بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Identification of Some Carcinogenic Compounds in Nicotiana rustica (Toombak) leaves

التعرف على بعض المركبات المسرطنة في أوراق النيكوتينا روستكا (التمباك)

A Thesis submitted in partial fulfillment for the requirements of master degree in chemistry

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الإستهلال

قال تعالى:

{ اللَّهُ نُورُ السَّمَاوَاتِ وَالأَرْضِ مَثَلُ نُورِهِ كَمِشْكَاةٍ فِيهَا مِصْبَاحٌ الْمِصْبَاحُ فِي زُجَاجَةٍ الزُّجَاجَةُ كَأَنَّهَا كَوْكَبٌ دُرِّيٌّ يُوقَدُ مِن شَجَرَةٍ مُّبَارَكَةٍ زَيْتُونَةٍ لّا شَرْقِيَّةٍ وَلا غَرْبِيَّةٍ يَكَادُ زَيْتُهَا يُضِيءُ وَلَوْ لَمْ تَمْسَسْهُ نَارٌ نُّورٌ عَلَى نُورٍ يَهْدِي اللَّهُ لِنُورِهِ مَن يَشَاء وَيَضْرِبُ اللَّهُ الأَمْثَالَ لِلنَّاسِ وَاللَّهُ بِكُلِّ شَيْءٍ عَلِيمٌ }

صدق الله العظيم

(سورة النور:الآية35)

Dedication

To ,…..

My Family,……

My Husband.

Acknowledgments

First would like to thanks Allah to get for giving me the strength and health to complete this research.

I would like to thank my Supervisor Prof. Elmugdad Ahmed Ali for his valuable efforts throughout all the steps of preparation of this dissertation .

ABSTRACT

Tobacco used for manufacturing of toombak is species of Nicotiana rustica leaves. Tobacco (Nicotiana rustica) contains carcinogenic N –nitrosamines. these are formed by N-nitrosation of the major tobacco alkaloids(nicotine and nor nicotine) during curing and fermentation of tobacco .

Among the N–nitrosamines formed during curing (processing) of tobacco : 4-(methylnitrosoamino)-1(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN) .

This study was conducted to Identify of carcinogenic N –nitrosamines (NNN and NNK) samples of Sudanese toombak were collected from five vendors in Bahri . **Separated funnel** was used to extract N-nitrosamines from the samples. **GC-MS Spectrometry** and **Thin-Layer Chromatography (TLC)** were used to detect and the identify N-nitrosamines extract. It was noted that, all the above carcinogenic compounds were detected in toombak .

صلختسملا .

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ا غنيتلا يزتعت)يئوزنكيالا اوالاور يئوزنكي . عنزاثأ زيولعجم غنيتلا رزنيمختور. نزم يزن غنبكرم

عايماز وررتيالا وكتملا عاثأ ولعجملال غبن :

4 - (methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN) .

أ نزير ه نتادلال حرنجت ة ى ن غنبكرم عايماز وررتيالا غزايجل نزم حغنبمتلا , نزجم نسمس

عاية م جئعس حغبمتلا ن ايدم رنحس . نزيح ا نزا مادخنتا فنزم خزصلأا كرختت غنبكرملا

نزم نزايجلا , انزبور مادختنزتا زغن **GC-MS Spectrometry** غنيادرغو تعمورركور نزونبلالا رلا نزنقي

(TLC) قــشك ة يو ختسم ص عايماز وررتيالا , يح ور د ه غنبكرملا ن حغبمتلا .
ل

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List of Abbreviations

TSNAs	Tobacco Specific N-nitrosamines
NNN	N-nitrosnornicotine
NAB	N-nitrosnoanabasine
NAT	N-nitrosnoanatabine
NDMA	N-nitrosodimethylamine
POB	pyridyloxobutylation
O6-mGua	O^6-methylguanine
O4-mTh	O^4-methylthymine
NNAL	4- (methylnitrosamino)-1 –(3-pyridyl)-1 – butanonl
Iso- NNAL	4- (methylnitrosamino)-4 –(3-pyridyl)-1 – butanonl
GC-MS	Gas Chromatography-Mass Spectrometry
TLC	Thin- Layer Chromatography
UV	Ultra Violet
TIC	Total Ion Chromatogram

Chapter One

Introduction and Literature review

1. Introduction and Literature review

1.1 Introduction

The oral use of smokeless tobacco either in the form of snuff as used in North America and Western Europe or mixed with lime and areca nut in the form of a betel quid as used in Asia has been unequivocally associated with human cancers, mainly of the oral cavity. However, little has been published about smokeless tobacco as it is used in Sudan and other African countries and any possible association with neoplastic diseases. In the Sudan the prevalent oral use of smokeless tobacco is in the form of snuff (saffa, toombak in local language), prepared from sun- dried tobacco leaves. The main tobacco species is Nicotiana rustica, the leaves of which are usually mixed with aqueous solution of Atron (sodium bicarbonate). An alternative base to slaked lime used in other parts of the world by tobacco chewers until saturated, the product left in a closed container for about 24 hr before use. A study at the River Nile state at the North of Sudan revealed that the prevalence of toombak dipping and cigarette smoking among men and women is in the range of 25-47% and 13-25% respectively. The use of snuff increases risk of cancer, also it is associated with oral mucosal lesions, including keratosis, oral precancer, leukoplakia, and other oral diseases such as gingival recession, dental caries. This interesting topic was chosen due to prevalence of toombak use in Sudan and its impact on health, social and economic situations (Idris et al., 1998).

1.1.1 Definition of Toombak:-

In the Sudan, snuff locally known as toombak was introduced approximately 400 years ago. It is always processed into loose moist form. Tobacco used for manufacturing of toombak is of species Nicotiana rustica. The fermented ground powder is mixed with an aqueous solution of sodium bicarbonate (Idris et al., 1998).

The commercial names for toombak include, El-sanf (of high quality) wad Amari (according to the person who was believed to have introduced it and Sultan El- kaif) the power to improve one's state of mind. Tobacco is
primarily consumed in the Sudan in two forms oral snuff and cigarettes. Tobacco species have high content levels of alkaloid (nicotine, anabasine, nornicotine) than Nicotina tabcum used for cigarettes (Idris et al., 1995) . a prime factor for the popularity of tobacco. Smokeless tobacco product (toombak) use in the Sudan, is widespread, especially in the northern, eastern and central parts (Idris et al., 1994). The use of toombak is particularly common among the Gaalen and Shiagia tribes who reside in these regions (EL -Besheir et al., 1989). So far only one study has estimated the prevalence of the use of tombak in the River Nile province in the north of Sudan (Iodris et al,. 1994) by 40% among adult male dip toombak including 9% who are also cigarette smokers among men aged 40 years or older. From many surveys performed randomly in the river Nile states to estimate the prevalence of tobacco use they found that among children and adolescents (47)was quite low (2%-1-2%) but there was an abrupt increase up to (25%) in late adolescence. Among the adult population aged 18 year and older the prevalence of toombak use (34%) and cigarette smoking (12%). which are significantly high than among females (2.5% and 0.9%respectively). The prevalence of toombak use

among the male population aged 18 years and older was significantly higher in rural than urban areas (35%to 24%), while cigarette smoking have a high prevalence in urban areas (18%to 12%). The highest rates of use were found in rural areas among the male population ages 30 years and older (Idris etal., 1998).

1.1.2 Botany of tobacco plant: -

The genus Nicotina is classified among the family Solanaceae which comprises about 100 species. The most famous species are largely cultivated in Virginia. USA Tobacco, Nicotina tobacum, Turkish tobacco and Nicotina rustica in Fig(1.1) (Broun and Massey 1929)

Figure(1.1) nicotiana rustica

Tobacco is believed to be native to tropical America and was cultivated and used by native inhabitants before the discovery of America. It is one of the few major contributions to civilization which the new world can claim. The first who used tobacco were the Indian of north and South

America and spread to other countries France 1556, England 1565and from these countries to different parts of the world (Hussain ,1984).

1.1.3 Family Solanaceae:-

Herbaceous or woody plant. Leaves are without stipules, alternate, simple flowers, hermaphrodites or very rarely unisexual. Usually actinomorphic, calyx 4.6 lobed persistent corolla monopetalous usually five lobed folder, contorted or valuate stamens inserted on the corolla lobes rarely two anther loculi par alley, ovary usually two lobular. The loculisometimes divided by a false septum style terminals .ovule very numerous exiles, Fruity capsule or berry (Andreus, 1951).

1.1.4 Nicotiana rustica:-

It is semi desert plant, grows in different areas in the Sudan but mainly in Darfour in the western region (Hiday - talla, 1983). the Herb is up to four feet high. Leaves pediculate ovate obtuse at the apex, sometimes subcordate at the base, up to one feet high long glandular pubescent.Flowers greenish yellow, in terminal subpaniculate. Racemes with or without bract. Capsule sub loose slight longer than the calyx (Broun and Massey, 1929).

