control	6.67±0.01^{a,A}	6.65±0.01^{a,A}	6.58±0.01^{b,A}
Acesulfame-K	6.67±0.01^{a,A}	6.62±0.01^{b,A}	6.54±0.01^{c,B}

Means in each row with different superscripts (a, b, c) were significantly different (LSD test, $P < 0.05$) from each other. Means in a column with different superscripts (A, B) were significantly different (LSD test, $P < 0.05$) from each other. Data are presented as means ± SEM (n = 5)

4.2.3 Textural parameters:

Texture is one of the most important characteristic that contribute to the overall quality of milk products. It is the property by which the consumer first identifies and judges the specific variety and its quality before assessing the flavour. The rate and extent of textural changes during storage can be monitored by measuring some of the quantifiable rheological characteristics in terms of viscosity, hardness, adhesiveness, springiness, cohesiveness, gumminess and chewiness. The changes in these characteristics as influenced by utilization of sweeteners are presented in this section.

4.2.3.1 Viscosity

Addition of sugar has a big part to play regarding the viscosity of the flavoured milk. The mean apparent viscosities for each sample are shown in table 4.8. Viscosity data obtained at 27°C confirmed the expected slight increase of viscosity for all samples during storage. The fresh samples (control) of flavoured milk had an average viscosity of 2.35 cp, which increased to 2.43 cp after the storage of seven days. Similar increase in viscosity was observed in flavoured milk sweetened with acesulfame-K. An analysis of variance of viscosity data revealed that the increase in viscosity as a result of storage was not significant ($P>0.05$). It was also observed that there was a significant ($P<0.05$) difference between the viscosity of the control and artificially sweetened flavoured milk during storage. The observations regarding the consistency and mouthfeel scores as recorded by judges for sensory evaluation (Table 4.5) do not corroborate these observations.

However, this difference was maximum in fresh samples as compared to other period of storage. Hence, it can be inferred that sweeteners significantly affected the viscosity of the product. The difference in viscosities of control and artificially sweetened flavoured milk may be due to the fact that sucrose has a higher water binding capacity which enhanced the viscosity of control.

Table 4.8 Viscosity of acesulfame-K sweetened flavoured milk during storage

Viscosity (cp)	Storage period (days)		
	0	3	7
Control	$2.35\pm0.01^{a,A}$	$2.40\pm0.012^{a,A}$	$2.43\pm0.01^{a,A}$
Acesulfame-K	$1.84\pm0.02^{a,B}$	$1.88\pm0.03^{a,B}$	$1.90\pm0.01^{a,B}$

Means in each row with different superscripts (a, b, c) were significantly different (LSD test, $P < 0.05$) from each other. Means in a column with different superscripts (A, B) were significantly different (LSD test, $P < 0.05$) from each other. Data are presented as means ± SEM (n = 5)

4.2.3.2. Hardness

Hardness is defined as the force needed to attain a given deformation. It can be obtained by measuring maximum force during the first deformation of the sample. The changes in the hardness of *burfi* and *kalakand* during storage are presented in table 4.9.

The hardness value were significantly (P<0.05) higher in sucrose sweetened *burfi* and *kalakand* than the acesulfame-K sweetened products throughout the storage period. These findings are in accordance with the findings of Gupta et al (1990) and Suresh and Jha (1994b), who reported that the increased hardness of khoa (base material for *burfi*) correlated highly with the total solids and by increasing the total solids, hardness also increased. Hence, the higher hardness value in control could be attributed to the higher amount of total solids mainly contributed by sucrose in comparison to acesulfame-K sweetened products.

Analysis of variance also revealed that there was significant increase (P<0.05) in the hardness of both control as well as in products with acesulfame-K for the entire storage period. The increase in hardness of both the *burfi* and *kalakand* samples during storage could be due to the fact that there is loss of moisture during storage. Utilization of acesulfame-K instead of sucrose resulted in decreased firmness of the product as it could not form a compact network as evident from the SEM plates (Plate 4.1- 4.4).

4.2.3.3 Adhesiveness

Changes in adhesiveness (N) of control and acesulfame-K sweetened *burfi* and *kalakand* during storage is depicted in table 4.9. It was observed that the adhesiveness reduced consistently (P<0.05) with the progress of storage in both the *burfi* and *kalakand* samples. Adhesiveness of control samples with sucrose were higher (P<0.05) as compared to acesulfame-K sweetened *burfi* and *kalakand* throughout the storage period. Our results are in accordance with the findings of Jha (2003) who reported that sugar had a positive linear effect on adhesiveness of Doda *burfi* (a milk- wheat based sweetmeat). However, Gupta et al (1990) noticed no significant effect of compositional

factors on adhesiveness of khoa. The decrease in adhesion in *burfi* and *kalakand* samples may be due to the concomitant decrease in free moisture during storage.

4.2.3.4 Springiness

Springiness expresses the rate at which the deformed food material returns to its original condition after removal of force. Changes in springiness (mm) of control and acesulfame-K sweetened *burfi* and *kalakand* during storage is depicted in table 4.9. It was observed that the springiness reduced consistently ($P<0.05$) with the progress of storage in both the *burfi* and *kalakand* samples. Springiness values of control samples were higher ($P<0.05$) as compared to acesulfame-K sweetened *burfi* and *kalakand* throughout the storage period. It may be due to the better defined porous texture in control samples as compared to acesulfame-K sweetened samples.

4.2.3.5 Cohesiveness

Cohesiveness expresses the strength of internal structure of a food and can be calculated as area of work during the second deformation divided by the area of work during the first deformation. Changes in cohesiveness of *burfi* and *kalakand* during storage are presented in table 4.9. The cohesiveness values were similar between control and the acesulfame-K sweetened *burfi* throughout the storage period. Analysis of variance also revealed that there was no difference in the cohesiveness of both control and the acesulfame-K sweetened *burfi* throughout the storage period.

Cohesiveness reduced significantly ($P<0.05$) upto the third day of storage in both the control and the acesulfame-K sweetened *kalakand* and remained constant up to the seventh day of storage. Garg et al (1989) also reported that cohesiveness value was significantly affected by moisture content in *khoa.* However, cohesiveness values were similar between control and the acesulfame-K sweetened *kalakand* throughout the storage period.

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4.2.3.6 Gumminess

The changes in gumminess during storage are shown in table 4.9. The gumminess expresses the force needed to disintegrate a semisolid food to a state ready for swallowing. It is the product of hardness and cohesiveness. There was a continuous increase in gumminess ($P<0.05$) in acesulfame-K sweetened *burfi* and *kalakand* samples on storage. However, in case of control *burfi* the increase was evident after three days of storage. The control *kalakand* showed a significant increase ($P<0.05$) throughout the storage period. Control samples of *burfi* and *kalakand* had higher values for gumminess ($P<0.05$) as compared to acesulfame-K sweetened *burfi* and *kalakand* at all stages of storage. Since gumminess is a secondary parameter derived from hardness and cohesiveness, hence slight change in these two textural parameters also affected it.

