ISOLATION AND IDENTIFICATION OF FLAVOURINGS
IN HERBS AND SWEETS

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DEDICATION

To the soul of my father
To my dear husband and daughter
To my mother and sisters
Who were very helpful throughout the study
To my dear friends and colleagues

With love and respect

Howida
Acknowledgements

First I am most grateful to Allah for assistance, health and patience that gave to complete this work.

I am greatly indebted to my supervisor Dr. Khogali Elnour Ahmed Ishag for his constructive and continuous guidance, friendly support and persistent encouragement.

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ABSTRACT

Steam extraction of volatile oils for four flavoured samples was carried out to determine the major active components in each. The samples included herb of peppermint (*Mentha piperita*) cinnamon bark spice (*Cinnamomum zeylanicum*) and two flavoured sweets.

The volatile oils were extracted by steam distillation where peppermint gave 0.0042% volatile oil and cinnamon bark gave 0.0035% using karlsruhe apparatus for determination.

The separation and identification of the components relied on the techniques of thin-layer chromatography (TLC) and Gas-liquid chromatography (GLC). The extract of the menthol – flavoured sweet sample was found to contain major component similar to that of the extract from peppermint herb. AGLC analysis was carried out for the flavoured sweet after hydrolysis and gave retention time (RT) for the main component comparable with that of the monoterpane menthol used as a reference compound. The RT values were 52.961 for menthol, 57.833 and 64.990 for flavoured sweet and 46.913 for peppermint herb extract.

The TLC analysis for the extracts from cinnamon bark and the flavoured sweets gave separation of similar main component. The comparative identification relied on colour reactions and RF-values
which was found to be 0.36 and 0.3 for the extracts from cinnamon bark and flavoured sweet respectively.

The results showed that steam distillation is efficient for extraction of volatile oils which contain flavoured compounds that are used as food flavouring additives. The techniques of TLC and GLC were quite satisfactory and reliable for the separation and identification of the flavouring substances.
بسم الله الرحمن الرحيم

خلاص الأطروحة

تناول البحث دراسة الزيوت العطرية الطبيعة لأربعة عينات وذلك بغرض تحديد طرق الاستخلاص والتعرف على محتواها من مواد النكهة، شملت العينات عشب النعناع Cinnamomum zeylanicum، تابل الفرصة Mentha piperita، ولحوى بنكهة الفرصة. تم استخلاص الزيوت العطرية من العينات بواسطة التقطير البخاري وكانت نسبة الزيت في عشب النعناع 0.042% أما في تابل الفرصة فكانت النسبة 0.0035% وذلك باستخدام جهاز Karlsruhe، استخدمت طرق الكروماتوغرافيا ذات الطبقة الرقيقة وكروماتوغرافيا الغاز السائلة لعملية الفصل والتعرف على المركبات الفعالة في الزيوت الطيارة. فقد إتضاح من نتائج التحليل أن الحلوي بنكهة النعناع تحتوي على مركبات مطابقة لتلك التي استخلصت من عشب النعناع حيث تم التحليل بكرماتوغرافيا الغاز السائلة بعد التحليل المائي للمستخلص وكان المركب الأساسي هو ميثوت أحادي التروبين وذلك عند مقارنته مع مركب مرجعي حيث كانت فترة الإستبقاء RT للمثلول المرجعي 52.961 والمستخلص من عشب النعناع 57.833 و 64.990. أما بالنسبة للمستخلص من تابل الفرصة والحلوى المنكهة فأعطى التحليل بكروماتوغرافيا الطبقة الرقيقة قراءات مشابهة للمكون الرئيسي في كل من مستخلص تابل الفرصة RF-value ومستخلص الحلوى حيث كانت قيمة 0.36 RF-value و 0.30 للمكون الرئيسي في كل على التوالي.

لقد أثبتت النتائج أن التقطير البخاري يمكن الإعتماد عليه لاستخلاص الزيوت الطيارة التي تحتوي على مركبات مسببات النكهة والتي تستخدم مضادات أغذية منكهة وكذلك وجد أن طرق كروماتوغرافيا الطبقة الرقيقة وكروماتوغرافيا الغاز السائلة ذات كفاءة عالية وكافية للتحلي والتعرف على المركبات الفعالة المسببة للنكهة.
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CHAPTER ONE
INTRODUCTION

Flavourings are usually prepared from natural and/or synthetic aromatic substances that may or may not be found in nature. The aim is to increase the acceptability of the end-product through the stimulation of smell and taste.

Flavouring material may be added to a product either to enhance a desirable flavour or to mask an undesirable one, to make the final product pleasant (Heath, 1981). Although, in the traditional medicine herbs and spices have been used since ages, the flavour industry has developed relatively recently from small companies specializing in the processing and marketing of natural herbs and spices, the distillation of essential oils and aromatic essences and the extraction and isolation of aromatic chemical to produce fragrances. Nowadays huge expanding industry is involved in offering a wide range of flavouring to the food specially for beverages, confectionary and related products as well as fragrances for toiletries and the cosmetic industry. This is an increasing demand for better flavour for food especially in terms of quality and safety (Kazeniac, 1977).
Flavour is the sum total of the sensory responses of taste and aroma combined with the general tactile and temperature responses to substances placed in the mouth.

Flavour can also mean any individual substance or combination of substances used for the principal purpose of eliciting flavour. Flavouring substances can vary considerably in complexity from a chemically simple component substance like ethyl butyrate to a chemically complex multiple component substance such as ginger oleoresin unlike many substances which are added to foods to achieve a desired effect, flavouring substances are added in amounts that are self-limiting and governed by the potency of the substance used to provide the necessary organoleptic characteristics of the food product. As a result, flavouring substances generally are used in low concentrations and add only small amounts to human intake.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) is in the forefront for the evaluation of the safety of flavouring substances. Several other organizations including the scientific Committee on Food (SCF), the Flavour and Extract Manufacturers Association (FEMA) Expert panel the committee of Experts on flavouring substances (CE) have recognized the importance of structure-activity relationships, metabolism and
exposure data in the safety evaluation of flavouring substances. The US Food and Drug Administration (FDA) has also used structure activity relationships to define concern levels for food additives in the Redbook (FDA, 1982).