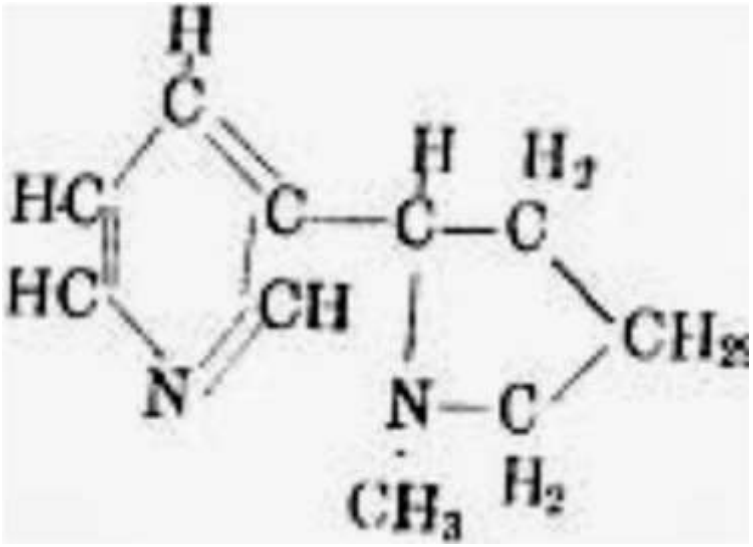
1.1.5 Chemical Composition of Tobacco:-

Natural tobacco contains at least 3050 different compounds (Roberts ,1988). Furthermore, smokeless tobacco may be enhanced by flavoring agents, added in the form of plant extracts and /or as chemicals (Roberts, 1988). Among 23 tumorigenic agents in smokeless tobacco (Wynder et al,1967), are volatile aldehydes and N-nitrosamines,Nitrosamine acids,

lactones, poly nuclear aromatic hydrocarbons pyrine,primanly benzo, and carbomates ,certain metals and the emitters, polonium-210 and uranium -235 and -238. The most abundant strong carcinogenic compounds in smokelesstobacco are the tobacco-specific N-nitrosamines (TSNA).these are formed by N-nitrosation of the major habituating tobacco alkaloid nicotine, and of minor nicotiana alkaloids during tobacco harvesting, curing fermentation and ageing.Seven N-nitrosamines(TSNA) have been identified in smokeless tobacco(Hecht et al.,1988).

Nnitrosonornicotine(NNN) and 4- (methynitrosamine) -1-(3-pyridyl-1-butanone (NNK) are the predominant carcinogens in smokeless tobaccos. Sudanese toombakcontains high concentration of TSNA, due to the use of N. rustica in its preparation. NNN and NNK levels in N. rustica leaves have been reported to be much higher than N. tobacum (Bhide et al., 1987).The active ingredient in tobacco is alkaloids of naturally occurring compound containing nitrogen and having the properties of an amine base, they have dramatic effects on the human system (Hommond, 1962). It was first isolated from the genus nicotina leaves in 1828 (Pavia et al., 1976), nicotine is a colorless oily liquid alkaloid, and it is considered as one of the most toxic drugs known to human, a dose of 60 mg is lethal in a few minutes (Pavaia et al,. 1976)Hussain, (1984) reported that nicotine constitutes 0,9 to 3,8% of Nicotiana. tobacum and between 7-12% of Nicotiana rustica. Nicotine is an organic compound, an alkaloid naturally contained in the tobacco plant. Although present throughout all the plant, it can be found in particularly high concentrations in the leaves, which contain 0.35% by dry weight. Nicotine is a mind-altering substance Liquid in Its pure state, it turns brown in contact with air. It is a powerful neural

poison. In low concentrations, the substance acts as a stimulant and is the main factor responsible for the dependence-forming properties of tobacco smoking. Nicotine molecule Fig(1.2) in small doses, nicotine is a stimulant: it increases activity, concentration and memory. It also increases heart rate and blood pressure and reduces appetite. In high doses it causes nausea and vomiting.



Figure(1.2) Structural formula of nicotine

1.1.6 Processing of Sudanese Toombak:-

Tobacco leaves, after cutting the trees, are dried in a big basket fermented and the color changes from yellow to brown after the fermentation process. The leaves were then milled to different particles size using an electric mill . this is mainly related to consumer taste consideration. Since in Eastern part of Sudan people prefer the coarse product while in Khartoum and central region, they prefer the fine or powdered product. The milling process is done in the same areas of

cultivation in Sudan. Most of milling machines are centered in El fashir town, Darfur province. Processing of toombak for sale is usually, carried out manually entombed shops by toombak vendors. It is performed by preparing four parts of a coarse powder of dried toombak Fig(1.3) leaves in a bowl and in another the concentrate of Atron (sodium bicarbonate) (1:4 Atron and water) is gradually added in small amounts to the tobacco (Idris, 1992). While adding the solution, the product is vigorously mixed by both hands Fig(1.4) and concurrently tested by sensation of the fingers tips until it becomes moist and hardened. The output is then transferred to special air tight tin containers which are then covered firmly for about 2 hour thereafter the product becomes ready for sale or use. Before buying users generally ask for a bit to smell or taste, since the aroma and taste decide the quality rank of the product.

1.1.6.1 Natron (sodium bicarbonate):-

Natron or atron (sodium bicarbonate, also called sodium hydrogen carbonate), is a mineral rock with the chemical formula $Na_2H(CO_3)_2.2H_2O$. Its colour is grey to yellowish white, and is of alkaline pH. There is no information on either the history or reasons behind use of atron as an additive to toombak. It may be used to homogenise the leaves to a fine sticky form as atron is used in the Sudan to homogenise vegetables during cooking.Atron, opposed to lime in other parts of the world, is probably added to toombak for its alkaline ects. It has been shown that at high pH (11.0±11.8) nicotine is completely protonated and its rate of absorption is increased. Studies of nass, a type of snus used in the former which contains lime and has high pH (11± 11.8), have shown that when the

product is placed in the mouth, nicotine reaches the central nervous system very quickly. Thus, pH value in tobacco products can influence the absorption and thereby the extent of pharmacological activity of nicotine . Atron probably quickens absorption of nicotine from toombak to the central nervous system.

Figure(1.3) Diagram of Dry Sunff

Figure(1.4) Diagram of addition of Atron to toombak

Figure(1.5) Diagram of moist snuff

1.1.6.2 Fermentation of Tobacco:-

Fermentation and aging of tobacco are common in the production of tobacco used in cigars and smokeless tobacco (e.g., moist and dry snuff, toombak, taaba). During fermentation or aging, the tobacco takes on a more agreeable flavor. For manufactured products, fermentation can occur in a partially insulated tank, which, because of increased microbial activity, can reach high temperatures (up to 65°C). Fermentation of toombak, a cottage industry product, occurs in a closed container at 30 to 45ºC for a few weeks, then the tobacco is aged for a year(Di Giacomo M, 2007).

Tobacco fermentation involves chemical and biochemical changes (bacteria-mediated reactions) (Fisher , 2012). During fermentation, a portion of nitrate in fire-cured tobacco is converted to nitrite, which then reacts with alkaloids to produce TSNAs.

Chemical markers indicative of bacterial and fungal growth have been identified in tobacco of various types and at various stages of production (Larsson , 2008) . In tobacco or tobacco products, a number of bacteria including Bacillus, Enterobacter, Staphylococcus, Corynebacterium, Clostridium, Serratia, and Escherichia species have been identified that are capable of converting nitrate to nitrite (nitrate reduction Additionally, several genera of fungi, such as Cladosporium, Alternaria, Candida, Fusarium, Aspergillus, and Acremonium are capable of nitrate reduction. Throughout production, the combined capacity of product microorganisms to generate nitrite is a key determinant of the levels of TSNAs and other nitrosamines in the final product. During one fermentation study, nitrite levels generated by bacteria resulted in almost threefold increase in TSNA levels (Rubinstein, 2002).

Pasteurization, or heat-treating of tobacco, is a very effective means of eliminating microorganisms during production, and thus preventing the reduction of nitrate to nitrite.Indeed, Swedish snus, a pasteurized product, generally has lower nitrite and TSNA levels than nonpasteurized products, such as moist snuff and khaini. It has been shown that a further increase in nitrite and TSNA levels can be prevented by cleaning fermentation equipment before use and ―seeding‖ the fermentation process with non-

nitrate-reducing bacteria. Together, these observations provide additional support for the idea that the levels of some carcinogenic and toxic agents in

tobacco products can be substantially reduced by changing tobacco processing methods (Leffingwell, 1999).

1.1.7 Absorption of Nicotine :-

Nicotine absorption occurring at different parts of body chiefly in the mucosal tissue of mouth, respiratory tract, intestine and skin (Hussain, 1984). There are few studies that have directly examined the effects of pH on nicotine absorption, (Beckett et al., 1972) found very little buccal absorption of nicotine from tobacco when the pH was 10% absorption at pH 7, and about 30% at pH 9.0.(Henning et al.,1990) found that rinsing with acidic beverages such as coffee or cola before chewing nicotine polacrilex nearly eliminated nicotine absorption. These results indicate that pH is an important determinant of buccal absorption of nicotine (Benefits et al., 1988). compared nicotine absorption from a moist snuff to that from cigarette smoking and nicotine gum. The nicotine-dosing potential of moist snuff is determined by at least three factors: The amount of nicotine in the product, the pH level of the product, and the size of the tobacco cutting. (Henning et al., 1995) found that the nicotine content of six moist snuff products ranged from (7.5mg/g to 11.4mg/g) and that the pH of these products ranged from (6.9 to 8.6). The pH of the snuff is important because nicotine most readily crosses the oral mucosa in the unionized form. The degree to which nicotine is unionized depends on the higher pH levels (more alkaline). The rate of absorption is highest when the snuff is first placed in the mouth and plasma concentration continued to rise until the snuff was removed from the mouth. Absorption continued even after the snuff was removed, presumably because of the slow release of nicotine from the mucosa into the plasma or absorption of swallowed nicotine in the gut.