4.2.3.7 Chewiness

Chewiness expresses the work needed to masticate a solid food to a state ready for swallowing. The changes in chewiness (N mm) during storage are shown in table 4.9. There was a decrease in the chewiness in both the *burfi* and *kalakand* samples and their corresponding control throughout the storage. Control had higher values ($P<0.05$) for chewiness as compared to acesulfame-K sweetened *burfi* and *kalakand* throughout the storage period. Since chewiness is a secondary parameter derived from hardness, cohesiveness and springiness, hence slight change in these textural parameters also affected it.

Table 4.9 Texture parameters of acesulfame-K sweetened *burfi* and *kalakand* during storage

Parameters	*Burfi*			*Kalakand*		
	Storage (Days)			Storage (Days)		
	0	3	7	0	3	7
Hardness (N)						
Control	62.21±0.11^{a,A}	68.28±0.04^{b,A}	82.18±0.07^{c,A}	51.31±0.15^{a,A}	68.68±0.58^{b,A}	75.30±0.15^{c,A}
Acesulfame-K	38.61±1.21^{a,B}	44.77±2.87^{b,B}	58.94±0.81^{c,B}	28.61±1.20^{a,B}	44.77±2.87^{b,B}	55.94±0.81^{c,B}
Adhesiveness (N)						
Control	0.39±0.01^{a,A}	0.35±0.02^{b,A}	0.28±0.01^{c,A}	0.42±0.01^{a,A}	0.35±0.02^{b,A}	0.22±0.04^{c,A}
Acesulfame-K	0.32±0.01^{a,B}	0.26±0.02^{b,B}	0.17±0.01^{c,B}	0.35±0.02^{a,B}	0.24±0.02^{b,B}	0.15±0.02^{c,B}
Springiness (mm)						
Control	1.66±0.02^{a,A}	1.34±0.01^{b,A}	1.10±0.05^{c,A}	4.17±0.09^{a,A}	3.07±0.08^{b,A}	2.25±0.11^{c,A}
Acesulfame-K	1.55±0.01^{a,B}	1.19±0.01^{b,B}	0.48±0.01^{c,B}	3.59±0.12^{a,B}	2.56±0.14^{b,B}	1.74±0.04^{c,B}
Cohesiveness						
Control	0.22±0.00^{a,A}	0.21±0.00^{a,A}	0.22±0.00^{a,A}	0.27±0.02^{a,A}	0.23±0.00^{b,A}	0.22±0.00^{b,A}
Acesulfame-K	0.18±0.00^{a,A}	0.19±0.00^{a,A}	0.18±0.00^{a,A}	0.25±0.00^{a,A}	0.19±0.00^{b,A}	0.20±0.00^{b,A}
Gumminess						
Control	13.68±0.02^{a,A}	13.65±0.01^{a,A}	16.44±0.01^{b,A}	13.85±0.01^{a,A}	15.71±0.01^{b,A}	16.55±0.01^{c,A}
Acesulfame-K	6.96±0.01^{a,B}	8.51±0.01^{b,B}	11.72±0.02^{c,B}	7.17±0.01^{a,B}	8.50±0.01^{b,B}	11.15±0.01^{c,B}
Chewiness (Nmm)						
Control	22.74±0.01^{a,A}	18.29±0.01^{b,A}	18.08±0.02^{b,A}	57.76±0.02^{a,A}	48.12±0.04^{b,A}	37.29±0.01^{c,A}
Acesulfame-K	10.77±0.02^{a,B}	10.12±0.01^{a,B}	6.23±0.02^{b,B}	25.69±0.01^{a,B}	21.77±0.01^{b,B}	19.38±0.01^{c,B}

Means in each row with different superscripts (a, b, c) were significantly different (LSD test, $P < 0.05$) from each other. Means in a column with different superscripts (A, B) were significantly different (LSD test, $P < 0.05$) from each other. Data are presented as means ± SEM (n = 5)

4.2.4 Colour Measurement

The colour of *burfi*, *kalakand* and *flavoured milk* was determined by using a hunter colour lab and the values were expressed in the form of colour L* (Black / white), colour a* (redness/greenness) and colour b* (yellowishness/ blueness). Table 4.10 indicated a trend of an overall decrease in lightness, increase in redness and no significant differences ($P<0.05$) in the yellowness during storage for *burfi* and *kalakand*. The objective measurement of colour could be correlated with the sensory attributes (Table 4.1 to 4.3). From the ANOVA analysis, it can be inferred that the change in L* values was not significant ($P>0.05$) during storage for both control and artificially sweetened *burfi* and *kalakand*. The slight change in these colour parameters was due to the maillard type of browning reaction in both control and acesulfame-K containing samples. It was also noted that the lightness was less in control samples than acesulfame-K sweetened *burfi* during the entire period of storage, which might be due to slight caramelization in presence of sucrose. Our results are in accordance with the results of Gothwal and Bhavadasan, (1991), who reported an increase in browining as a result of storage in khoa. In case of *Kalakand* a similar trend was observed for the colour parameters as that of burfi (Table 4.10).

Table 4.10 also indicated a trend of an overall increase in lightness (higher L* values) decrease in redness (more positive a* values) and no significant differences ($P>0.05$) in (b*) values on storage of both flavoured milk (control) and acesulfame-K sweetened samples. The objective measurement of colour cannot be correlated with the sensory attributes. The addition of the colour at the initial processing step and heating the mixture to 90˚C to achieve an even distribution of colour may have resulted in fading of colour due to certain chemical changes on storage which resulted in changes in these colour parameters. Lightness was observed to be more in acesulfame-K containing samples as compared to the control during the entire period of storage.

Table 4.10 Colour parameters of acesulfame-K sweetened, *burfi*, *kalakand* and flavoured milk during storage