Since the research in food flavouring is still young and relatively complex, the objective of this work will be confined to the extraction means and purification of some volatile components in some food products as well as the identification of the extracted components.
CHAPTER TWO
LITERATURE REVIEW

2-1 Classification of flavour

Flavours can be classified on the base of their mode of formation, either naturally by biogenetic path from known precursor or by processing in which biological, chemical or physical conditions are imposed on natural or artificial start materials. (Ohlo, 1972).

Natural flavours are substances derived from natural sources, These are Largely composed of essential oils and oleoresins. Artificial Flavours are substances that are chemically synthesized for flavour applications. (Lindsay, 1984)

Food flavourant are classified by the food sources in which they are normally detected, Table 1 (Flath et al. 1981).

2-2 Uses of flavour in food

Flavouring material may be added to product either to enhance desirable or to mask an Undesirable one, to make the final product pleasant (Heath, 1981).
<table>
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<th>Flavour class</th>
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<td>Citrus (terpene)</td>
<td>Grapefruit, Orange, Apple, raspberry, banana,</td>
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<td>Non citrus (non terpene)</td>
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<td>Tomato leather tobacco</td>
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<td>Spice flavours</td>
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<td>Lachr/matory</td>
<td>Onion, garlic</td>
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<td></td>
<td>Fermented</td>
<td></td>
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<tr>
<td></td>
<td>Compounded</td>
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Kazeniac (1977) showed that the success of a flavour depends on its recognition by consumer as being similar to a familiar. He
added that exotic, abstract or fancy flavours are not, usually well accepted by the consumer, when flavours are being developed it is necessary to take into account the compatibility between the new flavour and the inherent flavour of the product. Although, in the traditional medicine and culinary art, herbs and spices have been used since millennia, the flavour industry has developed only over the past 150 – 200 years from small companies specializing in the processing and marketing of natural herbs and spices.

Flavourings are those materials; added to substance to give it the flavour of the flavourings or to supplement or modify its own flavour or to cover up or mask the original flavour of the material.

For example orange to give orange identity to carbonated water, malt flavouring to modify the flavour of cereal grains, an ethol or anise to cover or mask the bitterness of medicine (Swine, 1964).

**2-3 Physical form of Flavouring agents**

Flavours can be obtained as solids, paste or liquids. Solid flavours may be provided in a variety of forms including powders or crystal. Semisolid or paste flavours are concentrated flavours such as oleoresins. Liquid flavours may be oily in nature or alcoholic or aqueous in character. Liquid flavours are prepared by dissolving
flavour materials into suitable solvent such as ethyl alcohol and propylene glycol (Lindsay, 1984).

2-4 Sources of natural flavour

2-4-1 Spices

Spice is a dried vegetable product derived from any part of plant whether it is root, stem, bark, fruit, bud, or seed. (Minifie, 1970) Some vegetables such as onion and garlic, can be considered spice. In food processing, spices are often used in the form of essential oils or oleoresins. Essential oil are prepared by steam distillation of the dried ground spices and contain the volatile flavour compounds. Oleoresins are the solvent extract of the spices and contain both volatile essential oils as well as non-volatile resinous material and more characteristic of the original ground spice (Flath et al 1981).

2-4-2 Herbs

Herb is dried product derived from the green leaves or herbaceous part of the plant (Minifie, 1970). Leaves are used whole or cracked. The aroma of the crushed leaves is delicate and fragrant. The taste is aromatic and bitter. Leaves are used principally in vinegar pickle pigs feet and lamb and park tongue. They are also used in flavouring of soups, stews, game dishes, fish and sauces, pickles and mixed pickling spice (Merory, 1960).
2-4-3 Fruit flavours

The tastes of fruit are a blend of the sweetness due to sugars (such as glucose, fructose and sucrose) and the sourness of organic acid (such as citric and malic). However, it is the aromas of the different volatile components of fruits that allow us to distinguish between them. Fruit aromas vary widely. Citrus, such as grape fruit, orange, lemon and lime are rich in terpenoids whereas most non-Citrus Fruits such as apple raspberry, cranberry and banana are characterized by esters and aldehydes- (Flath et al 1981).

Lindsay (1996) reported that Citrus flavours are among the most popular fresh fruit as well as flavours for beverage. Most information about the flavour chemistry of natural citrus flavours stem from research on processed juices, peel essential oils, and aqueous essences used to flavour juice product. Several classes of flauvor components serve as major contributors to citrus flavours, including terpenes, aldehydes, esters and alcohols and large numbers of volatile compound have been identified in the various extracts from each citrus fruit.

2-4-4 Smoke flavouring

FAO/WHO (1992) reported that smoke flavouring is complex mixture of components of condensed smoke derived from pyrolysis of
hard woods in the absence of or in a limited amount of air-source materials must be free of pesticides, wood preservatives and other extraneous matter which may result in hazardous constituents in wood smoke. The major flavouring principles of smoke flavouring are carboxylic acid compounds with carbonyl groups and phenolic compounds. During manufacture condensed wood smoke is treated by procedures for isolation, fractionation or purification with the intention of eliminating hazardous constituents of smoke, such as polycyclic aromatic hydrocarbons. Commercial smoke flavouring products may be dilution of concentrated smoke flavouring. Liquid smoke flavouring products may be diluted in solvent media such as water, propylene glycol or in vegetable oils, with or without emulsifiers. Solid smoke flavouring products may be prepared with carriers such as salt.