1.1.7.1 Metabolism of Nicotine in the Body:-

When nicotine is absorbed, immediately distributed into different parts of the body brain, lungs, liver, intestine, spinal cord and adrenal gland (Hussain, 1984). Liver is the site of breaking down of nicotine into harmless compounds which passed in urine with small amount of un metabolized nicotine.

1.1.8 Chemistry of Tobacco Spescific N_nitrosamines(TSNA):-

1.1.8.1 Definition of N_nitrosamines(TSNA):-

Nitrosamines are organic compounds with the chemical formula R_1NNOR_2, where R_1 and R_2 may be alkyl or aryl groups, or part of a ring. The term N-nitrosamines (NAs) is used for a wide variety of chemical substances of different molecular weights that appear as reaction products of amines (especially secondary) with nitrosating agents.

Most nitrosamines show carcinogenic potential, besides presenting teratogenic and mutagenic action, although not all are carcinogenic, and their carcinogenic potential depends on their molecular structure.

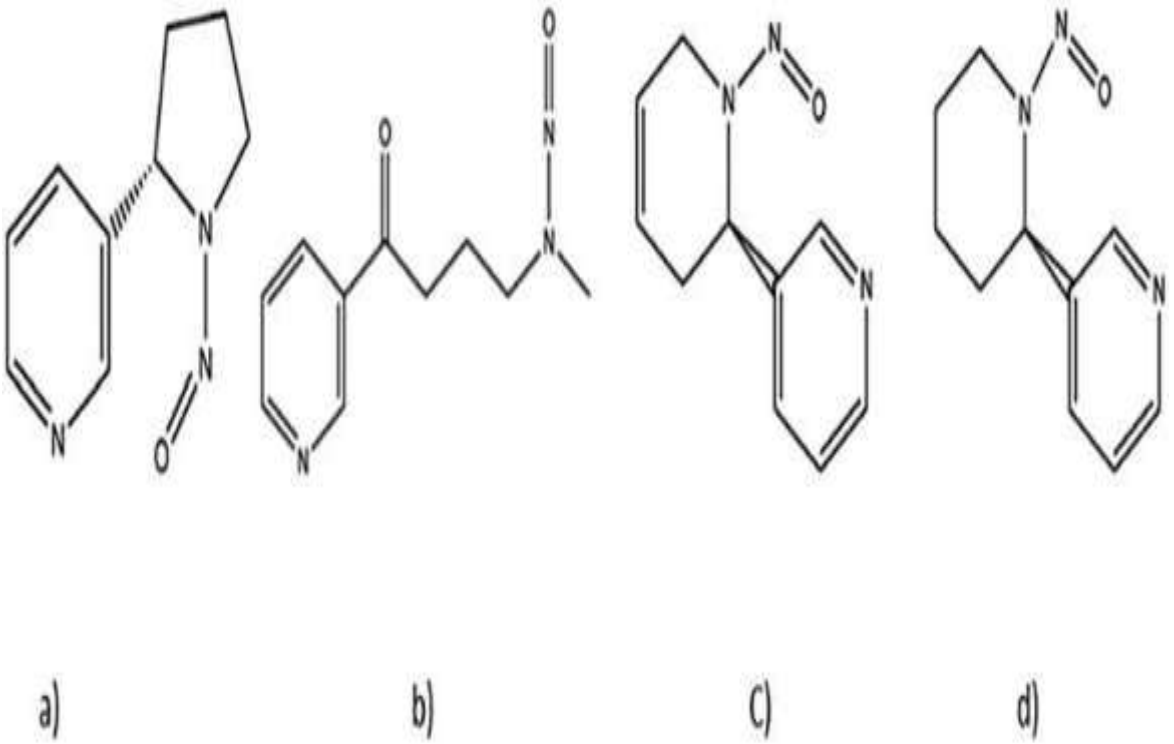
N-nitrosamines have become the subject of intensive toxicological studies from 1956, when Magee and Barnes frst reported the induction of tumors in the liver of rats fed with feed contaminated with N-nitrosodimethylamine (NDMA). `Since then, many studies have been conducted with experimental animals, seeking to evaluate the toxicological effects of this class of compounds.Tobacco is a matrix of special interest due to the fact that a greater incidence of several disabling diseases is attributed to smoking, such as bronchitis, lung emphysema, respiratory problems, arterial aneurysm and cancer. This caused the World Health

Organization (WHO) to indicate smoking as a risk factor for six of the eight leading causes of death worldwide.Seven nitrosamines have been identified in both tobacco and smoke·

Four tobacco-specific N-nitrosamines (TSNA), namely, 4-(methylnitrosamino)-1-(3pyridyl)-1-butanone (NNK),N'-nitrosonornicotine (NNN), N'-nitrosoanabasine (NAB) and N'-nitrosoanatabine (NAT) were considered by a previous Working Group in October 1984 (IARC, 1985).

Two (NNN andNNK) are potent carcinogens classified as human carcinogens by the International Agency for Research on Cancer.NNN, NNK and NAT levels are generally higher compared to other nitrosamines. /AKnowledge of the composition of the products present in tobacco is an important issue that has been widely studied by different researchers, in order to obtain information on cigarette chemistry and toxicity, and, currently, a trend is in place in the tobacco industry to develop products that produce lower amounts of nitrosamines.Thus, in this context, monitoring of nitrosamines is of paramount importance.

As TSNA concentrations are in the ng cigar range, extraction and pre-concentration steps are necessary prior to determination. In all studies regarding TSNA determination in tobacco, as well as in reference compendiumsTSNA extraction is conducted by leaching a mass of homogenized tobacco, usually with the aid of an extraction solution, such as ascorbic acid, acetic acid or sodium hydroxide.



Figure(1.6) Structural formula of (A) NNN, (B) NNK, (C) NAT and (D) NAB.

1.1.8.2 Formation of (TSNAs):-

TSNA are identified as the major carcinogens in tobacco. Generally, TSNA are not present in freshly harvested green leaf and they are formed during curing due to nitrosation of tobacco alkaloids. The nitrosating agent is nitrite derived from tobacco nitrate. Bacteria (Enterobacter*;* Agrobacterium radiobacter) and tobacco enzymes take part in the reduction of nitrate.