Parameters	Storage period (days)		
	0	3	7
Burfi			
L (light ness)			
Control	60.21±2.47^{a,A}	59.53±3.92^{a,A}	57.56±3.31^{a,A}
acesulfame-K	63.55 ±0.41^{a,B}	63.84±0.11^{a,B}	62.58±0.22^{a,B}
A (red ness)			
Control	7.63±0.22^{a,A}	8.41±0.40^{b,A}	8.85±0.03^{b,A}
acesulfame-K	7.34±0.31^{a,A}	7.88±0.19^{a,A}	8.28±0.20^{b,A}
B (yellow ness)			
Control	31.47±0.60^{a,A}	30.51±0.27^{a,A}	30.41±0.28^{a,A}
acesulfame-K	30.08±0.32^{a,A}	29.75±0.32^{a,A}	29.33±0.27^{a,A}
Kalakand			
L (light ness)			
Control	65.72±0.23^{a,A}	64.75±0.31^{a,A}	62.13±1.10^{b,A}
acesulfame-K	67.26 ±0.54^{a,A}	66.04±0.51^{a,A}	65.43±0.69^{a,B}
A (red ness)			
Control	8.49±0.10^{a,A}	8.87±0.07^{a,A}	9.86±0.08 ^{b,A}
acesulfame-K	8.36±0.07^{a,A}	8.63±0.25^{a,A}	9.48±0.18^{b,A}
B (yellow ness)			
Control	31.79±0.10^{a,A}	30.57±0.19^{a,A}	30.43±0.15^{a,A}
acesulfame-K	30.50±0.03^{a,A}	30.32±0.07^{a,A}	30.08±0.06^{a,A}
Flavored Milk			
L (light ness)			
Control	73.60±0.01^{a,A}	73.95±0.016^{b,A}	74.33±0.017^{c,A}
acesulfame-K	74.67 ±0.03^{a,B}	75.89 ±0.03^{b,B}	76.99 ±0.03^{c,B}
A (red ness)			
Control	25.17±0.01^{a,A}	24.17±0.009^{b,A}	24.03±0.01^{b,A}
acesulfame-K	24.25±0.01^{a,B}	24.13±0.01^{a,A}	24.05±0.01^{a,A}
B (yellow ness)			
Control	-1.32±0.01^{a,A}	-1.28±0.01^{a,A}	-1.25±0.01^{a,A}
acesulfame-K	-1.06±0.01^{a,B}	-1.04±0.01^{a,B}	-1.03±0.01^{a,B}

Means in each row with different superscripts (a, b, c) were significantly different (LSD test, $P < 0.05$) from each other. Means in a column with different superscripts (A, B) were significantly different (LSD test, $P < 0.05$) from each other. Data are presented as means ± SEM (n = 5)

4.2.5 Stability of Acesulfame-K

4.2.5.1 Solid Phase Extraction of Acesulfame-K from Dairy Products

The sample preparation procedure used for isolation of acesulfame-K was essentially based upon the method of BSEN (1999):12856 cited by wood et. al. (2004). In case of flavoured milk the method was applicable as such, However, in case of *burfi* and *kalakand* due to their higher fat content (24-28% fat), weight of sample standardized was 1.75 gm to ensure the complete fat free extraction through C18 cartridge. The amounts of Carrez solutions 1 and 2 required for the precipitation of protein in flavoured milk were 2 ml each. The amounts of Carrez solutions 1 and 2 required for the precipitation of proteins in *burfi* and *kalakand* were standardized as 6 ml each.

4.2.5.2 HPLC Analysis of acesulfame-K and acetoacetamide

Acesulfame-K and acetoacetamide gave λmax at 220 and 200 nm respectively. Fig 4.1 represents HPLC chromatogram of these two components under the standardized analytical conditions (section 3.4.6).