**2-4-5- Oleoresins**

These complex mixtures are obtained by extracting, concentrating, and standardizing the essential oils and nonvolatile constituents from spices. They are commonly paste or solid extracts and possess power full flavouring properties (Lindsay, 1984).

Merory (1960) reported that oleoresins are prepared by extraction of comminuted seeds, roots, barks, leaves, and fruits with a
solvent of high chemical purity and low boiling temperature. The extraction is performed either by maceration or percolation immediately after the pulverization of the botanical material to reduce loss of volatile aroma.

Swaine (1964) showed that oleoresins are normally thick, viscous, highly coloured substances. They impart less colour to the product than the corresponding spices because they are used in small quantities. Oleoresin is very similar to the spice from which it is derived. Certain spices are extracted to yield their oleoresins, not for the flavouring effects, but for intense colour that is produced. Examples of this are paprika and turmeric, which are used heavily in the manufacture of French – type salad dressings.

2-4-6 Essential oils

These flavours are composed of only volatile fraction obtained from distillation of a spices and similar plant materials that contain aroma compounds which are present in high concentration like anise, nutmeg, celery and cinnamon (Lindsay, 1984). Volatile oils derived from plants, which usually carry essential odor or flavour of the plant are called essential oils. They are as a rule in soluble in water. The yield of essential oil usually is not high (Merory, 1960). Essential oils may occur in different plant organs like flower petals as in rose, bark
as in cinnamon, leaves as in peppermints, wood as in sandal, roots as in angelica, rhizomes as in ginger, fruit as in bergamot and seeds as in anise (Claus, 1965).

2-5 Extraction of volatile oils

The manufacturing process employed for extraction of volatile oils may be classified according to Ainely and Jamed (1977) as follows:

2-5-1 Distillation

In the distillation method odoriferous material is separated from the raw material by means of boiling water or stem. The distillate, which consists of a mixture of oil and water, is condensed and collected in a suitable receiver.

2-5-2 Expression

Some volatile oils are highly sensitive to distillation and so are prepared if possible by mechanical means as in the case of citrus oils from the peel of lemon, orange and bergamot.

2-5-3 Solvent method

These methods are mainly applied for unstable aromatic substances. It is applied mainly to flowers such as jasmine. The method of extraction is carried out by using non-volatile or fixed
solvents such as animal fats (lard) or vegetable oils (olive oils) or extraction with volatile solvents such as petroleum ether, pure benzene and alcohol.

2-6 Extraction and Isolation methods

Aromatic components as their precursors are generally present in aqueous solution or as droplets in the cell sap, although some essential oils may exist in oil sacs, glandular hairs, it is necessary to extract or to isolate the odor/flavour complex as completely as possible from the mass of inert cellular matter with the minimum amount of chemical change. This may be achieved by several techniques depending on the nature of the start material. These include.

2-6-1 Expression

The physical extraction of aqueous juices from plant tissues of particular interest in the studies of fruit flavours.

2-6-2 Solvent Extraction

The solvent used may be either water from which the aromatic components may be recovered by high vacuum vaporization, or low-boiling point non polar solvents such as ether, methylene dichloride,
hexane or liquefied gaseous solvents such as liquefied carbon dioxide. The solvent depends on the physical nature of start material and its susceptibility to oxidation.

2-6-3 Steam distillation at Atmospheric pressure

Is the most used method of isolation and recovery of aromatic compounds from plant materials, although precautions must be taken to limit thermal degradation of components.

2-6-4 Vacuum Distillation

Is used for distillation of high molecular weight substances, which need high temperatures for their distillation at atmospheric pressure, resulting in chemical decomposition.

2-6-5 High vacuum Degassing.

Applicable to the recovery of volatiles from fixed oils and foods having a high lipid content.

2-6-6 Head space vapour Collection

This technique is important for the examination of the low volatile components. If the material under examination is allowed to stand in a suitable vessel, the low–boiling volatile components will achieve the equilibrium in the head-space. These vapors can then be used for direct identification using gas chromatography. The results
obtained are, of coarse not representative of the full odour / flavour of the start material (Quirin, 1995).

2-7 Flavour Enhancers

Compounds eliciting this unique effect have been utilized y humans since the inception of food cooking and preparation but the actual mechanism of flavour enhancement remains largely unknown (Lindsay, 1996). Flavour enhancers include monosodium glutamate (MSG), rib nucleotides disodium inosinate, and disodium guanylate (Olney, 1969).

2-7-1 Uses of flavour Enhancers:

Olney (1969) and Bindoni (1990) reported that monosodium glutamate and the ribonucleotides disodium inosinate and disodium guanylate are commonly used as flavour enhancers in processed foods. These substances contribute a delicious taste to foods when used at levels in excess of their independent detection threshold, and they simply enhance flavour at levels below their independent detection threshold. Their effect are prominent and desirable in the flavours of vegetables, soups, dairy products, meats, poultry, fish, and other sea foods. Although most attention has been directed to wards the 5-ribonucleotides and monosodium glutamate, other flavour-enhancing compounds have been claimed to exist. Maltol and ethyl
maltol are worthy of mention because they are used commercially as flavour enhancers for sweet goods and fruits (Lindsay, 1996).

2-7-2 Monosodium Glutamate (MSG).

Monosodium glutamate is used very broadly. In most commercially prepared frozen foods containing meat or fish, in almost all dry soup mixes, and in many canned foods, MSG is an ingredient. In addition it is used by many housewives to enhance the flavour of foods. (Sjstrom, 1964).

Laboratory evidence has linked monosodium glutamate with the Chinese restaurant syndrome a tightening of the muscles of the face and neck, occasionally accompanied by headache, nausea and giddiness. Experienced by some people who have eaten in restaurants where monosodium glutamate has been used in large amounts. Many countries have therefore restricted the use of monosodium glutamate or required its presence in food to be prominently stated on the label (Kermode et al., 1972).