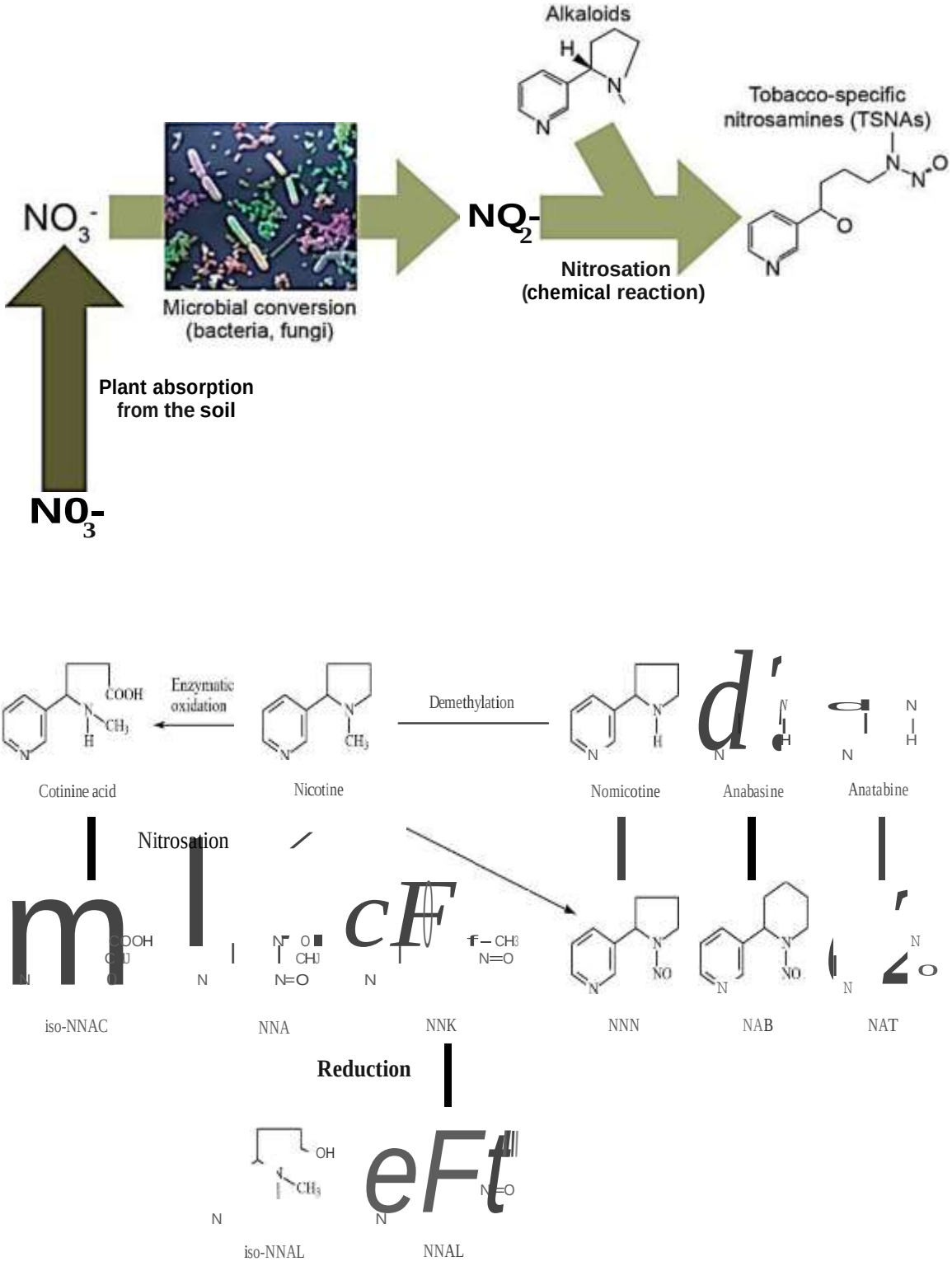


Figure (1.7) Formation of N-nitrosamines

1.1.8.3 Carcinogenicity of Tobacco Specific N_nitrosamines:-

Cellular DNA damage that is misrepaired or not repaired constitutes a necessary, although not sufficient prerequisite for induction of cancer. Thus, IARC (2007) underlines the important mechanistic role of DNA adducts derived from tobacco specific nitrosamines (TSNA) in the induction of cancer by smokeless tobacco products. The main purpose of this review is to examine the dose-response relationships between exposure to TSNA and formation of DNA and hemoglobin (Hb) adducts in the rodent, and to compute on basis of such data the adduct concentrations to be anticipated in humans exposed to tobacco. The expected adduct levels are then compared with those that have, actually, been found in the corresponding human tissues, while taking available epidemiological evidence into consideration.

NNK and NNN induce three types of crucial DNA damages: nucleotide methylations, pyridyloxobutylations(POB) as well as pyridylhydroxy butylations. In addition, DNA phosphate POB adducts have been identified (Haglund et al., 2002). 7-N-methylguanine (7-mGua) is the predominant adduct found in target tissues induced by NNK, followed by O^6-methylguanine (O6-mGua), whereas very low levels of O^4-methylthymine (O4-mTh) are present (Belinsky et al., 1986). Depending on the presence of activating enzymes, adduct formation from NNK show great variations between rodent tissues (Deilhaug et al., 1985; Belinsky et al., 1987a, 1988; Jansen et al., 1996).

The capacity of various DNA adducts to induce mutations and chromosomal aberrations vary extensively. O6-mGua appears to play a major role in lung tumorigenesis induced by TSNA in rodents (Belinsky et

al., 1987b, 1990; Peterson and Hecht, 1991; Upadhyaya et al., 2009), as well as in several types of human cancers (Margison et al., 2002).

Although the concentrations of O4-mTh induced by methylating agents in rat are more than one order of magnitude below those for O6-mGua (Den Engelse et al., 1986; Belinsky et al., 1986), they may contribute to a limited extent to the over all cancer risk from TSNA because of their mutagenic potential. On the other hand, after NNK treatment O6mGua persisted, while O4-mTh was removed rapidly in the lung, suggesting operation of different repair pathways (Belinsky et al., 1986).

1.1.8.3.1 Haemoglobin adducts:-

Haemoglobin adducts of NNK and NNN are formed upon reaction of a common intermediate, POB-DZH, with aspartate or glutamate in haemoglobin. In the case of NNK, POB-DZH is generated by CYP-mediated hydroxylation of the methyl group to give ⍺HOMeNNK. The POB-aspartate and -glutamate esters in haemoglobin can readily be hydrolysed by base treatment to release HPB, which can be quantified by gas chromatography‑mass spectrometry (Carmella et al., 1990a; Hecht, 1998). The presence of HPB-releasing haemoglobin adducts in humans provides strong evidence for the metabolic activation of NNK and/or NNN, although another possible source − nitrosation of myosmine − has been proposed (Wilp et al., 2002).

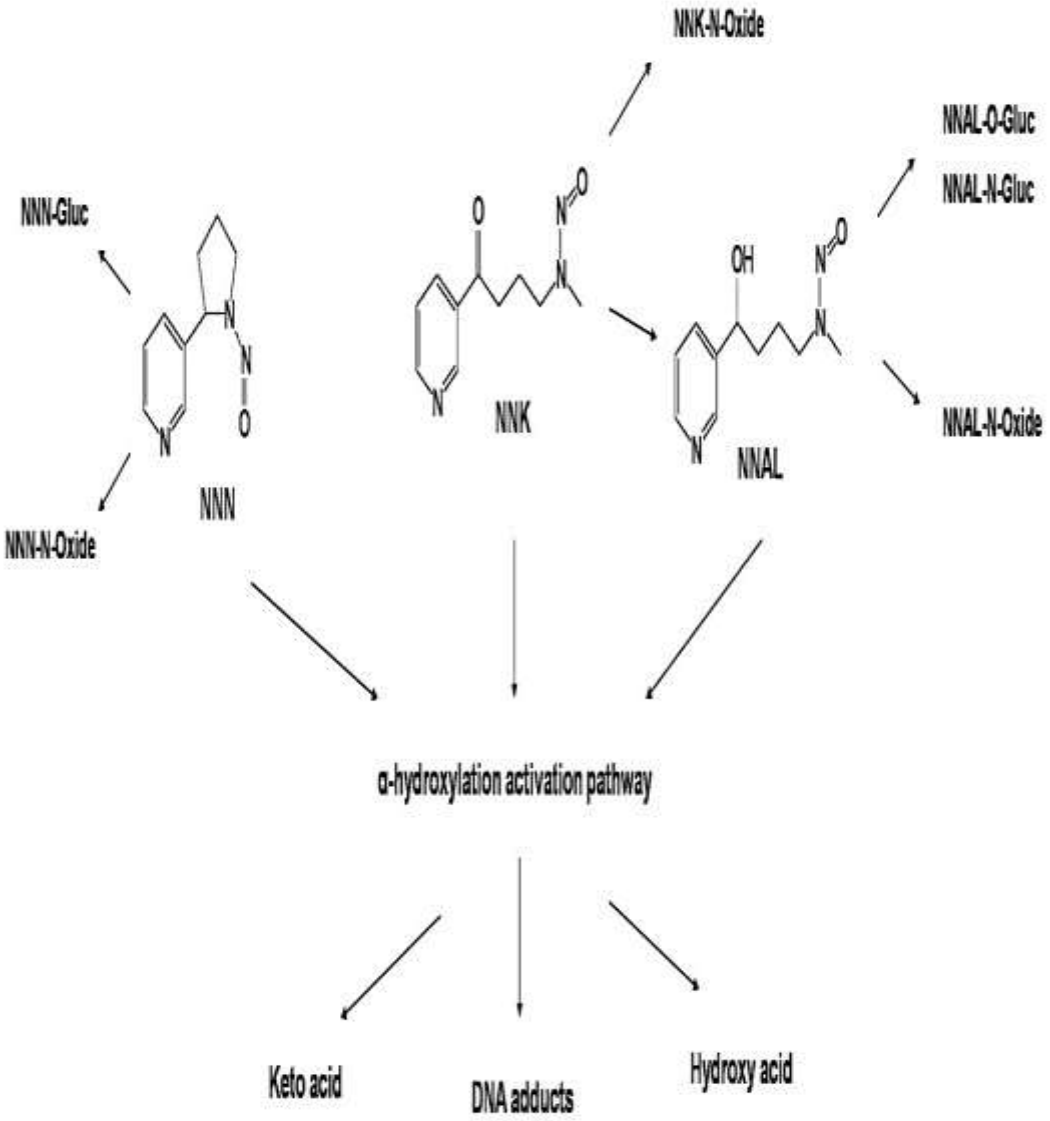
The highest levels of HPB-releasing haemoglobin adducts have been found in smokeless tobacco users. Mean levels (mol/g haemoglobin) were 517 in snuff-dippers, 236 in nasal snuff users and 148 in toombak users (Carmella et al., 1990a; Falter et al., 1994; Murphy et al., 1994). Lower levels were reported in smokers. Mean levels (fmol/g haemoglobin) in smokers

and nonsmokers in four studies were 79.6 and 29.3 (Carmella et al., 1990a), 54.7 and 26.7 (Branner et al., 1998), 61 and 34 (Falter et al., 1994) and 26 and 19 (Atawodi et al., 1998). Levels of HPB-releasing haemoglobin adducts were not high in nonsmokers exposed to secondhand tobacco smoke than in non-exposed nonsmokers (Branner et al., 1998).

1.1.8.4 Metabolism of Tobacco Specific Nitrosamines:-

Of all the TSNAs identified, NNK and NNN are the most prevalent strong carcinogens in tobacco products as documented by Hecht and Hoffmann, 1988, Spiegelhalder and Bartsch, 1996 and Hoffmann et al., 1995. Moreover, the International Agency for Research on Cancer, 2007 classifies NNK and NNN as the only TSNAs carcinogenic to humans. Thus, the discussion on the metabolism of TSNAs will be mainly focused on these two compounds.