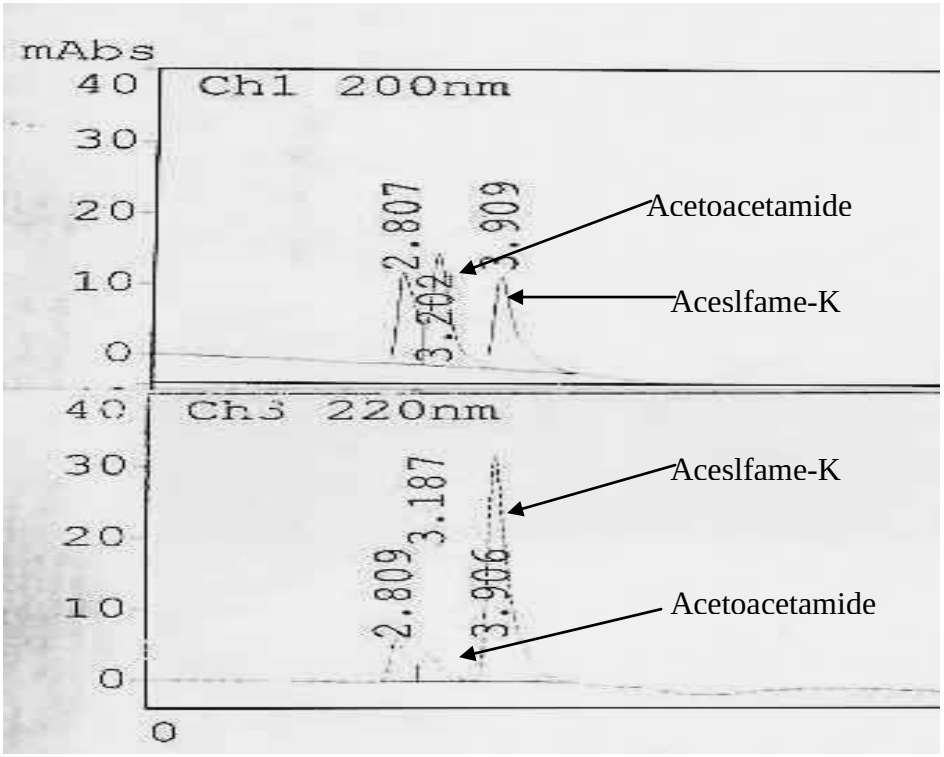


Figure 4.1: HPLC chromatogram of standard acesulfame-K and acetoacetamide

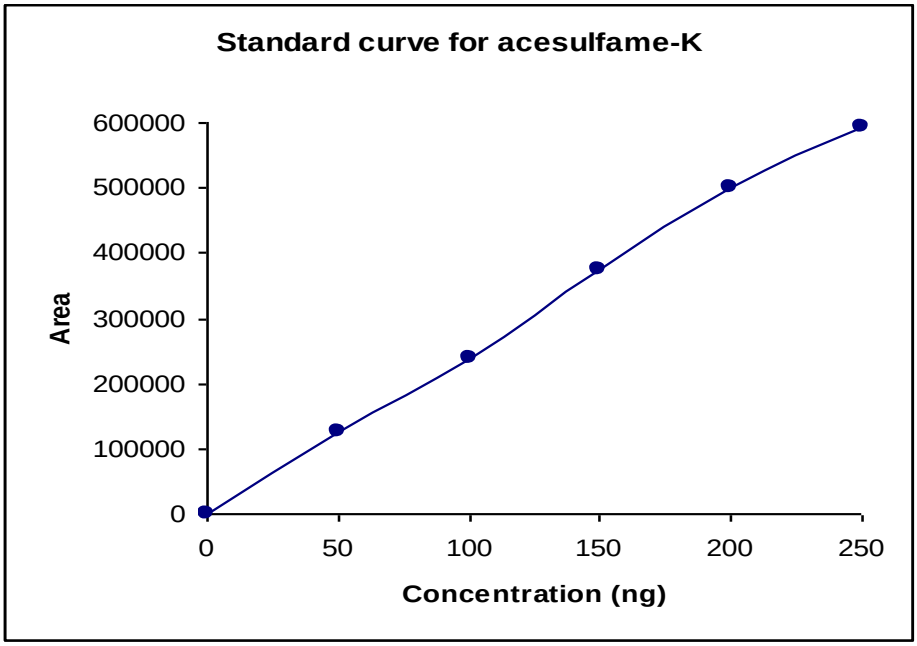


Figure.4.2 Standard curve for acesulfame-K

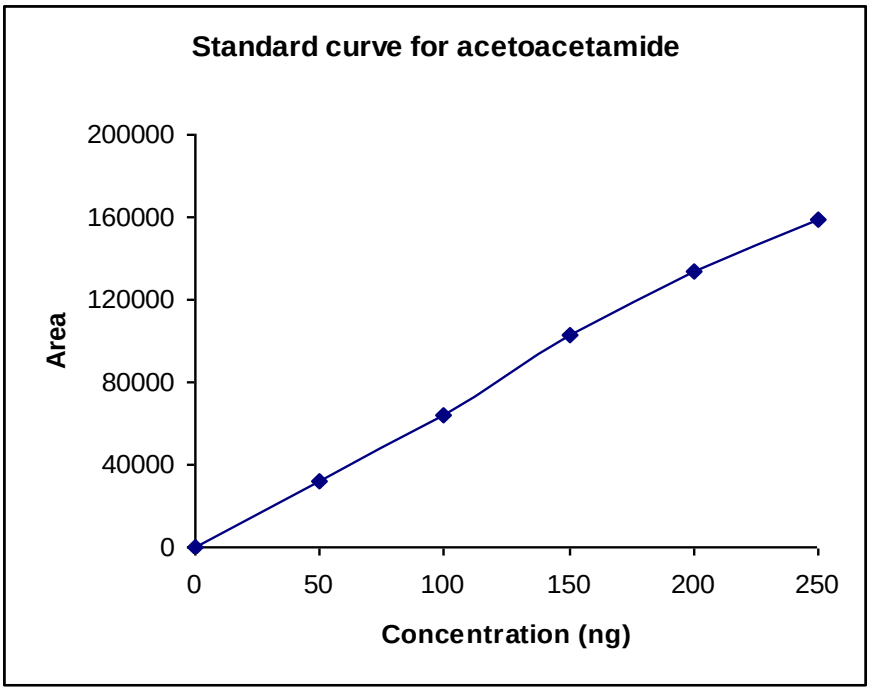
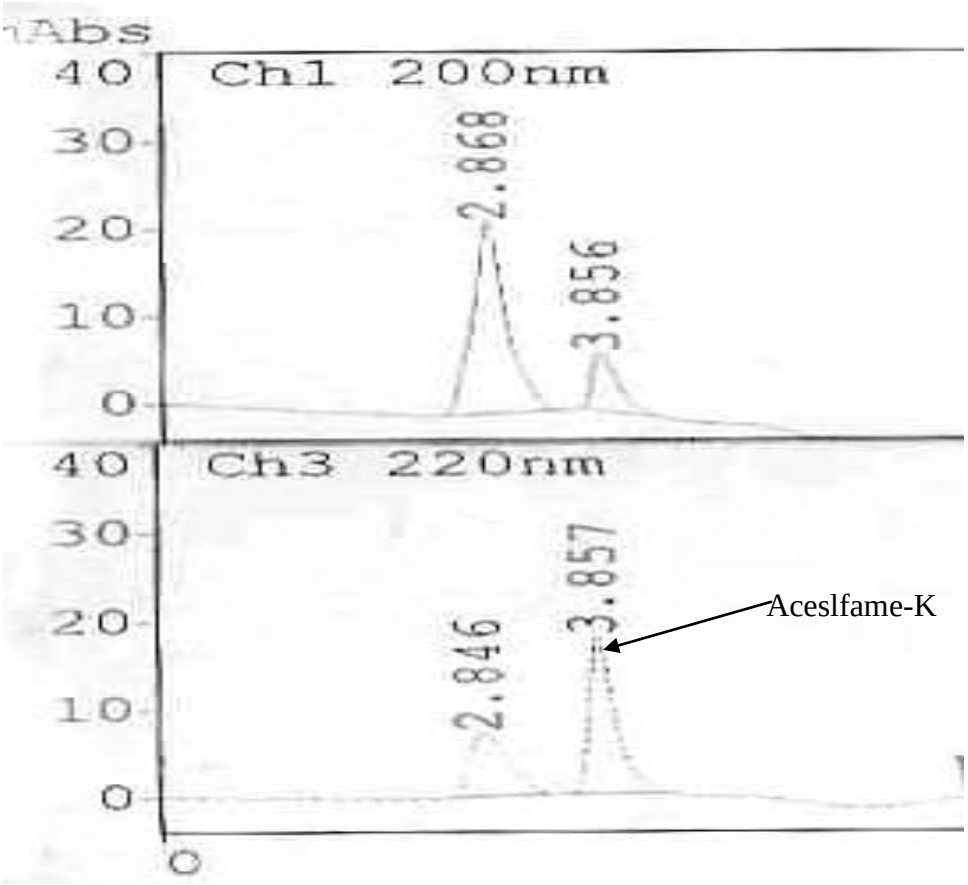


Figure.4.3. Standard curve for acetoacetamide

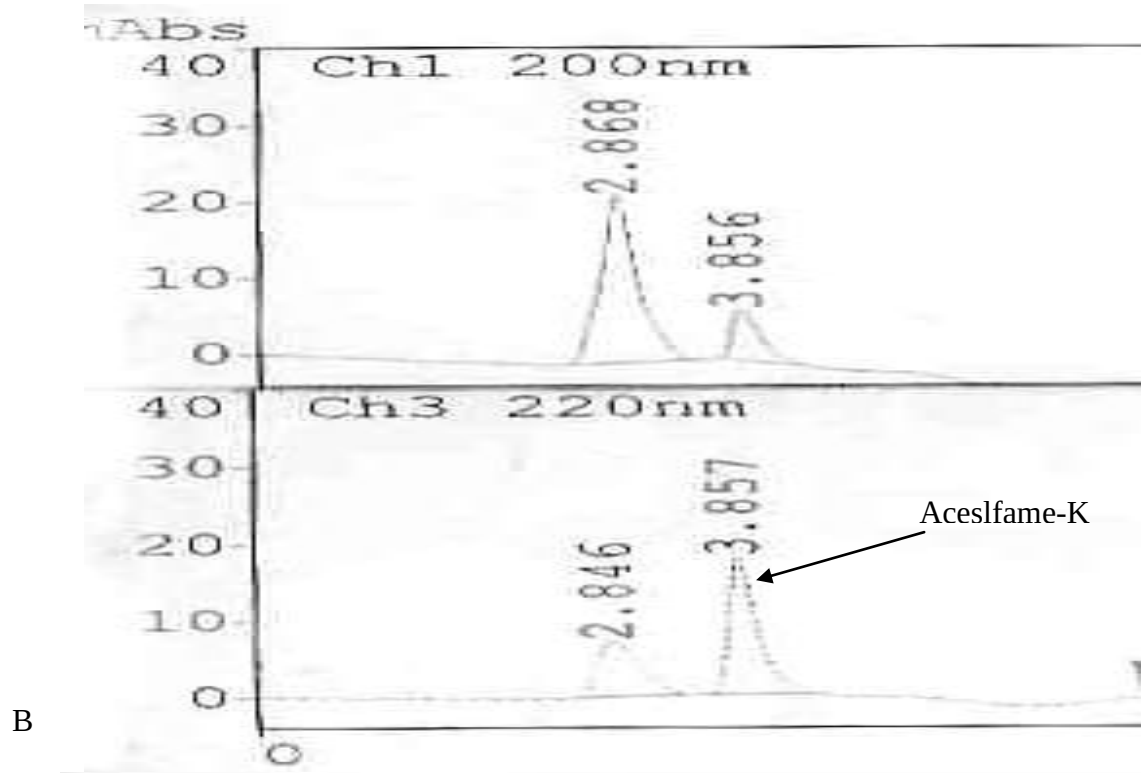
Fig 4.2 and 4.3 illustrate the 5- point calibration curved plotted for acesulfame-K and its degradation product acetoacetamide representing 50, 100, 150, 200, 250 ng concentrations against their corresponding peak areas. The regression equation and correlation coefficient obtained for acesulfame-K were $Y = 2422.1X + 3155.2$and 0.9978 respectively and the corresponding values for acetoacetamide were $Y = 650.04X + 739.33$ and 0.9969 respectively. The correlation coefficient of 0.99 for both acesulfame-K and acetoacetamide showed linearly of the system. The detection limits of both acesulfame-K and acetoacetamide were 15 ng and 20ng respectively.