Olney (1969) and Belluardo (1990) pointed out that monosodium glutamate is converted into the amino acid, glutamic acid, which is known to have neuroexcitatory properties, and glutamate is also implicated in neurotransmission. Monosodium glutamate was stimulated after some people has experienced symptoms such as
tightening of the face and chest muscles and also burning sensation in
the upper body as well as headaches. This was called Chinese
restaurant syndrome, because it was frequently experienced by patrons
of Chinese restaurants and was traced to the liberal use of (MSG) by
Chinese. Other symptoms associated with (MSG) and dizziness,
diarrhoea, nausea and stomach cramps. Some children experience
shudder attacks which may be mistaken for epilepsy, and long- term
exposure in mice can lead to obesity. The most serious side effect
which also effects the endocrine function. MSG has been reported to
depress growth hormone levels, and levels of prolation and sex
hormone and also effected Guillemin (1984), Martin (1981) and Olney
(1971) reported that such finding should make one extremely cautious
of using (MSG) and replacement products such as protein
hydrolysates should also be avoided as they also contain large
amounts of glutamate and thus have potential to elicit similar effects.

2.8. Safety assessment of flavour ingredients:

2.8.1 Basic principles of safety evaluation:

Assessing the safety of flavouring substances is similar to that
of other food additives and includes the consideration of inherent
toxicity of the substance, exposure to the substance, chemicals
structure and structure activity relationship, and natural occurrence.
Over the last 45 years, the joint FAO/WHO Expert Committee for food additives (JECFA) has been in the forefront of developing a procedure to evaluate the safety of flavouring substances and has recognized the unique issues surrounding their evaluation. Other organizations, commission of the European Communities, Scientific Committee on Food (SCF, 1991), the Flavour and Extract Manufactures Association (FEMA) Expert panel (Wood and Doull, 1991), and the Committee of Expert on flavouring substances of Council of Europe (CE, 1974, 1981, 1992, 2000a, 2000b) have recognized the importance of structure-activity relationships, metabolism, and exposure data in the safety evaluation of flavouring substances. Historically flavouring substances have been tested for safety by testing representative members of a chemically similar group. This means that many flavouring substances have not been subjected to detailed and comprehensive toxicity testing programs. This is also due in part to the large number of flavouring substances that are used in food. Many flavouring substances are self-limiting and are typically used at very low concentration to impart desired effect, and exposure from their use in food is, generally, very low—Munro et al., (1998) stated that the intake of 95% of flavouring substances used in food in the US is 1mg/day or less. Many methods
for calculating intake have been suggested, however estimating the intake of flavouring substances historically has been performed in two ways. One method involves multiplying that level of use of the flavouring substances in a particular food category and multiplying this value by the amount of that particular food category eaten per person and dividing by the total population to estimate the per capita intake. A second method of estimating the intake of flavouring substances is to assume that the total amount of a particular flavouring substances produced has been added to broad categories of foods and is completely consumed by the total population. This method uses poundage data obtained from surveys of substances reported to be intentionally added to foods by ingredient manufacturers and food processor in the US and Europe. In the US, these surveys have been conducted by the US National Academy of Sciences/ National Research Council (NAS/NRC) between 1970 and 1987. The International Organization of the Flavour Industry (IOFI, 1995) has provided similar data for Europe. A more accurate method for determining intake, but time consuming and expensive is to use individual consumer dietary surveys which is referred to as the detailed dietary analysis, involves recording in detail the eating habits of group of individual consumer. Hall et al. (1999). Conducted a
comparison between the use of poundage data on a per capita basis and the use of a detailed dietary analysis in calculating the intake of flavouring substances. Ten flavouring substances were selected as representative example and intakes for these substances were calculated using both methodologies. The results clearly showed that the detailed dietary analysis provided good estimates of the distribution, of intakes across the population as well as patterns of intake of individuals, but it was both expensive and labor intensive. The poundage method resulted in intake values that were comparable to, if not greater than the detailed dietary analysis and was considered to be amore practical approach to intake estimations (Smith et al., 2001).

Chemical structure determines the inherent toxicity of a substance its metabolic profile and its pharmacokinetics.

In 1978 Cramer et al. Incorporate chemical structure, pharmacokinetics and knowledge of metabolic fate to produce a decision tree for classification of flavouring substances into toxicity concern level. The US Food and Drug Administration (FAD) has also used structure–activity relationships to define concern levels for additives (FAD, 1982). JECFA has repeatedly demonstrated to use of structure–activity relationships and known metabolic path ways in the
evaluation of flavouring substances and has recognized that generation of extensive toxicity data is not necessary when toxicity data are available for one or more members of a homologous series of chemical substances (WHO, 1987). Another consideration in the estimation of the intake of flavouring substances internationally added to foods in the natural occurrence of the substance in food.

The natural occurrence of a particular ingredient does not necessarily prove the safety of the substance. In the safety assessment the ratio of the natural occurrence of a flavouring substance to the intentional addition to food, referred to as the consumption ratio (CR) (Stofberg and Kirschman, 1985). A CR of greater than one indicates that the substance is nature predominant, and ACR of greater than 10, which according to Stofberg and Grundschober (1987) applies to most flavouring substances, indicates that the added substance contributes an insignificant amount to the diet. Example of the importance of natural occurrence is in the evaluation of process flavours. Process flavours generated from interactions between protein, nitrogen carbohydrate, fat or fatty acid sources during thermal processing. They mimic flavour naturally present in cooked foods, particularly cooked meat. As such, process flavours are a potential dietary source of heterocyclic amines (HCA₅). Which are reported to be potent
mutagens and animal carcinogens (Munro et al 1993). Although toxicological data are available on HCA, the studies are inadequate to characterize dose-response relationship and do not represent the low levels found in foods. Since HCA, do occur naturally, particularly in cooked meats, a comparison of exposure to HCA from use of process flavour in food can be made with exposure of HCA found naturally in the diet. This comparison has shown that the intake to HCA naturally occurring in food and it was concluded that the use of process flavours would not pose a significant health risk to humans. Richling et al. (1998), has demonstrated the ubiquitous occurrence of HCA in numerous foods. Safety assessment of flavouring materials as potential elicitors of allergic reaction involves consideration of the chemistry of the material, the source, the method of manufacture and an evaluation of use level and subsequent exposure. (Toylor and Dormedy, 1998).