The main routes of NNK and NNN metabolism are shown in Figure(8) below . NNK is rapidly metabolized in animals and humans via three main routes: carbonyl reduction, pyridine oxidation and α-hydroxylation (Hecht, 1998b). In humans, carbonyl reduction of NNK forms NNAL which is also a potent lung carcinogen having similar carcinogenicity and metabolic pathway as NNK. The pyridine-N-oxidation pathway results in the formation of NNK-N-oxide and NNAL-N-oxide. Metabolic activation of NNK and NNAL to DNA adducts proceeds via α-hydroxylation pathways. The end products of this pathway are keto acid and hydroxy acid. NNN metabolism is similar to NNK metabolism (Hecht, 1998b) and is depicted in Figure(1.8)



Figure(1.8) Metabolism of NNN and NNK

1.2 Literature Review:-

Tobacco-specific N-nitrosamines, including 4-(methylnitrosamino)-1-(3-pyridyl)-1butanone (NNK), N′-nitrosonornicotine (NNN), N′-nitrosoanabasine (NAB) and N′-nitrosoanatabine (NAT), occur widely in tobacco and tobacco smoke. They are formed by the nitrosation of nicotine and other tobacco alkaloids and have been detected in green tobacco leaves from Nicotiana tabacum and Nicotiana rustica species; however, the largest quantities of tobacco-specific N-nitrosamines are formed during tobacco curing and processing and additional amounts are formed during smoking. Tobacco-specific N-nitrosamines occur in all commercially and non-commercially prepared tobacco products including cigarettes, cigars, *bidis*, pipe tobacco and smokeless tobacco products. N- nitrosamines occur in a wide variety of both food and non-food products, but the amounts of tobacco-specific N-nitrosamines in all tobacco products exceed the levels of other *N*-nitrosamines in other commercial products by several orders of magnitude. The highest levels of tobacco-specific *N*-nitrosamines are measured in smokeless tobacco products. For example, levels of NNK up to 17.8 μg/g have been measured in North American and European smokeless tobacco products; up to 245 μg/g have been measured in products used in India; and up to 7870 μg/g have been measured in Sudanese toombak. Levels of NNN up to 135 μg/g have been measured in North American and European smokeless tobacco products; up to 1356 μg/g have been measured in products used in India; and up to 3085 μg/g have been measured in Sudanese toombak. These compounds are also present in secondhand tobacco smoke. The degree of exposure to tobacco-specific N-nitrosamines depends not only on the levels of these compounds

in tobacco products or smoke, but also on the manner in which the products are used.

Tobacco Specific Nitrosamines (TSNA) are identified as the major carcinogenic compounds in tobacco. In view of the increasing health consciousness, monitoring and reduction of this group of compounds is attracting attention of researchers all over the world today, more than ever before. Analysis of different types of tobacco produced under varying agro-ecological situations adopting different production practices in India has revealed that Burley tobacco in East Godavari district of Andhra Pradesh has higher levels of TSNA (6-6 to 9.3 ppm). The investigations carried out for developing suitable techniques to reduce the levels of these compounds in Burley tobacco are presented. A significant reduction in TSNA ranging from 11.1 to 28.2% was observed due to removal of midrib and subsequent air-curing. Further, highly significant differences were observed in the levels of TSNA among the treatments due to foliar spray of manganese sulphate 0.20% on a 150-day old Burley tobacco field crop and a significant reduction (50.2%) in TSNA could be achieved after 30 days after spray. Similarly, exogenous application of ascorbic acid 0.25% on the harvested Burley tobacco green leaves and subsequent air-curing resulted in highly significant reduction (46.5%) in TSNA at 30 days after spray.

Using gas chromatography with thermal energy analysis, the TSNA types; N-nitrosonornicotine (NNN), 4(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosoanatabine (NAT) and N-nitrosoanabasine (NAB) were quantified in toombak and saliva of toombak users(Idris AM 1991). Exceptionally high levels (mean; range, mg/g toombak/dry wt) of NNN (1.13; 0.50±3.08), NNK (2.31; 0.62±7.87; 31), NAT (0.08; 0.02±0.2) and NAB (0.22; 0.02±2.37) were found. Previously, the highest levels of

NNN and NNK in any oral tobacco found were 0.154 and 0.014 mg/g dry wt, respectively. Two additional TSNAs, 4-(methylnitrosamino)-1(3-pyridyl)-1-butanol (NNAL) and 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL) were found in the saliva of toombak users for the first time and were conformed by gas chromatography mass spectrum. Exposure to TSNAs has also been assessed by measuring the content in the saliva of 21 toombak users, where nine out of 10 subjects had detectable levels of TSNAs in their saliva before dipping (total TSNAs: 0.01±1.0 mg/ ml). During toombak use, TSNAs concentrations have reached g/ml levels (range: number of positive subjects) for NNN (0.6±1.2, 12/12), NAT (0.6±6.7, 2/12), NAB (0.05±1.9, 12/12), NNK (0.06±6.7, 8/12), NNAL (0.05± 3.3, 11/12) and iso-NNAL (0.07±0.4, 8/12). These high levels of TSNAs found in toombak were partially attributed to the use of tobacco Species, Nicotiana rustica, fermentation of toombak at elevated temperature, prolonged storage, and contamination during processing (Anderson RA1989).Levels of nicotine and TSNAs in snus and toombak were previously compared(Idris, 1994). tables (1.1) and(1.2) show quantitative data on nicotine and TSNAs in various snus brands and saliva of snus dippers and compare that with concentrations of nicotine and TSNAs in various samples of toombak and saliva of toombak dippers. These data clearly document that toombak contains concentrations of TSNAs 100-fold higher than those found in snus . The level of NNN and NNK in the saliva of toombak dippers is also significantly higher than those found in the saliva of snus dippers . It has been estimated that more than 80% of the carcinogenic TSNAs are extracted from the toombak by saliva and negative pressure during sucking of dipped toombak quid(Prokopczyk 1992). The concentrations of TSNAs in the saliva of

toombak dippers exceeded their concentration in a solution that produced mouth tumours in rats swabbed with this solution twice daily(Idris , 1992). Whereas epidemiological studies suggest low incidence of oral cancer in Sweden(Ostman ,1960 ,1989) .both high relative frequencies of oral cancer as well as increased risk of oral cancer development among toombak dippers in the Sudan have been reported(Idris 1970_1985). These data strongly support the widely held idea that TSNAs NNN and NNK play an important role in the carcinogenicity of toombak.

Table 1.1: Levels of the tobacco-specific N-nitrosamines in the saliva of snus and toombak dippers

Product	Year	No. of samples		TSNA (ng/ml saliva)		
			NNN	NNK	NAT	NAB
Snus	1988	4	3±140	nd±16	4±85	
Toombak	1991	12	582±21 000	63±6690	nd±471	46±1944
	1993	6	14.8±105.7	20±135	2.3±20.4	2.6±14.2

Table 1.2 :Prevalence of use of snus in Sweden and toombak in the Sudan

Survey	Year	Age (years)	No.	%
Snus University of Malmo	1970	15	20333	15.9
Toombak University of Kartoum	1992	52	2868	41.6
Toombak Toombak Research Centre and Oral Cancer Campaign, Khartoum	1998	17	3795	

A wide range of TSNA concentrations are found in cured tobacco, regardless of the type. In each category of tobacco type, the range reflects the diversity of the tobacco variety, production year, climate, country of origin, agricultural practices including fertilization, post-harvesting and curing technologies, post-curing handling and storage conditions, as well as the analytical methods used and the reporting of the analytical results (e.g. ng/g dry tobacco wt versus ng/g wet tobacco wt).

The levels of total TSNA are highest in air-cured Burley tobacco and lowest in sun cured Oriental (Turkish) tobacco. The highest levels of NNN were reported in Burley laminae and midribs (up to 8620 and 9080 ng/g dry tobacco, respectively). The highest reported concentrations of NNN were 1700 ng/g dry wt in flue-cured Bright tobacco and 420 ng/g dry wt in sun-cured Oriental tobacco. (MacKown et al ., 1988) also reported levels of NNN up to 3400 ng/g dry wt in reconstituted tobacco sheets that are used in cigarette blends.

The highest levels of NNK were reported in midribs of Burley tobacco (6660 ng/g dry tobacco) and laminae of Bright (Virginia) tobacco (2690 ng/g dry tobacco). It should be noted that levels of NNK in Burley midribs exceed those in the laminae (6600 versus 1370 ng/g dry wt). It should also be noted that NNK is a predominant TSNA in Bright tobacco (2690 ng NNK compared with 1370 ng NNN) while NNN is predominant in Burley tobacco (1370 ng NNK compared with 8620 ng NNN).

1.3 Techniques Used:-

1.3.1 GC-MS Spectrometry:-

GC-MS a combination of two different analytical techniques, Gas Chromatography (GC) and Mass Spectrometry (MS), were used to analyze complex organic and biochemical mixtures. GC can separate volatile and semi-volatile compounds with great resolution, but it cannot identify them. MS can provide detailed structural information on most compounds such that they can be exactly identified and quantified, but it cannot readily separate them. Therefore, it was not surprising that the combination of the two techniques was suggested shortly after the development of GC in the mid-1950's. Gaschromatography and mass spectrometry are, in many ways, highly compatible techniques. In both techniques, the sample is in the vapor phase, and both techniques deal with about the same amount of sample (typically less than 1 ng). This article was prepared with an aim to review different aspects GC-MS, such as principle, types, instrumentation and applications in food science.