HPLC chromatograms (Fig 4.4-4.6) obtained on 0, 3rd and 7th days for storage also support this observation as no extra peak appeared other than acesulfame-K in these chromatograms. This established that the acesulfame-K was not degraded during storage in the dairy products under investigation.

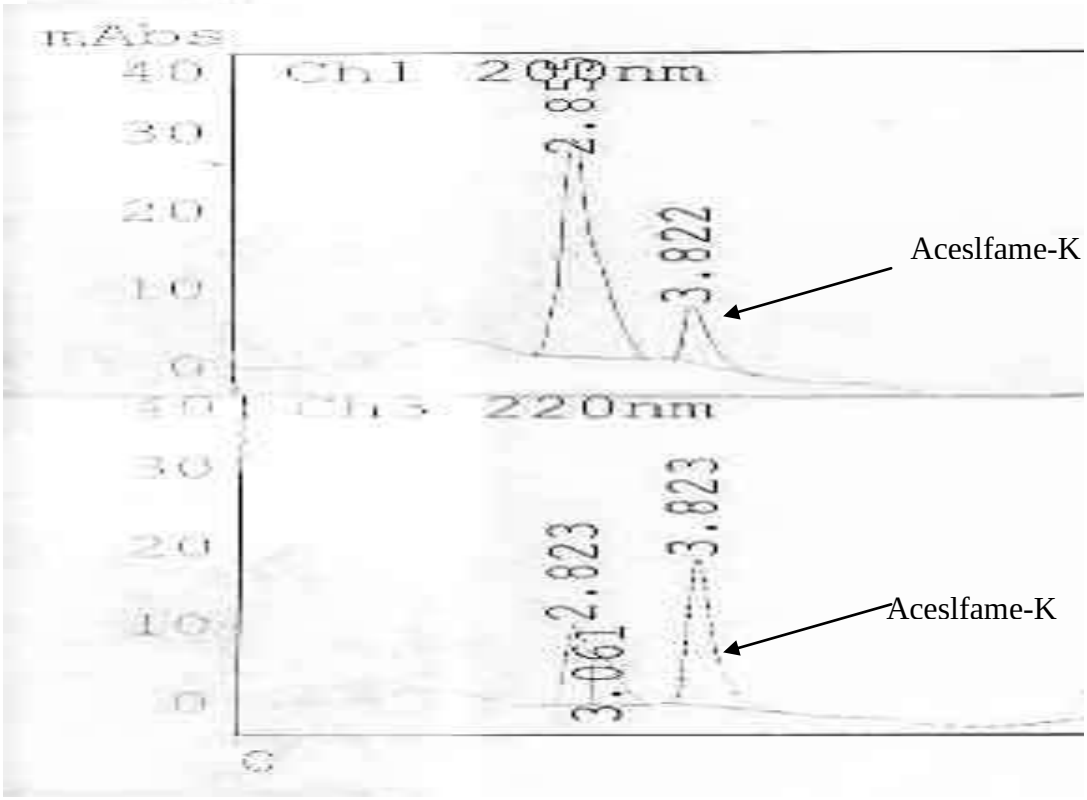
The results are in accordance with the observations of Von Rymon Lipinski and Hanger (2004) reporting stability of acesulfame-K at pasteurization and baking temperatures.


Abs
40
30
20
10
0
Ch1 200nm
2.868
3.856
40
30
20
10
0
Ch3 220nm
3.857
2.846
Aceslfame-K
0

A



B



C

Figure.4.4 HPLC chromatograms of sample isolates of acesulfame-K from flavoured milk during storage
A: 0 day; B: 3rd day; C: 7th day