2-8-2. Biotechnology and Flavour Production:

Advances in modern molecular techniques now allow the development of new genetically modified (GM) plant and animal sources from which flavouring substances can be derived—also microorganisms with specifically engineered pathways used to produce specific flavour enzymes and chemicals. A basic premise in the safety
assessment of new food products is idea of substantial equivalence safety of the new products can be judged by comparing it to its traditional counterpart which has had a history of safe use (FAO/WHO, 2000).

Food and flavour ingredient production from GM materials safety assessment could be made by determining whether the ingredient had a prior history of safe use in approved foods whether the GM substance was the same as that conventionally produced (IFBC, 1990). The FEMA Expert panel reconsidered the criteria employed in making a Generally Recognized as safe assessment for a flavouring substances in light of GM technology (Hallagan and Hall, 1995b). Oser and Ford (1977) reported certain for judgments on evaluation of food flavours to be generally recognized as being safe (GRAS). Similar Hall (1960), Hall and Oser (1961, 1965, 1968) state that the requirements include evidence for the identity and purity of the substance, its chemical and pharmacological relation to structurally analogous substances, its presence and level as a naturally occurring constituent of foods, intended use levels, and any pertinent metabolic or toxicologic data. Oser and Ford (1979) pointed out that the considerable knowledge gained from the isolation and characterization of components of natural flavours have paved the
way for preparation of synthetic flavouring substances to be used as food improvers and must be GRAS type. The maximum level of these compounds can be taken as good manufacturing practice (GMP) since they are GRAS or according to food additives regulations (Miles, 1979).

2-9 Detection method of odour:

Teranishi et al. reviewed different techniques on food aroma separation, detection and identification (1967). They mentioned the techniques of gas chromatography (GC), Infrared spectrometry (IR), mass spectrometry (MS) and proton magnetic resonance (PMR)—practical application for the use of GC in food flavours separation and detection have been made to follow autoxidation of potato granules (Buttery 1961), storage of fruits and vegetables (Buttery and Teranishi 1961, Teranishi and Buttery 1962, Teranishi et al., 1962), ripening of bananas (Mc Carthy et al., 1963) According to Walsh and Merritt (1960) a simple rapid and general method for functional group classification has been developed which can be used any conventional gas chromatograph equipped with non destructive detectors e.g. thermal conductivity detectors, where functional groups like alcohols, aldehydes, ketones, esters and aromatic hydrocarbons. Could be detected. Also direct coupling of GC–TIC was found to be effective
for flavours identification (Casu and Cavallotti 1962, Janak 1963, Kaiser 1964, and Nano et al., 1965). Anon (1962, 1963) reported that infrared spectra can be used as good tools for identification of compounds in food flavours relying on finger-print and functional groups zones. Teranishi et al. (1967). The application of the GC-MS combination is a major advance in the analysis of the volatiles contributing to the aroma of foods. A large number of compounds can be easily identified with a good degree of certainty with sub-microgram quantities. Unknown compounds or mixtures are indicated to guide subsequent work. Thus, the GC–MS method promises to yield a wealth of information very difficult or impassible to obtain previously in aroma research. Stone et al. (1965) pointed out that, the properties of the stimulus and the uniqueness of alfactory perception have motivated some workers to devise instruments, for the detection of odours several assemblies for alfactometry were devised never the less all relied on dilution of odour and observing the response of individuals. The most well known physical and chemical method is gas liquid partition chromatography with flame ionization and electron capture detections. Webb and Kepner (1962) Found 23 components, mainly esters and alcohol, definitely present, and 5 others probable esters present in the volatile aroma of flor sherry. More than 30
volatile components of coffee were identified by Zlatkis and Sivetz (1960). Twenty-one volatile carbonyl compounds were identified or tentatively identified in cooked chicken by Pippen and Nonaka (1960). In cabbage the volatile sulfur compounds alone were found to number 20 (Bailey et al., 1961). No less than 50 components were revealed Bernhard (1961), as volatile constituents of certain California oranges, and Walford et al. (1963). Found 40-50 individual flavour and aroma components in Florida oranges. Jennings and Wrolstad (1961). Found at least 30 volatile components in black pepper. Using some of the most advanced equipment, Teranishi et al. (1963). Found over 150 volatile constituents in strawberries. Gold and Wilson (1963). Working with celery, have so far found and identified 38 volatile compounds. Dairy butter was found by Winter et al. (1963) to contain at least 12 volatile carbonyl compounds. Many of the volatile constituents in foods may be in amounts for below human detection and due to a complex mixture of components which are individually undetectable (Singleton and Ough 1962). Other working on the effects of subthreshold mixtures have reached similar conclusions (Nawar and Fagerson, 1962; Guadagni et al., 1963b).

Monerieff (1961) tried to use adsorption technique in olfaction with simple polymer films such as polyvinyl chloride, cellulose,
acetate, regenerated cellulose, calcium alginate, casein, and peanut protein. He reported that his trials were of limited application. Hartman (1954) studied vegetables flavours use of sensitive bridge circuit with a microelectrode that could respond to odours compounds. Several other physical methods of measuring odorous compounds have been developed. Borsanyi et al (1963), Borsanyi and Blanchard (1962), and Moncrieff (1963) have suggested the use of psychogalvanic skin response (PGSR) coupled with odour presentation.

CHAPTER THREE
MATERIALS AND METHODS

3-1-Materials

Fresh pepper mint herb, Cinnamon bark spice and sweets were purchased from the different market.

3.2. Methods

3.2.1. Extraction of volatile oils:

The volatile oils of peppermint and cinnamon were obtained by steam distillation method as described by Stahl (1969).

Prior to distillation each sample was cleaned and cut into small pieces. About 300g of peppermint and 285g of cinnamon were
introduced separately into 3 litre round bottom flask with ½ litre of distilled water distillation was carried out for about four hours. The apparatus was allowed to cool down to room temperature. The oil was collected and dried over an hydrousodiumsulphate then kept in bottle under refrigeration until analysis.

3-2-2 Extraction of volatile oils from sweets:

The volatile oils from menthol flavoured and cinnamon flavoured sweets, samples C and F respectively were obtained by a modification method for the distillation technique (Stahl, 1969). About 18g of each sample were weighed and placed separately into 250 ml flask, 50ml of distilled water were added and shaken for about 15 minutes, then 1 ml of HCl was added. The mixture was placed on water bath at 65° for one hour, filtered using filter paper No 12.

After that about 10 ml of toluene was added, then shaken, and two layers were formed using separately Funnel the upper layer was collected which contained volatile oil, this layer was kept in a bottle under refrigeration until analysis.

3.2.3. Determination of the oil contents:

The percentage of volatile oil in the pepper mint was determined by steam distillation method using karlsurhe apparatus as described by Stahl (1969).
Fig. (1) Karlsruhe apparatus
100 g of the peppermint were accurately weighed introduced into 1Litre distilling flask, and 200 ml of distilled water were added. The distillate was received in a specially constructed receiver where 1ml of toluene solvent was added. Distillation was continued for four hours after boiling until no further increase of the oil volume was observed.

3.2.4. Determination of volatile oil from cinnamon:

The percentage of volatile oil in the cinnamon was determined by steam distillation method Stahl (1969). 95g of sample were weighed introduce into 1 litre distilling flask and 200 ml of distilled water were added. The distillate was received in a specially constructed receiver. Distillation was contained for four hours after boiling until no further increase of the oil volume was observed.

3.2.5. Chromatographic Technique:

3.2.5.1. Thin-layer chromatographic.

The chromatography was done according to the method of Stahl (1969).

3.2.5.1.1. Preparation of the plate:

A 30g of silica gel G60 were shaken for 2 minutes with 60 ml distillated water in a 250 ml Stoppered conical flask. The slurry was Spread using spreader making 0.25mm thick layer on 5 glass plates 20 × 20cm. The coated plates were allowed to dry at room temperature
then activated at 105°C for one hour. The hot plates were stored and allowed to cool down in a desiccators over blue gel.

3.2.5.1.2. Stationary phase waste:

Silica gel G60.

3.2.5.1.3. Method of development:

One dimensional ascending development at chamber saturation was used.

Mobile phase – for menthol.
Solvent (1) Benzene: methanol 19:1.
Solvent (2) Toluene: ethylacetate.

Mobile phase for cinnamon.

(1) Benzene

(2) Benzene : ethyl acetate : acetic acid 18:1:1.

(3) Chloroform.

3.2.5.1.4. Application of sample:

The test sample to be separated was applied by means of capillary tubes 10ul.

3.2.5.1.5. Preparation of locating reagents:

The following locating reagents were used.

a- Ansialdehyde sulphuric acid:
The reagent was freshly prepared as a solution of 0.5ml anisaldehyde in 50 ml acetic acid and 1 ml conc sulphuric acid. (Lisboa 1964. Stahl 1961).

b- Vanillin Sulphuric acid:
1g vanillin was dissolved in 100 ml concentrated sulphuric acid (Tyihak et al, 1963).

3.2.5.1.6. Visualisation

The chromatograms were initially examined under the long wave of ultraviolet (366nm) after being freed of solvent. The chromatograms were then sprayed with suitable reagent and heated at 105°C until the spots attain maximum colour intensity the RF-values of developed compounds was calculated as follow:

\[ R_f = \frac{\text{Distance moved by spot for component}}{\text{Distance moved by solvent}} \]

3.2.6. Gas chromatography:

Equipment: gas chromatograph. 6890 series Agilent

Column: Capillary column 60m X 250\(\mu\)m.

Stationary phase: Capillary column 60m.

Detector: Flame Ionization detector (FID).

Carrier gas: He.

Flow rate: 30ml /1 min.
Combustion mixture: Hydrogen and oxygen (flaw rate 60ml/min).

Oven temperature: Programmed 60 – 130° C (Rate 10° C /min).

60° C for 5 minutes
90° C for 10 minutes
120° C for 15 minutes
130° C for 45 minutes

Injector temperature: 180° C.

Detector temperature 200° C

Computing integrator: GC 6890 series Agilent

Chart speed: 1cm/min.

Injection: Manually.

Injection volume: 0.5 ml.

Concentration of standard: 0.05%.
CHAPTER FOUR
RESULTS AND DISCUSSION

4.1. Yield of volatile oils:

The percentage yield was 0.004 of volatile from fresh peppermint herb and 0.003 of volatile oil from cinnamon bark spice.

4.2. Separation of peppermint volatile oils on silica gel by TLC:

Various solvent system commonly applied for the resolution of volatile oils were tested to obtain the optimum system for separation of the oils of fresh peppermint herb. The following solvent systems were found to give acceptable separation on TLC.

S -I Tolune: Ethyl acetate 93 + 7.
S-II Benzene: Methanol 95 + 5.

The solvent system S-I (Tolune: ethyl acetate) gave a good separation for the components of the extracts. Table (1) and Plate No (1) showed that sample A (extract of volatile oil from peppermint) gave four spots on TLC indicating presence of four components in the volatile oil in the extract.