1.3.1.1 Principle of GC-MS:-

GC-MS a combination of two different analytical techniques, Gas Chromatography (GC) and Mass Spectrometry (MS), is used to analyze complex organic and biochemical mixtures (Skoog et al., 2007). GC-MS instrument consists of two main components. The gas chromatography separates different compounds in the sample into pulses of pure chemicals based on their volatility (Oregon State University, 2012)by flowing an inert gas (mobile phase), which carries the sample, through a stationary phase fixed in the column (Skoog et al., 2007). Spectra of compounds are collected as they exit a chromatographic column by the mass spectrometer, which identifies and quantifies the chemicals according their mass-to-charge ratio (m/z). These spectra can then be stored on the computer and analyzed (Oregon State University, 2012)

1.3.2 Thin-Layer Chromatography:-

Thin ‑Layer Chromatography (TLC) is a method for analyzing mixtures by separating the mixture ,the identity of compounds,and the purity of a compound . by observing the appearance of a product Or the disappearance of a reactant ,it can also be used to monitor the progress of a reaction . TLC is a sensitive technique ‑microgram quantities can be analyzed by TLC ‑ and it takes little time for an analysis (about 5-10 minutes).

TLC consist of three steps ‑ spotting , development ,and visualization. First the sample to be analyzed is dissolved in a volatile(easily evaporated) solvent to produce a very dilute (about 1%) solution.

Spotting consist of using a micro pipet to transfer a small amount of this dilute solution to one end of a TLC plate , in this case a thin layer of powdered silica gel that has been coated onto a plastic sheet .The spotting solvent quickly evaporates and leaves behind a small spot of the material .

Development consist of placing the bottom of the TLC plate into a shallow pool of a development solvent, which then travels up the plate by

capillary action . As the solvent travels up the plate , it moves over the original spot.

Visualization of colored compounds is simple- the spots can be directly observed after development . because most compounds are colorless however , a visualization method is needed. The silica gel on the TLC plate is impregnated with a fluorescent material that glows under ultraviolet (UV) light . a spot will interfere with fluorescence and appear as a dark spot on a glowing background. While under the UV light , the spot can be outlined with a pencil to mark their locations.

1.4 Objectives:

- Extraction of N-nitrosamines from Nicotiana rustica leaves (Toombak)
- Chemical identity of the main carcinogenic N-nitrosamines (NNN and NNK) by GC-MS and TLC.

Chapter Two

Materials and Methods

2. Materials and Methods:-

2.1 Materials:-

2.1.1 Sampling:-

The sample represents five types of moist snuff (toombak) mixed to form the test sample according to the standard methods .

The coning and quartering method was used to first divide the sample into four segments , the diagonally opposite of which are rejected.quartering was continued until a suitable sample volume is achieved.

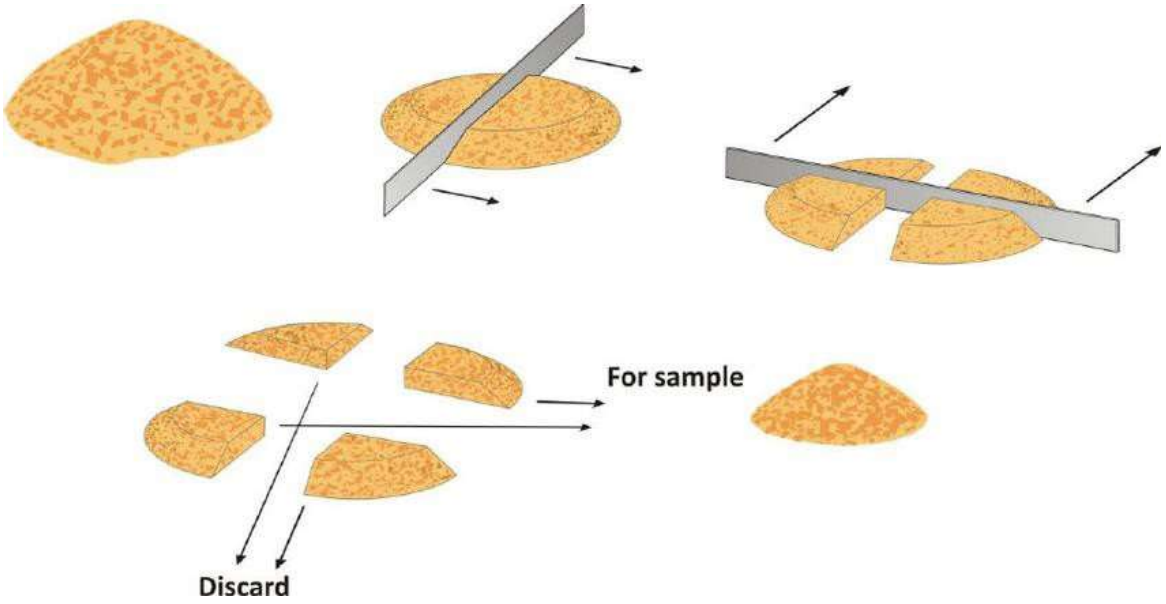


Fig (2.1)The coning and quartering method of sample collection

2.1.2 Chemicals:-

-Methanol (CH_3OH)-99.5% - Loba Chemie Pvt , Ltd ., India.

-Dichloromethan (CH_2Cl_2) - 99%- Loba Chemie Pvt , Ltd ., India.

-Sodium hydroxide (NaOH) -99.6%. Lab tech chemicals.

- Ethylacetate ($CH_3COOCH_2CH_3$) -99.5% - Loba Chemie Pvt , Ltd ., India.

- Chloroform ($CHCl_3$) - 99.5% - ALPHA CHEIKA -

India.

-Ammonia (NH_3) - 25% S D fine CHEM - Limited -

India.

2.1.3 Equipments:-

2.1.3.1 Separated Funnel:

Figure (2.2)Separated Funnel

2.1.4 Instruments:-

2.1.4.1 GC-MS Spectrometry:-

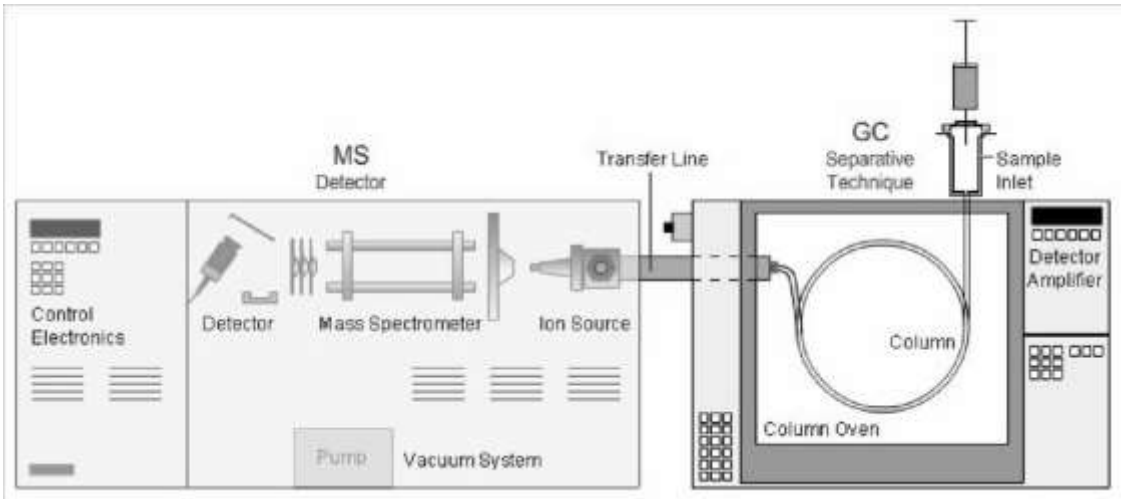


Figure (2.3) schematic Diagram of GC-MS

2.2 Methods:-

2.2.1 Extraction of TSNAs :-

The five Samples of moist snuff were mixed and transferred to a mortar with dry ice ,and homogenized with the aid of a pestle.20g portion of the homogenized snuff was transferred to separation funnel , followed by the addition of 150ml of dichloromethane and 20ml of sodium hydroxide (10% w/w) this mixture was vortexed three times (90s stirring , 20s interval), after the last stirring ,the mixture was allowed to stand until settling .The extract was removed and the eluted fraction was dried at room temperature for 24 hours .after solvent evaporation , the concentrated extract was dissolved in 10ml of methanol.

2.2.2 Method of analysis:-

2.2.2.1 GC-MS Method:-

The sample(liquid extract) was placed put in the cell of GC-MS spectrophotometer.

2.2.2.3 GC -MS Conditions:-

The qualitative and quantitative analysis of the sample was carried out by using GC-MS technique model (GC-MC-QP2010-Ultra) from japans Simadzu Company,with serial number 020525101565SA and capillary column (Rtx-5ms-30m×0.25mm ×0.25µm) The sample was injected by using split mode ,helium as the carrier gas was passed at flow rate of 1.61 ml/min ,the temperature program was started from 60c, with rate 10c/min to 300c as final temperature degree with 5 minutes hold time , the injection port temperature was 300c , the ion source temperature was 200c and the

interface temperature was 250c . the sample was analyzed by using scan mode in the range of m/z 40-50 charges to ratio and the total run time was 29minutes. Identification of components for the sample was achieved by comparing their retention index and mass fragmentation pattern with those available in the library , The National Institute of Standard and Technology (NIST). ,results were recorded.

2.2.2.2 TLC Method:-

Table 2.1 : shows the types of solvent system used for TLC process

Sample	**Solvent System**	**Ratio (V/V)**
NNN	Chloroform: Methanol : Ammonia	6: 5 : 1
NNK	Chloroform : Methanol	14: 1

Plates were marked at bottom and top for spotting sample ,to stop running of solvent beyond end of the slides marking done also at the top.

Capillary tube dipped into respective test solution so that solution rises up in the tube. Prepared plate where briefly touched by the capillary tube at the start line so that the test solution get absorbed. Plates were placed at the developing chamber containing solvent of interest after sometime solvent front reached at the top near the marked line and hence plate was taken out and dried to evaporate the solvent at the stationary phase .

Spots were observed after development by placing TLC plate under ultraviolet(UV) light .while under the UV light ,the spots were outlined with a pencil to mark their locations .

Chapter Three

Results and Discussion

3. Results and Discussion:-

3.1 GC-MS analysis of Toombak(Nicotiana rustica):-

The extract of sample was analyzed by GC- MS and the total ion chromatogram revealed the presence of 36 constituents.Different constituents were quantified and identified by their retention times and mass spectra. extract constituents are presented in Table (3.1) . Total ions chromatogram is displayed in Fig (3.1).

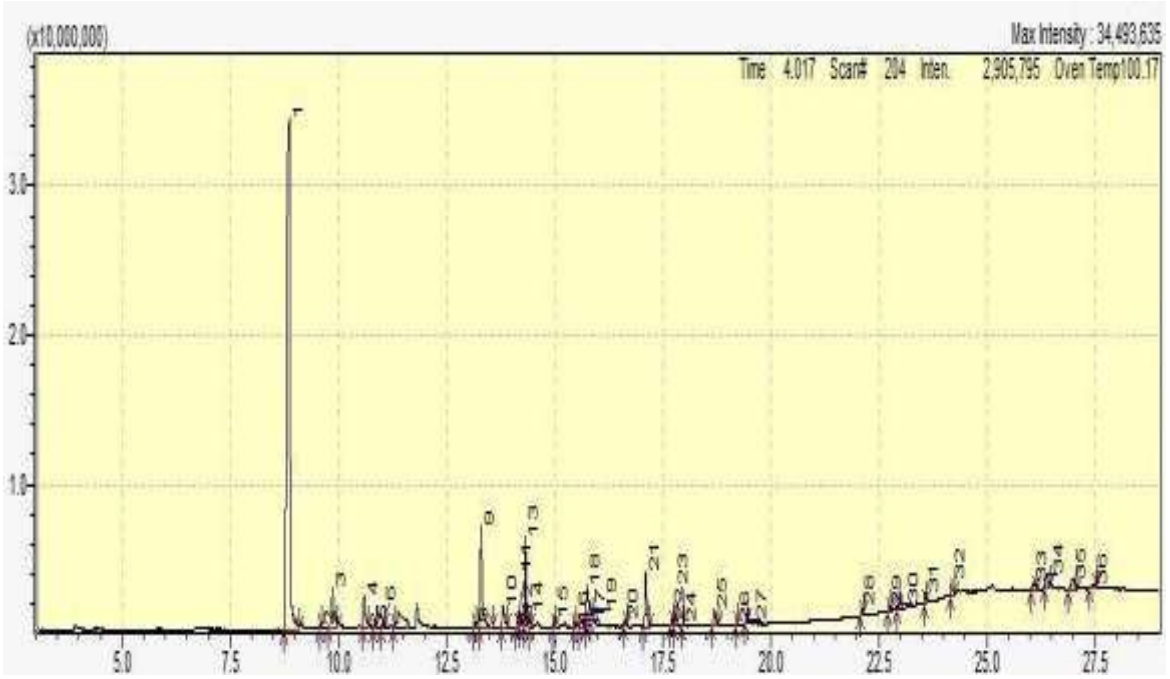


Figure (3.1)Total ions chromatogram

Table 3.1: Total Ion Chromatogram (TIC)

ID#	Name	Ret.Time	Area	Area %
1.	Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-	8.877	201420500	62.37
2.	2,5,6-Trimethylbenzimidazole	9.588	615430	0.19
3.	Pyridine, 3-(3,4-dihydro-2H-pyrrol-5-yl)-	9.868	12210350	3.78
4.	Nicotyrine	10.598	8147303	2.52
5.	Phenol, 2,4-bis(1,1-dimethylethyl)-	10.844	850706	0.26
6.	1,2,3,6-Tetrahydro-2,3'-bipyridine	11.034	862387	0.27
7.	2,3'-Dipyridyl	11.299	461272	0.14
8.	Cedran-diol, (8S,14)-	13.156	657524	0.20
9.	Cotinine	13.303	22477895	6.97
10.	N-Nitrosonornicotine	13.829	5957061	1.85
11.	Nicotine, 1'-demethyl-, (.+/-.)-	14.150	2298437	0.71
12.	2-Cyclohexen-1-one, 4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl)-	14.196	2263055	0.70
13.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	14.331	9483911	2.94
14.	2-Pentadecanone, 6,10,14-trimethyl-	14.432	1703105	0.53
15.	2-Pyrrolidinemethanol, 1-methyl-	15.005	1598029	0.50
16.	2-[2-(1-Methylpyrrolidinyl)]ethylphosphine	15.467	859722	0.27
17.	5,9-Dimethyl-2-(1-methylethylidene)-1-cyclodecanol	15.618	2211415	0.69
18.	4-(N-Nitroso-N-methylamino)-1-(3-pyridyl)-1-butanone	15.754	11101654	3.44
19.	1-Heneicosanol	15.858	1061000	0.33
20.	7,11-Epoxymegastigma-5(6)-en-9-one	16.629	827485	0.26
21.	Phytol	17.113	11006197	3.41
22.	Bromoacetic acid, octadecyl ester	17.708	772197	0.24
23.	Racemethorphan	17.781	382349	0.12
24.	Phytol, acetate	17.973	308038	0.10
25.	Nerolidyl acetate	18.701	3701623	1.15
26.	4,8,12,16-Tetramethylheptadecan-4-olide	19.214	283840	0.09
27.	2,6,10,14-Hexadecatetraen-1-ol, 3,7,11,15-tetramethyl-, acetate, (E,E,E)-	19.438	606416	0.19
28.	Pyrrol-3(2H)-one, 5-amino-1-benzyl)-4-(1-methyl-2-benzimidazolyl)-	22.104	1646027	0.51
29.	Squalene	22.743	750229	0.23
30.	trans-Geranylgeraniol	22.942	437267	0.14
31.	Oxirane, 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19-heneicosapentaenyl)-, (all-E)-	23.577	1244495	0.39
32.	N'-Propylnornicotine	24.186	3287640	1.02
33.	Ergost-5-en-3-ol, (3.beta.)-	26.089	1985257	0.62
34.	Stigmasterol	26.385	3706238	1.15
35.	.gamma.-Sitosterol	26.993	3858536	1.20

The following components were detected as major constituents in the total ions chromatogram:

i)pyridine,3-(1-methyl -2- pyrolidinyl) (nicotine)(62.37%)

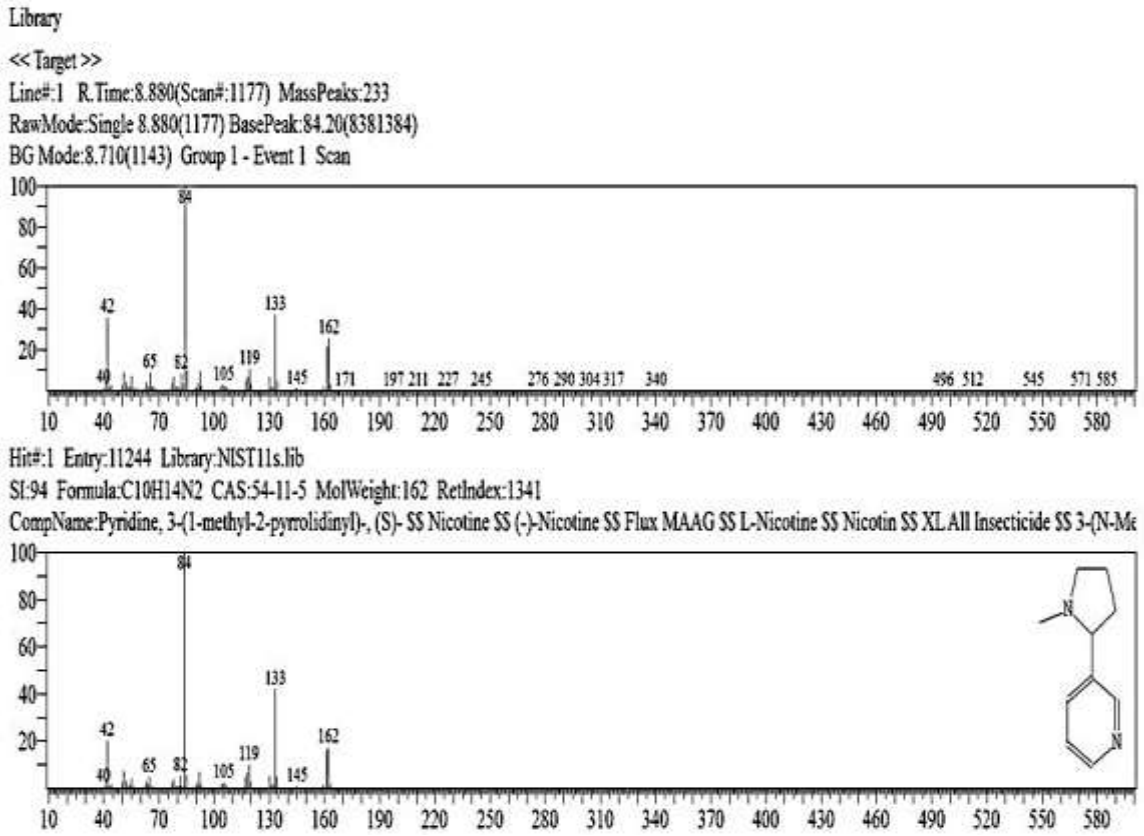


Fig (3.2): Mass spectrum of Pyridine, 3-(1- methy-2-pyrolidinyl) (nicotine)

The mass spectrum of pyridine ,3-(1-methyl -2-pyrolidinyl) (nicotine) is shown in fig (3.2).The peak at m/z 162, which appeared at R.T.8.880 in total ions chromatogram,corresponds to M^+ $[C_{10}H_{14}N_2]^{\cdot+}$. The peak at m/z 133 corresponds to loss of $(-N^+-CH_3)$.

ii)N-Nitrosonornicotine (NNN)(1.85%)

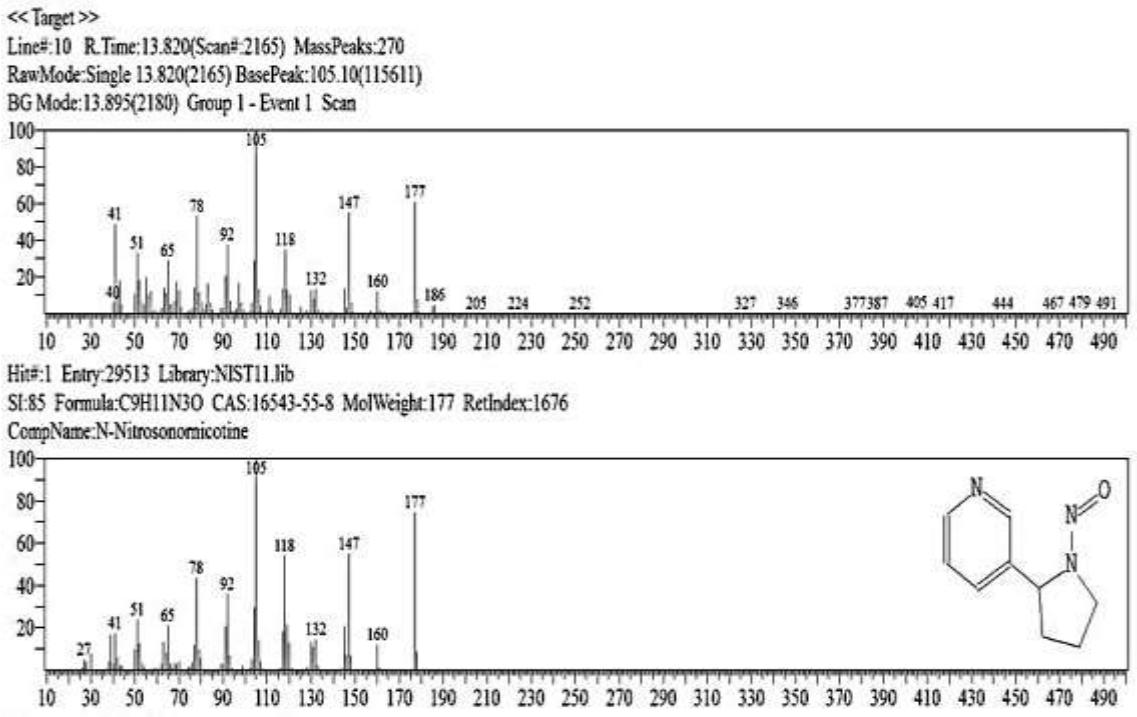


Figure (3.3): Mass Spectrum of N'-nitrosonornicotine (NNN)

The mass spectrum of N-nitrosonornicotine (NNN) is shown in Fig(3.3) . The peak at m/z 177,which appeared at R.T.13.820 in total ion chromatogram, corresponds to M^+ $[C_9H_{11}N_3O]^+$. The peak at m/z 147 corresponds to loss of (-N=O).

iii)4- (methylnitrosamino)-1 – (3pyridyl)-1 – butanone (NNK)(3.44%)

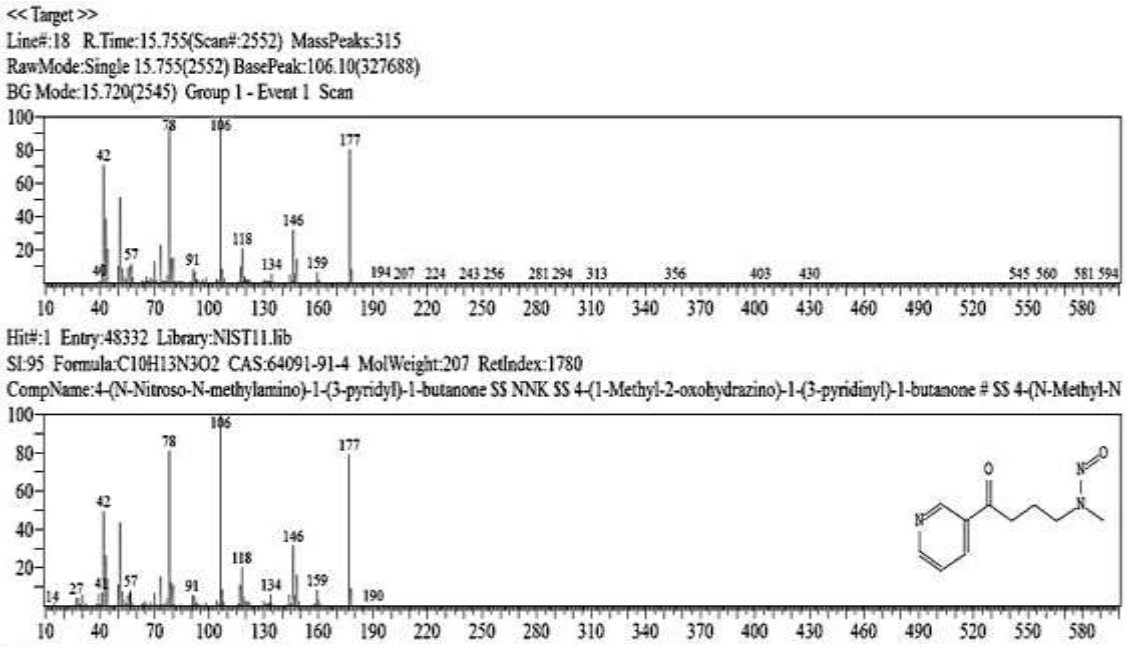


Figure (3.4): Mass Spectrum of 4- (methylnitrosamino)-1 – (3pyridyl)-1 – butanone (NNK)

The mass spectrum of 4- (methylnitrosamino)-1 - (3pyridyl)-1 - butanone

(NNN) is shown in Fig (3.4) . The peak at m/z 207,which appeared at R.T.15.755 in total ion chromatogram, corresponds to M^+ $[C_{10}H_{13}N_3O_2]^+$.

The peak at m/z 177 corresponds to loss of(-N=O).

3.2 Thin- Layer Chromatography (TLC) analysis of Toombak:-

By TLC, during analysis of sample, it was observed that clear indication from the spots intensity of TLC plates. The intensity of color is more and clearly spotted on the plates which also indicate the presence of compounds in toombak. Two spots tell that in all cases of sample, carcinogenic N-

nitrosamines (NNN and NNK) are present during running the TLC plates as shown in Fig (3.5 and 3.6).

Fig (3.5) TLC plate of N-nitrosnornicotine (NNN)

Fig (3.6)) TLC plate of 4- (methylnitrosamino)-1 – (3pyridyl)-1 – butanone (NNK)

Conclusion:-

- **GC-MS** analysis of toombak was conducted and the identification of nicotine and carcinogenic N-nitrosamines (NNN and NNK) was initially accomplished by comparison with the **Mass Spectrum** libarary and further confirmed by interpreting the observed fragmentation pattern . hence can be concluded that nicotine alkaloid and carcinogenic N-nitrosamines (NNN and NNK) are present in toombak.
- **TLC** analysis of toombak was conducted, Two spots tell that in all cases of sample, carcinogenic N-nitrosamines (NNN and NNK) are Present in toombak.

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