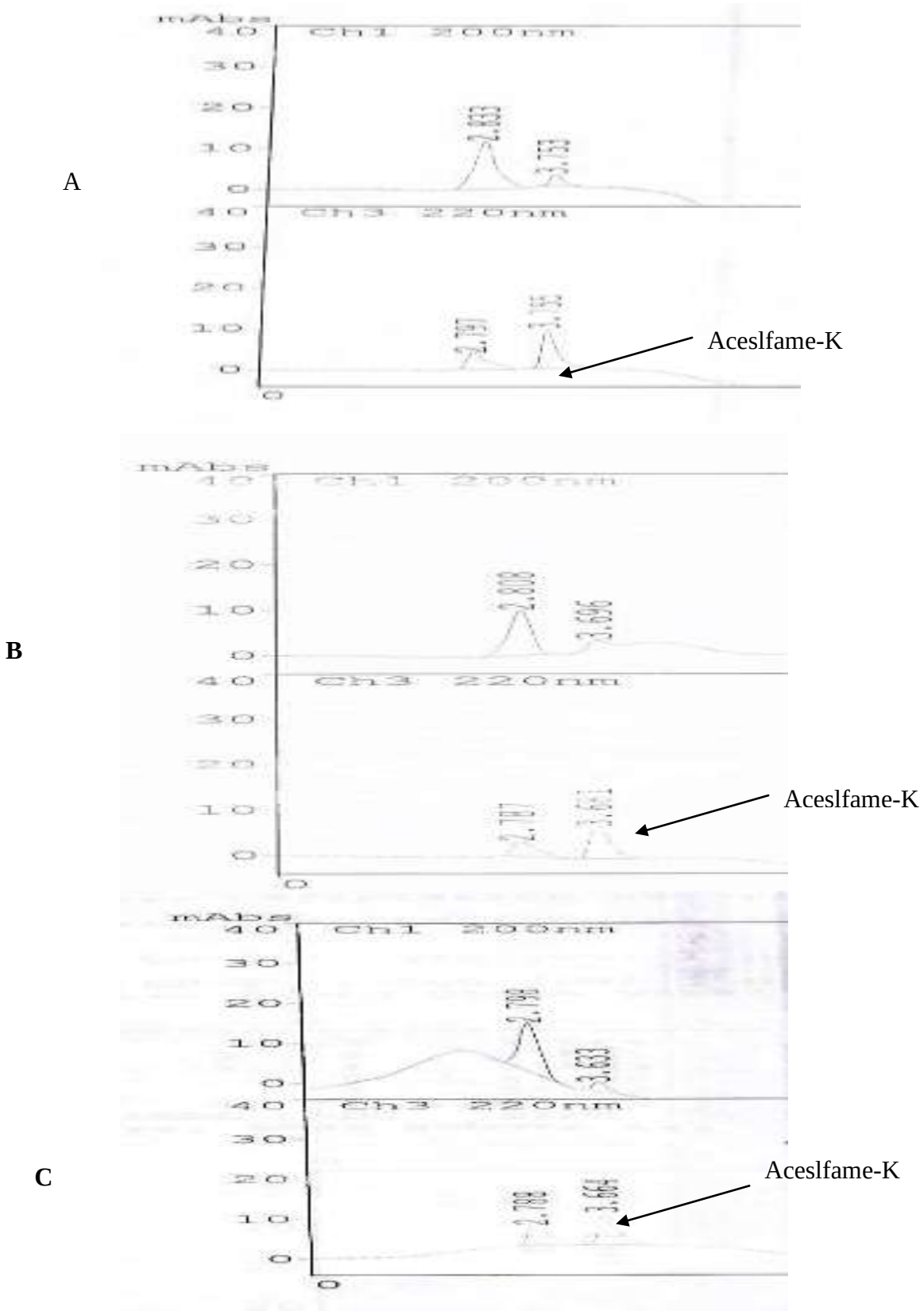


Figure.4.4.5 HPLC chromatograms of sample isolates of acesulfame-K from ***burfi*** during storage
A: 0 day ; B: 3rd day; C: 7th day

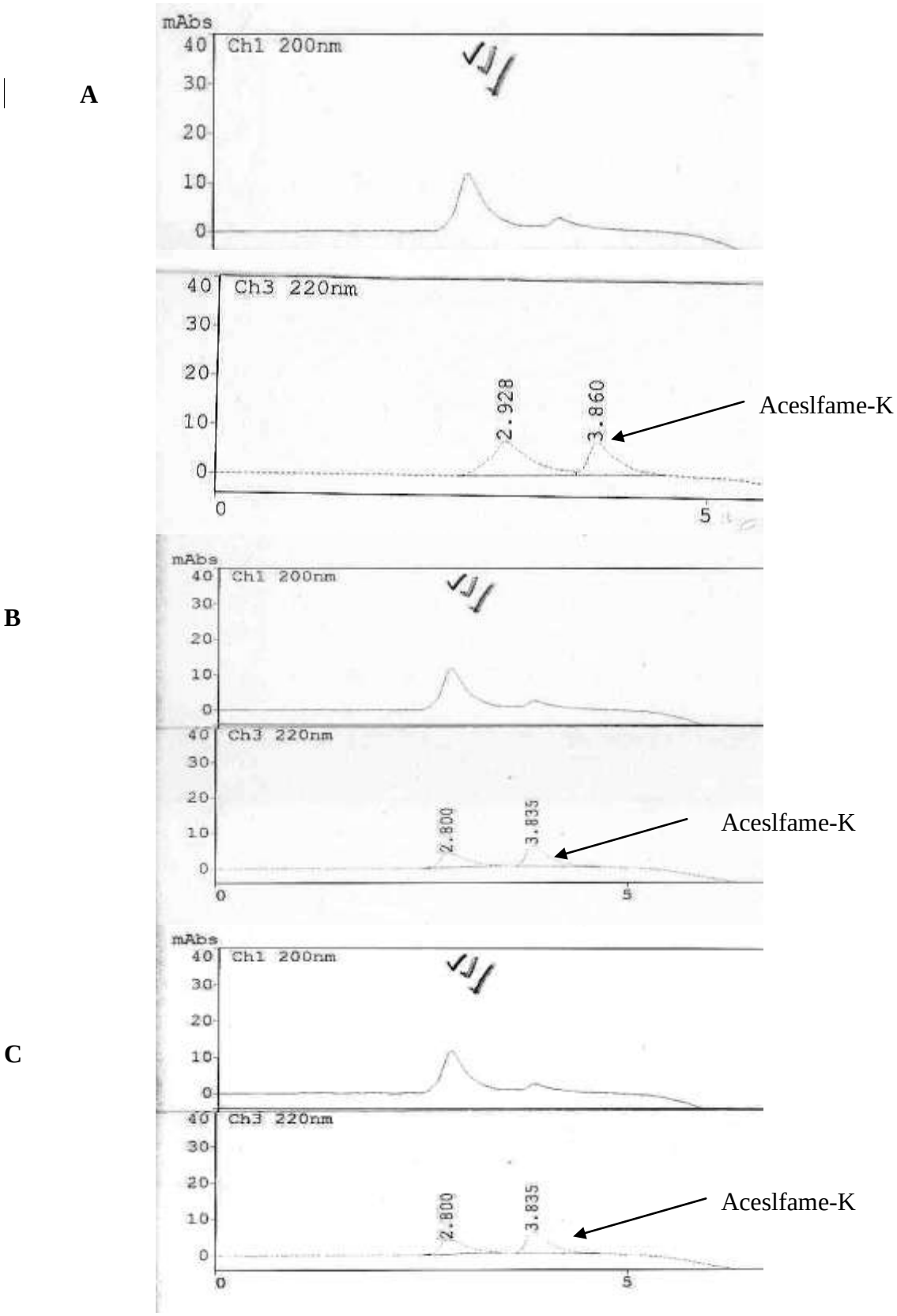


Figure.4.6 HPLC chromatograms of sample isolates of acesulfame-K from ***Kalakand*** during storage
A: 0 day ; B: 3rd day; C: 7th day

4.2.5.3 Stability of acesulfame-K in dairy products during storage as analyzed over HPLC

Table4.11 shows the stability of acesulfame-K in flavoured milk *burfi* and *kalakand*. It is evident from the table that acesulfame-K levels remained unchanged even up to 7 days of storage in all the three dairy products, establishing thereby the stability of acesulfame-K during storage.