The Rf values for the components in that solvent system were found to be 0.147, 0.223, 0.279, 0.412 with brown, blue, brown and violet colours respectively when the plate was sprayed with anisaldehyde– sulphuric acid reagent. The standard methanol is
sample B had Rf value 0.229 appeared as blue colour when treated with anisaldehyde–sulphuric acid spray reagent. The blue spot with Rf value 0.223 for sample A identified as menthol. Sample C (extract oil volatile from flavoured sweet) did not give spot on TLC but, when resolved this sample by gas chromatographic analysis gave two peaks perhaps to lower concentration of volatile oil extract from sample.
Table (2). Components of peppermint volatile oil extract and flavoured sweet on TLC using Toluene:ethyl acetate as solvent

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spot No.</th>
<th>Rf value</th>
<th>Colour reaction with spray reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>0.147</td>
<td>Brown</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.223</td>
<td>Blue</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.279</td>
<td>Brown</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.412</td>
<td>Violet</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>0.227</td>
<td>Blue</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Where:

A: extract of volatile oil from peppermint herb.

B: standard.

C: extract of volatile oil from flavoured sweet.
Plate 1. Thin layer chromatogram of pepper mint volatile oils using toluene ethyl acetate as solvent

A: Extract of volatile oil from peppermint
B: Standard
C: Extract of volatile oil from flavoured sweet
Plate No (2) and table (2) demonstrate the separation of the same samples in plate No (1) except that the solvent system S-II (Benzene methanol 95 + 5) was used instead. The spots were found to be more than those of solvent S-I, indicating that solvent S-II was better resolving than solvent S-I. This solvent system revealed four components in sample A (extract of volatile oil from peppermint). The Rf values for the components were found to be (0.117, 0.161, 0.205, 0.412) and appeared as brown, blue, brown violet colours respectively when treated with anisaldehyde-sulphuric acid spray with anisolaldehyde-sulphuric acid spray reagents. Furthermore table (2) shows that the standard menthol in sample B had Rf value 0.161 and appeared as blue colour when sprayed with reagent. The blue spot which have Rf value 0.161 for sample A identified as menthol. Sample C (extract of volatile oil from flavoured sweet) did not gave spots on TLC but when this sample was resolved by gas chromatographic analysis give result indicating lower concentration of volatile oil in this sample.
Table (3). Components of peppermint oil extract and flavoured sweet on TLC using Benzene: methanol as solvent.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spot No.</th>
<th>Rf value</th>
<th>Colour reaction with spray reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>0.117</td>
<td>Brown</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.161</td>
<td>Blue</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.205</td>
<td>Brown</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.412</td>
<td>Violet</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>0.161</td>
<td>Blue</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Where:

A: extract of volatile oil from peppermint.

B: standard.

C: extract of volatile oil from flavoured sweet.
Plate 2. Thin layer chromatogram of pepper mint volatile oils using benzene methanol as solvent

A: Extract of volatile oil from peppermint
B: Standard
C: Extract of volatile oil from flavoured sweet
4.3. Components of cinnamon bark Spice Separation of volatile oil extract on TLC:

The following solvent systems were found to give fairly good separation for the volatile oils. Solvent S-I chloroform. Solvent S-II Benzene: ethyl acetate: acetic acid 19: 1: 1 Solvent S-III: Benzene.

The solvent system S-I (Chloroform) gave good separation for components of the extract. It can be inferred from table (3) and plate No (3) that sample D (extract of volatile oil from cinnamon bark spice) gave three spots and E (extract of volatile oil from flavour sweets) gave one spot on TLC plates.

All spots under UV 366nm were observed as gray spots. Component D₁ (Rf0.46) appeared as pink spot while D₂ (Rf 0.32) was violet and D₃ (Rf 0.078) was gray and components E (Rf 0.071) appeared as gray spot when the plate was sprayed with vanillin-sulphuric acid spray reagent:
Table (4). Components of volatile oils from cinnamon bark spice and flavoured sweet on TLC using chloroform as solvent.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spot No.</th>
<th>Rf value</th>
<th>Colour reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>UV 366 nm</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>0.456</td>
<td>Grey</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.320</td>
<td>Grey</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.078</td>
<td>Grey</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>0.071</td>
<td>Grey</td>
</tr>
</tbody>
</table>

Where:

D: extract of volatile oil from cinnamon bark spice.

E: extract of volatile oil from flavoured sweet.
Plate 3. Thin layer chromatogram of cinnamon bark spice volatile oil using chloroform as solvent

D: extract of volatile oil from cinnamon bark spice.
E: extract of volatile oil from flavoured sweet
Plate No (4) and table (4) demonstrate the separation of samples D and E used for these separation in plate No (3) excepted that the solvent system S-II (Benzene : ethylacetate : acetic acid 19: 1: 1) was used instead. More spots were obtained indicating that the solvent system S-II was better resolving than. Solvent S-I. This solvent system revealed four components in sample D (extract of volatile oil from cinnamon bark spice) and three components in sample E (extract of volatile oil from flavoured sweet) in both samples appeared as gray fluorescent under UV 366 nm. When the plates were sprayed with vanillin – sulphuric acid reagent the spots which have Rf values 0.47, 0.40, 0.36, 0.13 for sample D appeared as violet, pink, orange and gray colour respectively where the spots with Rf values 0.45, 0.4, 0.014 for sample E appeared as gray, yellow and violet colour respectively.
Table (5): Components of volatile oils from cinnamon bark spice and flavoured sweets on TLC using Benzene: ethylacetate: acetic acid as solvent system SII

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spot No</th>
<th>Rf value</th>
<th>Colour reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>1</td>
<td>0.47</td>
<td>Gray Violet</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.40</td>
<td>Gray Pink</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.36</td>
<td>Gray Orange</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.13</td>
<td>Gray Gray</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>0.45</td>
<td>Gray Gray</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.40</td>
<td>Gray Yellow</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.014</td>
<td>Gray Violet</td>
</tr>
</tbody>
</table>

Where:

D: Extract of volatile oil from cinnamon bark spice.

E: Extract of volatile oil from flavoured sweets.
Plate 4. Thin layer chromatogram of cinnamon bark spice volatile oil using benzene ethyl acetate acetic acid as solvent

D: extract of volatile oil from cinnamon bark spice.
E: extract of volatile oil from flavoured sweet
Plate No. (5) and table (5) shows separation of the same samples D and E on TLC excepted that the solvent system S-III (Benzene) was used instead. The spots were found to be more than those obtained when solvent S-I and S-II were used, indicating that the solvent S-III was best resolving than solvent S-I and S-II. This solvent system revealed five spots in sample D and four spots for sample E. All the components in samples D and E appeared as gray spots under UV 366nm. The spots which have Rf values 0.059, 0.089, 0.33 for sample D and Rf value 0.35 for sample E appeared as gray colour when treated with vanillin–sulphuric acid spray reagent.

The spot which had Rf value 0.3 for sample E appeared as yellow colour when the plate was sprayed with vanillin–sulphuric acid reagent, while the spots which had Rf value 0.24, 0.4 in samples D and E respectively gave violet colour after being sprayed with vanillin–sulphuric acid spray reagent.
Table (6): Components of volatile oils from cinnamon bark spice and flavoured sweets on TLC using benzene as solvent

<table>
<thead>
<tr>
<th>sample</th>
<th>Spot No</th>
<th>Rf value</th>
<th>Colour reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>uv 366nm</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>0.059</td>
<td>Gray</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.089</td>
<td>Gray</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.24</td>
<td>Gray</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.33</td>
<td>Gray</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.48</td>
<td>Gray</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>0.124</td>
<td>Gray</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.30</td>
<td>Gray</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.35</td>
<td>Gray</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.40</td>
<td>Gray</td>
</tr>
</tbody>
</table>

Where:

D: Extract of volatile oil from cinnamon bark spice.

E: Extract of volatile oil from flavoured sweets.
Plate 5. Thin layer chromatogram of cinnamon bark spice volatile oil using benzene as solvent

D: extract of volatile oil from cinnamon bark spice.
E: extract of volatile oil from flavoured sweet
4.4. Gas chromatographic analysis of peppermint herb extract and flavoured sweet volatile oils:

The gas chromatographic (GC) scanning for the volatile oils extracted from peppermint and flavoured sweet are displayed in figures 2 and 3 and summarized in table (6). The results were studied against the reference compound menthol. Fig. (2) shows gas chromatogram for the reference compound with retention time (Rt = 52.961).

Fig (3) shows gas chromatogram for the volatile oil extracted from peppermint. It can be inferred from table (6) that the sample A (extract of volatile oil from peppermint herb) gave one peak at retention time (Rt = 46.913) which could be related to the reference compound used.

The chromatogram Fig. (4) shows a gas chromatographic separation of volatile oil from flavoured sweet. The sample C (extract of volatile oil from flavoured sweet) gave two peaks. The first peak at retention time (Rt = 57.833) was similar to reference compound. Second peak at retention time (Rt = 64.990) which remained unidentified. This sample (C) gave result after made hydrolysis.
Table (7) Gas chromatographic analysis of volatile oils from peppermint and flavoured sweet

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak No.</th>
<th>Rt</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>46.913</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>52.961</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>57.833</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>64.990</td>
</tr>
</tbody>
</table>

A: Extract of volatile oil from peppermint herb.
B: Reference compound (menthol).
C: Extract of volatile oil from flavoured sweet.
Fig. 2. Gas chromatogram of the reference compound (methanol)
Fig. 3. Gas chromatogram of volatile oil of peppermint
Fig. 4. Gas chromatogram of volatile oil of flavoured sweet
CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusion:

The work presented in this thesis covers extraction and identification of aromatic components of volatile oils from peppermint herb, cinnamon bark spice and flavoured sweets.

The plant material was extracted by steam distillation which showed variation for volatile oils content for the different samples, the percentage of volatile oil was 0.0042 and 0.0035 for peppermint and cinnamon bark spice respectively.

Thin-layer chromatography examination revealed that the extract of volatile oil from peppermint contained menthol which was identified against standard. The best solvent systems for separation were found to be Toluene: Ethyl acetate (93+7) and benzene: methanol (95+5). Anisaldehyde –sulphuric acid reagent was found to be reliable locating reagent for menthol.

The TLC examination for volatile oils from cinnamon bark spice and flavoured sweet revealed that both samples contain cinnamaldehyde. The acceptable solvent systems for separation were found to be chloroform, Benzene: Ethylacetate: acetic acid (19:
1: 1) and Benzene. Vanillin sulphuric acid reagent was found to be reliable locating reagent.

Gas chromatography determination for the volatile oils from peppermint herb and flavoured sweet revealed that both samples contained similar compounds. These compounds were identified according to their retention time using standard (menthol).
5.2. RECOMMENDATIONS:

The following can be inferred as recommendations:

1. Steam distillation is quite satisfactory for extraction of flavourings from food materials.

2. Thin-layer chromatography can be taken as a reliable technique for separation and identification of aromatic flavouring compounds.

3. The flavourings in food materials are recommended to be hydrolysed before identification since some may be in abound form.

4. Anisaldehyde – sulphuric acid reagent is recommended for menthol and its derivatives while vanillin – sulphuric acid is suitable for cinnamoldehyde identification.

5. The best recommended means of analysis for flavouring compounds is GC specially for quantitative analysis.
REFERENCES


International Food biotechnology Council (IFBC). (1990). Biotechnologies and food: assuring the safety of foods


spectral identification of the more volatile components. J. Food Sci. 28, 478.