Table 4.11 Stability of acesulfame-K in dairy product during storage as analyzed by HPLC

Product(level added-ppm)	0 day		3rd day		7th day	
	Level Recovered-ppm	% Recovery	Level Recovery	% Recovery	Level Recovery	% Recovery
***Burfi* (450)**	435.15	96.70	441.04	98.50	430.43	95.65
***Kalakand* (450)**	448.97	99.77	420.52	93.45	423.68	94.15
Flavoured milk (400)	352.80	88.20	336.04	84.01	338.04	84.51

4.2.6 Microbiological changes in during storage

4.2.6.1 Total plate counts

The changes in total plate counts during storage are presented in table 4.12. Total plate counts were higher ($P<0.05$) in acesulfame-K sweetened products than their corresponding control throughout the storage period as there is no preservation/protection effect of these sweeteners unlike sugar, which ultimately lead to higher microbial counts.

There was an increase ($P<0.05$) in the total plate counts in both the control *burfi* and the acesulfame-K sweetened products during the storage period. Analysis of variance revealed that there was a significant ($P<0.05$) difference in total viable counts in control and acesulfame-K sweetened products after 3 days of storage.

Table 4.12 Total plate count (CFU/ml) of *burfi*, *kalakand* and flavoured milk during storage

Total plate count	Storage period (days)		
	0	3	7
Burfi			
Control	$2.13 \times 10^3 \pm 0.30^{a,A}$	$4.95 \times 10^3 \pm 0.48^{b,A}$	$6.85 \times 10^3 \pm 0.62^{c,A}$
Acesulfame-K	$2.4 \times 10^3 \pm 0.46^{a,A}$	$6.08 \times 10^3 \pm 0.52^{b,B}$	$10.0 \times 10^3 \pm 0.58^{c,B}$
Kalakand			
Control	$2.03 \times 10^3 \pm 0.56^{a,A}$	$6.7 \times 10^3 \pm 0.34^{b,A}$	$14.6 \times 10^3 \pm 0.68^{c,A}$
Acesulfame-K	$1.99 \times 10^3 \pm 0.38^{a,A}$	$9.23 \times 10^3 \pm 0.32^{b,B}$	$20.75 \times 10^3 \pm 0.85^{c,B}$
Flavoured milk			
Control	$25.75 \pm 0.85^{a,A}$	$34.0 \pm 2.86^{b,A}$	$37.0 \pm 0.58^{c,A}$
ACESULFAME-K	$28.2 \pm 0.18^{a,A}$	$46.75 \pm 1.70^{b,B}$	$56.0 \pm 1.68^{c,B}$

x- Dilution Factor

Means in each row with different superscripts (a, b, c) were significantly different (LSD test, $P < 0.05$) from each other. Means in a column with different superscripts (A, B) were significantly different (LSD test, $P < 0.05$) from each other. Data are presented as means ± SEM (n = 5)

4.3 Ultrastructure by scanning electron microscopy

4.3.1 Scanning Electron Microscopy (SEM)

Scanning electron microscopy is an important tool in the study of surface topography as it produces a 3-D impression of a rough surface. This technique is especially useful in the study of various microstructural changes taking place during the various stages of processing of a product. Casein, organized in micellar form in fluid milk, in conjunction with other milk components is capable of forming structures widely ranging in density depending on the manufacturing conditions. During the preparation of sample for SEM, they were chemically dehydrated by passing through a graded series of alcohol-water mixture which resulted in removal of fat. The void spaces as evident from the photographs might have developed as a result of the fat extraction during sample preparation for electron microscopy.

SEM micrographs 4.1 and 4.2 revealed the microstructure of fresh *kalakand* with sucrose (control) and *kalakand* sweetened with acesulfame-K, respectively. The resulting microstructure of control was a spongy 3-D structure due to a networking of casein micelles alternating with very small pockets. *Kalakand* manufactured using acesulfame-K lacked a well defined 3-D microstructure. The microstructure of protein matrix differs from the matrix in control. The spongy texture was replaced with a loosely held structure with fewer pockets of larger size as compared to control. Hence, it is clearly evident that sucrose is responsible for maintaining the structure of *kalakand* (control) due to its water binding ability and thus providing enhanced stability. This property is lost in *kalakand* manufactured using alternative sweetener acesulfame-K.

A similar pattern in the formation of microstructure (SEM micrographs 4.3 and 4.4) can be noticed in control and *burfi* made with acesulfame-K. The control *burfi* had a well defined compact globular structure while replacement of sucrose with acesulfame-K resulted in development of microstructure which had lost most of its globular nature. The microstructure appeared to be open or loosely held. This difference can be ascribed to the presence of sugar in the control samples.

The compactness of the network decreased with the use of alternate sugar (acesulfame-K). The loose network in these samples may explain the low hardness, cohesiveness and accordingly gumminess and chewiness in these samples. Hence, each of the two systems resulted in a distinct type of microstructure in the finished product i.e. *burfi* and *kalakand*.

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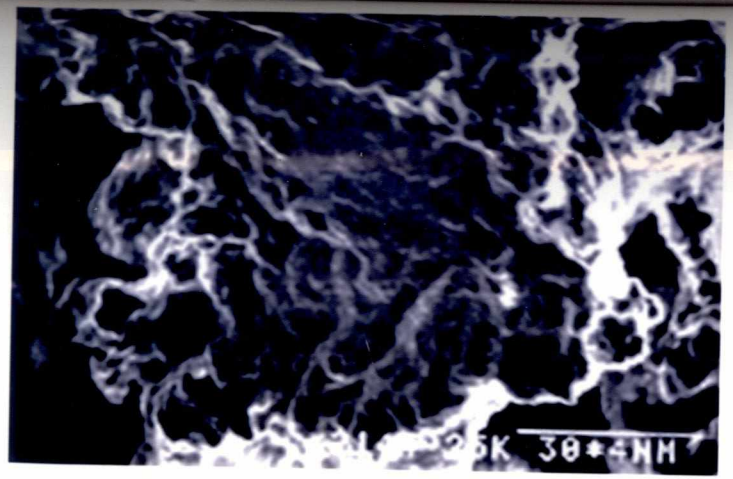

Plate 4.1. Scanning electron micrograph of control kalakand. (X 100)

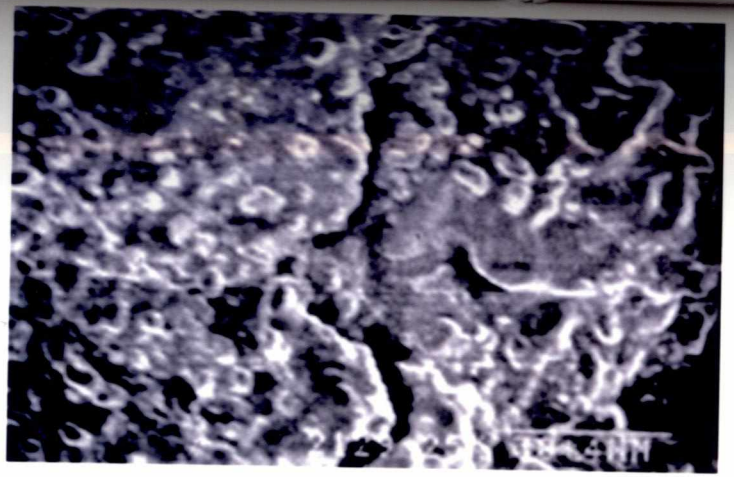

Plate 4.3. Scanning electron micrograph of control burfi (X 100)

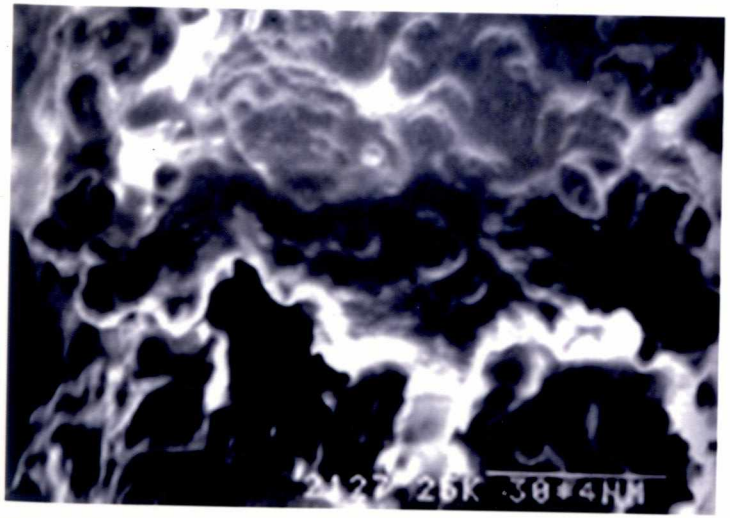

Plate 4.2. Scanning electron micrograph of kalakand with ace-K (X 100)

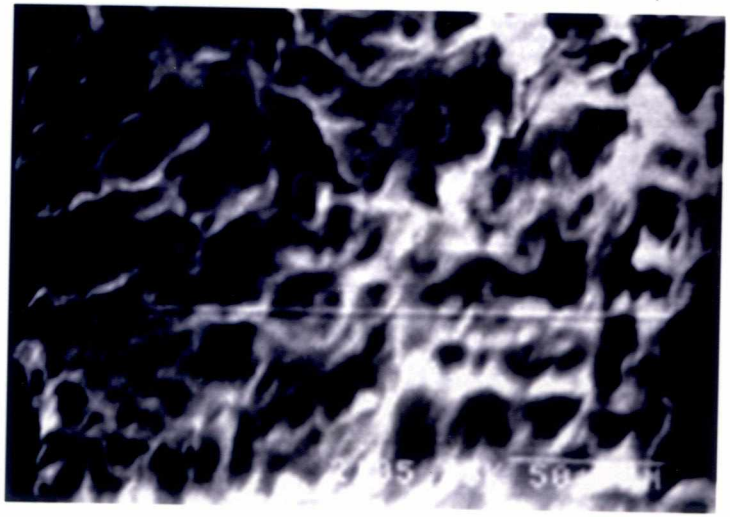

Plate 4.4. Scanning electron micrograph of burfi with ace-K (X 100)

CHAPTER - 5

Summary and Conclusions

5. SUMMARY AND CONCLUSIONS

This chapter deals with the major findings of the investigation. *Burfi, kalakand* and flavoured milk, were prepared using acesulfame-K. The optimum level of acesulfame-K was ascertained in all these products. These products were stored for seven days (6-8˚C) and their physicochemical, textural, sensory, microbial and microstructural attributes were studied. Effect of storage on the stability of acesulfame-K was determined by HPLC analysis.

5.1 SUMMARY

Optimum level of acesulfame-K was found to be 0.015% in *burfi, kalakand* and flavoured milk on the basis of sensory evaluation.

A solid phase extraction (SPE) method was standardized for the isolation of acesulfame-K in dairy products.

HPLC analytical conditions were standardized for the separation of acesulfame-K and its degradation product 2-sulfobenzoic acid.

The compactness of the network decreased with the use of acesulfame-K in *burfi* and *kalakand* as evident from SEM. The loose network in these samples explains the low hardness, cohesiveness and accordingly gumminess and chewiness in these samples.

5.1.1 Storage studies revealed:

Acesulfame-K sweetened products possessed the same desirable sweetness, colour, body & texture/consistency & mouthfeel even after 7 days of storage. Acesulfame-K sweetened dairy products ranked slightly lower ($P<0.05$) than the control on sensory evaluation at all periods of storage.

There was a significant increase ($P<0.05$) in acidity of control as well as acesulfame-K sweetened products during storage. The titratable acidity was

more in acesulfame-K sweetened products than the corresponding control samples.

pH of control and sweetened *burfi* showed non significant differences($P>0.05$), whereas the corresponding differences were significant ($P<0.05$) in case of *kalakand* and flavoured milk on seventh day of storage. However, a decrease in pH was observed in both control as well as acesulfame-K sweetened products on storage.

The viscosity of flavoured milk sweetened with acesulfame-K was significantly lower ($P<0.05$) than the corresponding control with sucrose.

Acesulfame-K sweetened *burfi* and *kalakand* ranked lower in various textural attributes at all period of storage.

Lightness was less in control samples with sucrose than the acesulfame-K sweetened *burfi* and *kalakand* during storage. However, in flavoured milk lightness was observed to be more in acesulfame-K containing samples as compared to control.

Acesulfame-K sweetened dairy products showed high total plate counts compared to control. These counts increased linearly for both acesulfame-K sweetened products and control during storage.

HPLC analysis showed no degradation of the added sweetener acesulfame-K establishing thereby the stability of acesulfame-K and hence its sweetness on storage of dairy products under investigation.

5.2 CONCLUSION

The results of the present investigation have established the successful use of acesulfame-K in the preparation of indigenous dairy products. The sweetener was very stable in *burfi, kalakand* and flavoured milk as no degradation was observed on HPLC analysis during storage establishing thereby the stability of sweetness also. Manufacture of indigenous dairy products with acesulfame-K will provide a successful outlet for traditional milk products and also provide an alternate variety to the health conscious consumers.

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APPENDIX

APPENDIX I

9- POINT HEDONIC SCALE FOR BURFI / KALAKAND

Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

Characteristics		Sweetener levels				
	Control					
Sweetness						
Colour & Appearance						
Body & Texture						
Overall Acceptability						

Remark if any for aftertaste:

Signature:

Date:

APPENDIX II

9- POINT HEDONIC SCALE FOR FLAVOURED MILK

Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

Characteristics		Sweetener levels				
	Control					
Sweetness						
Colour & Appearance						
Consistency & Mouthfeel						
Overall Acceptability						

Remark if any for aftertaste:

Signature:

Date: