Analytical Method Development for the Compositional Investigation of Khat (*Catha edulis* Forsk) Leaves with Respect to Its Active Constituents, Selected Secondary Metabolites and Inorganic Nutrients

Minaleshewa Atlabachew

A Thesis Submitted to

The Department of Chemistry

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (Chemistry)

Addis Ababa University

Addis Ababa, Ethiopia

January 2013

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by Minaleshewa Atlabachew entitled: *Analytical Method Development for the Compositional Investigation of Khat (Catha edulis Forsk) Leaves with Respect to Its Active Constituents, Selected Secondary Metabolites and Inorganic Nutrients* and submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy (Analytical Chemistry) complies with the regulations of the University and meets the accepted standards with respect to originality and quality

Signed by the Examining Committee

Name	Signature	Date
Prof. Robert I. McCrindle External Examiner	_____	30 January 2013
Dr. Negussie Megersa Examiner	_____	30 January 2013
Dr. Merid Tessema Examiner	_____	30 January 2013
Prof. Bhagwan S. Chandravanshi Advisor	_____	30 January 2013
Dr. Mesfin Redi Advisor	_____	30 January 2013

_____

Chair of Department

ABSTRACT

Analytical method development for the compositional investigation of khat (*Catha edulis* Forsk) leaves with respect to its active constituents, selected secondary metabolites and inorganic nutrients

Minaleshewa Atlabachew
Addis Ababa University, 2013

The rapid growth of khat use and misuse in the countries of its origin and other parts of the world has led to the development of methods for extraction, clean-up, detection and quantification of its psychoactive alkaloids and other nutrients. However, the extraction and clean-up methods reported so far have a number of drawbacks and there is paucity of quality data with regard to the concentrations of its psychoactive alkaloids, phenolic compounds and mineral nutrients of Ethiopian khat accessions. Thus, this study addressed some of the challenges facing researchers in the area, such as the need to use easily accessible, simple, fast and plausible sample preparation techniques for khat alkaloids and tannins analysis. Furthermore, total polyphenols, selected essential and non-essential elements and fluoride accumulation in various khat cultivars were also investigated.

Matrix solid phase dispersion (MSPD), molecularly imprinted polymer for solid phase extraction (MIP-SPE) and the modified quick, easy, cheap, effective, rugged and safe (QuEChERS) based methods were investigated for extraction and clean-up of khat alkaloids prior to HPLC-DAD detection. Under optimum conditions, good recoveries, selectivity and reproducibility were obtained.

There was an assertion that drying khat leaves could significantly (if not completely) decompose cathinone to cathine. However, this study demonstrated that cathinone was

persistently existed in dried plant material and it is wrong to assume that sun or oven drying the leaves could completely convert cathinone to cathine.

This study also presents the quantitative determination of total phenolic compounds, flavonoids, tannins and their related *in-vitro* antioxidant activities of the young leaves, matured leaves and tips of tender stem near the young shoots. Simplified, rapid and robust method was also developed for the analysis of total tannins using egg albumin as a precipitating agent and Folin Denis reagent and $FeCl_3$ solution for color developing moieties for spectrophotometric assay.

In addition, the concentrations of selected major, minor and toxic metals as well as fluoride distribution in various khat cultivars and the corresponding soil samples were investigated by flame atomic absorption spectrometry and fluoride ion-selective electrode potentiometry, respectively. It was noticed that khat leaves could be a good sources of most of essential mineral nutrients and free from toxic metals like Pb and Cd. Analysis of fluoride in the edible portion of the khat leaves resolved the equivocal information about khat chewing and dental staining due to fluoride. It was found that the plant accumulated significantly lower concentrations of fluoride compared with similar stimulant plants.

ACKNOWLEDGMENTS

A journey is easier when we travel together. This thesis is the result of four and half years of work whereby I have been accompanied and supported by many people. It is a pleasant aspect that I now have the opportunity to express my gratitude for all of them. Though there are numerous people over the years who have brought me to this point, but due to space limitation I shall only highlight a few. First and foremost I would like to express my sincere appreciation to my supervisors Prof. Bhagwan Singh Chandravanshi and Dr. Mesfin Redi for their guidance and encouragement. Prof. Chandravanshi who was also my mentor and supervisor for my M.Sc. study, has special place in my academic life for taking the initiative for pursuing my PhD study in collaboration with Dr. Mesfin Redi. I am deeply appreciative of his frankness and unlimited concern as well as guidance for my success and achievements of today. Equally it is worth to mention the dedicative continuous support, guidance, expertise and valuable advice made by Dr. Mesfin Redi throughout the study period.

I am also very grateful to Prof Nelson Torto for giving me the opportunity to do part of my research work under his supervision at the Department of Chemistry, Rhodes University, South Africa. During, my stay at Rhodes, I was accessing all the available facilities in his group and within the department so that the skills and experience I have got were beyond my expectation.

I am grateful to Bahir Dar University for giving me sponsorship and financial support. I greatly appreciate the Department of Chemistry, Addis Ababa University, and all staff members for their sincere cooperation during my study. My special thank should go to Prof. Ermiyas Dagne for this kind cooperation to use the NMR and UV-Vis Spectrophotometer as well as expensive solvents. I am also thankful to Dr. Feleke Zewge, Dr. Nigist Asfaw and Dr. Yonas Chebude for their willingness to allow me to use their laboratory facilities. I am also indebted to express my appreciation to Dr. Merid Tessema, Dr. Negussie Megersa, Dr. Ahmed Mustafa, Dr. Shimelis Admassie and Dr. Ashebir Fiseha for their encouragement, appreciation, and resource sharing and/or evaluating my seminar courses.

Let me extend my gratitude to the staff of Addis Ababa University, Food Science Program: Dr. Gulilat, W/t Weynishet, Ato Ashagrie and Ato Daud for their valuable mentee and resource sharing.

I specially thank with gratitude the staff members of Ethiopian Health and Nutrition Research Institute (EHNRI) for generously providing specialized chemical standards and my special thank should go to Prof. Eyasu, Head of the Department of Pharmacology for allowing me to use lyophilizer in his lab.

I am thankful to Dr. Atakilit Abebe, Ato Aleminew Geto and PhD students of our research group (Ato Kebede Nigussie, Ato Bewketu Mehari and Ato Tesfaye Bekele), the other chemistry research groups, Aster, Megabit and Lemlem, technicians and everyone else. You have been awesome!

Maereg Amare: What a friend! It has been awesome! Traveling from Akaki campus to Arat Kilo campus every day would not have been easy without you! You are one of a kind! Thanks for always being there for me and with me.

I am thankful to F12 (Prof. N. Torto research group): Dr. Samuel Chigome, Kehinde, Boitumelo, Phumelele Kedemetsie and Bellah are greatly acknowledged for your endless mentor and encouragement during my stay at Rhodes

My most sincere appreciation goes out to my parents who have disciplined and nurtured me throughout life to give me the strength and tools to achieving any goals I may set for myself. I could not have completed this research without the love and support of my wife, Elsabet Ferede. You showed great endurance in taking care of me and our son, Natan; I thank you so much for your patience, understanding and encouragement during the ups and downs we have passed. May God give you his rewards for your sacrifices! Love you so much!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION AND MOTIVATION

Khat (*Catha edulis* Forsk) is an evergreen shrub or tree found growing wild or cultivated in the east of a region extending from Southern Africa to the Arabian Peninsula.

In the past, khat was grown in Ethiopia on a limited scale in certain geographical locations and used to be chewed only by a small number of people (especially elderly) either for religious or entertainment reasons. Its cultivation and use is now gradually becoming an omnipresent phenomenon in the country (Zeina, 1988; Selassie *et al*., 1996; Kebede, 2002, Dhaifalah *et al*., 2004). Currently, with the exception of children, all categories of people and ages, including college and university students are exposed to this potential psychostimulant plant (Zeina, 1988; Kebede, 2002).

Recent studies revealed that khat chewing can cause various health related problems to the chewers. Short term effects of chewing khat are clearly identified but the long-term health impact of chewing khat is not well established. Scattered reports from Yemen and elsewhere indicate that chronic khat consumption may be a leading cause of cancer and other metabolic disorders in the Yemen population (Kassie *et al*., 2001; Lukandu *et al*., 2008; Ahmed and El-Qirbi, 1993). Some authors tried to link the disorders with the alkaloids fractions (Ahmed *et al*., 1993; Cox *et al*., 2003; Feyissa *et al*., 2008; Karunamoorthi *et al*., 2010) while others link with the other components of the plant in addition to the alkaloids fraction of the plant (Kennedy *et al*., 1983; Karunamoorthi *et al.,* 2010). Thus, urgent studies of the components of this plant are required to conduct *in-vivo* and *in-vivo* studies to clearly quantify the effect of particular khat component on the chewer.

Ethiopia is one of the countries where khat is largely cultivated for domestic consumption and export market. It is believed that more than 50 khat types are available in the country and it is the third largest cash crop to the country. In addition, though compressive studies have been made on toxicological effect of khat chewing, concentration based studies are few in number. This might be due to the fact that there was no systematically investigated report on the active principles of khat in different cultivars of the plant grown in different

parts of the region. Therefore, the compositions of Ethiopian khat need to be systematically investigated for further clinical studies using simplified, inexpensive and environmentally friendly analytical techniques.

Currently there are commercially available hyphenated analytical instruments with quantification and detection limits that suit the monitoring of bioactive phytochemicals in the area of forensic toxicology and biomedical sectors. Despite these advances, sample preparation is still a vital part of the analytical process.

Since 1980, after the discovery of the khat alkaloids in the plant by the research group of WHO, various analytical techniques (both extraction, clean up and quantification techniques) have been reported (Szendrei, 1980). Ultrasonic assisted extraction and maceration are the extraction protocols reported so far with an extraction time of up 30 min to 24 h using organic solvents (Geisshusler *et al*., 1987; Brenneisen *et al*., 1985; Gambaro *et al*., 2012; El-domiaty *et al*., 1994; Mathys *et al*., 1993). Except the work reported by (Mathys *et al*., 1993) using C_{18} as SPE sorbet, most of the authors used liquid –liquid extraction (an acid-base type extraction) so as to clean up the analytes of interest and requiring large volume of organic solvents.

Hence, all the reported extraction and clean up methodologies have extensive draw backs requiring lengthy extraction time, large amount of organic solvent, sample size and multistep procedures. They are also susceptible to analyte loss. Therefore, there is a quest among the researchers to minimize the aforementioned limitations and find simpler ways to combine extraction and clean-up in a single step that can be applicable in pharmacological and forensic laboratories.

Furthermore, there is strong controversy about cathinone stability during khat preservation for the analysis. Previous studies on the khat plant illustrated the importance of using freshly harvested young shoots and leaves such that cathinone, the principal active component contained within the plant, could be suitably isolated and identified. Upon drying with sunlight and storage of the cut plant material, cathinone readily converts to the reduced product, cathine, which necessitates rapid extraction and chemical analysis for cathinone identification. Furthermore, a common belief among

pharmacologists working on khat is that cathinone is highly unstable once the plant is harvested, and may be undetectable upon drying and prolonged storage. On the other hand a recent report indicated that drying the plant material could significantly retard the decomposition of cathinone. Thus, controlled various drying methods need to be investigated so as to quantify the kinetics of cathinone upon drying, storage and extraction in order to recommend the plausible condition for preservation of the plant without appreciable change in the alkaloids content.

Most researchs on khat nowdays concentrate on the chemical analysis of the psychoactive components and their pharmacological and pharmacokinetic prosperities. However, other aspects of the plant like inorganic nutrients and bioactive phenolic compounds have had limited attention.

Phenolic compounds constitute a large group of secondary metabolites widely distributed throughout the plant kingdom (Makkar *et al*., 2007). Natural products rich in phenolic compounds are attracting particular interest for potential medicinal related benefits of their consumption and products produced from them.

Increasing epidemiological data suggest that a high intake of fruits and leafy vegetables offers a number of health benefits against degenerative diseases (Alia *et al*., 2003; Dai *et al*., 2010). These disease prevention properties are due in part to the phenolic content of the plant (Makkar *et al*., 2007; Wollgast *et al*., 2000). Numerous studies have suggested that the antioxidant activity, due to the phenolic composition of a food product, contributes to their protective effects against chronic and degenerative diseases (Heinonen *et al*., 1998; Record *et al*., 2001). The phenolic composition of a plant is generally unique to the plant species and can vary with its growth climate (Manach *et al*., 2004). It has also been shown that the antioxidant activity varies with the types of phenolic compounds present in the fruit and that certain types of phenolic compounds show greater antioxidant activity than others (Rice-Evans *et al*., 1995; Amic *et al*., 2003).

On the other hand, some polyphenolic compounds like tannins have side effects when consumed in larger quantity. Gastric complaints among khat chewers, preventions of bioavailability of antibiotics as well as observable body weight loss effect on chronic

khat chewers might be a direct of khat tannins. However, tannin concentration of khat varieties is scares in the literature. Thus, the characterization of the chemical composition and phenolic profile of khat varieties would lead to conduct further research on medicinal important of khat

Concerning mineral accumulation in khat leaves, there is paucity of reported literatures. It is apparent that heavy metal contamination of the food chain is becoming a serious issue, globally due to various factors related to human activities like rapid industrial growth, the widespread use of chemicals in agriculture, and increasing urbanization (Chandravanshi, 2011). Furthermore, trace metals are vital for normal function of body enzymes and their excess concentration is harmful (National Research Council, 1989). Thus, commonly taken food materials should be evaluated for their metal contents so as to ensure the dietary safety of the individuals as well as to monitor source of contaminations for those minerals whose concentration exceeds the guideline. Khat is becoming more popular all over the country and other parts of the world and farmers are accustomed in using chemicals like insecticides and fertilizers (Lemessa, 2011) to increase their yield. Having detail documents focusing on essential and non-essential minerals in khat is very important to evaluate the total mineral intake of the individual who is using this plant regularly. Furthermore, analyzing the soil where khat is largely grown is important to correlate accumulation of a particular metal in the plant and in the soil. This intern has an advantage to control the soil parameters and locate source of pollution based on the available data so as to take monitoring action. Furthermore, contribution of khat chewing to dental fluorosis due to fluoride in not well understood and there is confusion among dentists.Thus, different varieties of khat from different geographical locations need to be investigated so to come up with concrete evidence about the contribution of khat in terms of fluoride.

In this work, we developed various sample preparation protocols for extraction and clean up of khat alkaloids and for the quantitative determination of tannins in plant extract. Furthermore, the composition of various Ethiopian khat and the respective soil samples were studied in terms of alkaloids concentration, total flavonoids, total polyphenols, total

tannins, antioxidant activities, selected essential and non-essential metals and fluoride accumulation.

We have also developed a protein precipitation method based on egg albumin for quantification of tannins. Then various khat types/varieties were investigated for their composition of total tannins, total flavonoids, total polyphenols and their antioxidant activities. Furthermore, the composition of fluoride, and selected metals in khat and the respective soil samples have been studied.

The main objective of our work was to develop analytical methods for the compositional investigation of the active principles, selected secondary metabolites and selected inorganic nutrients of khat (*Catha edulis* Forsk) chewing leaves. Our work has three major parts

Part I: Method development for the compositional investigation of khat alkaloids.

Part II: Compositional investigation of selected secondary metabolites

Part III Compositional investigation of Ethiopian khat with respect to selected inorganic nutrients

Specific objectives of this thesis work were:

Part I

- To optimize matrix solid phase dispersion (MSPD) and applied for extraction and clean up of alkaloids from khat
- To investigate the distribution of khat alkaloids in different Ethiopian khat varieties/types after extraction with MSPD and HPLC-DAD detection
- To investigate the effect of various sample preservative conditions (freezing, freeze drying, air drying, sun drying and oven drying) on cathinone degradation
- Synthesized, characterized and optimized different molecularly imprinted polymers for the application of solid phase extraction clean up of the extract prior to HPLC determination

- To modify and optimize the conventional Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) based methods were for extraction and clean up of khatamines from khat samples.

Part II

- To optimize colorimetric method for tannin determination based on protein precipitation using egg albumin
- To investigate the distribution of total tannins, total flavonoids, total polyphenols and antioxidant activity of Ethiopian khat were studied

Part III

- To investigate the distribution of selected essential and non-essential metals in Ethiopia khat and the respective soil samples
- To conduct soil-plant correlation analysis for each metals studied
- To investigate the distribution of total and water soluble fluoride in various khat samples collected from rift valley and non-rift valley regions of Ethiopia

2. LITERATURE REVIEW

2.1 Khat plant origin, history, cultivation and geographical distribution

Khat is a natural stimulant derived from the *Catha edulis* Forsk plant, which is cultivated in Yemen and most East African countries. Its young shoot and tender leaves as well as the tender stems are chewed to attain a state of euphoria (Karunamoorthi *et al*., 2010). Khat-chewing originated in Ethiopia and subsequently spread through Kenya, Somalia, Djibouti, Uganda, Tanzania, Zimbabwe, Zambia, South Africa, Yemen and others as well (Nencini *et al*., 1989; Halbach, 1972; Elmi, 1987; Lemessa, 2001; Feyissa *et al*., 2008; Karlson, 2006).

The plant is known by different names depending on its origin: chat/Jimaa in Ethiopia, qat inYemen, qaad/jaad in Somalia, miraa in Kenya, mairungi in Uganda and Muhulo in Tanzania (Halbach, 1972; Elmi, 1987; Lemessa, 2001; Feyissa and Kelly, 2008). The dried leaves of khat are known as Abyssinian tea or Arabian tea (Odenwald, 2007).

The use of khat has traditionally been confined to the regions where it is grown (Feyissa *et al*., 2008), because the shoots must be used fresh for the desired effects (Kriseviski *et al*., 2007). In recent years, however, the economic importance and consumption of khat leaves have increased dramatically (Odenwald, 2007). This change is due to improved road and air transport, which has allowed a much wider distribution (Lemessa, 2001). Furthermore, immigrants from East Africa and the Arabian Peninsula, who continue to use khat, has resulted in the importation of khat to countries where these immigrants have settled. These include Europe, Australia and the United States (Fitzgerald, 2009; Karlsson, 2006; Cox *et al.,* 2003; Toennes *et al.,* 2004 Balint, 2010). Although khat can be freely obtained in East Africa and Arabian Peninsula, its use in western countries, such as the United Kingdom, Australia, Canada, and the United States has recently become restricted, and it is now classified as a controlled substance. Nonetheless, individuals from such regions as, Ethiopia, Somalia and Yemen, continue the habit even after immigration to the West (Balint, 2010; Lemessa, 2001).

In areas with frost, the shrub can grow higher than 1.5 m but in places with more rainfall, such as the highlands of Ethiopia and areas near the equator, khat trees can reach up to 20 m (Lemessa, 2001). Khat can also survive droughts, when other crops fail. It grows at altitudes of 1500 m – 2000 m. The plant requires well drained field with pH range of 6.0 to 8.2 and annual rainfall ranges from 1000 to 1500 mm for its growth (Lemessa, 2006).

2.2 Distribution of khat tree in Ethiopia

In Ethiopia khat is an important and potentially lucrative cash crop and Ethiopia is the world's largest producer of khat, which is the country's fastest growing export. Thus, khat has becoming the country's second largest export item. Currently, it is believed that almost all regions of Ethiopia are cultivating khat for domestic consumption and export markets. The major khat cultivating regions of Ethiopia for local consumption, export market to the capital city Addis Ababa and other neighboring countries are the Oromiya region, Southern Ethiopia, and the Amhara region. From the Oromiya region , eastern and western Harargie as well as the Harari district are the major and the ancient khat cultivating regions. Districts like Awadai, Gelemso, Kerti, Hirna, Alemaya Habro and Asebeteferi are few among them. Other parts of the Oromiya region like Hageremariyam (Borena Zone), Sebeta, and Woliso (West Shoa Zone) and Anferara (Guji Zone) also cultivate khat for domestic consumption (Lemessa, 2001; Karunamoorthi *et al*., 2010; Karlson, 2006; Dessie *et al.,* 2008).

The major khat cultivating areas of southern Ethiopia are in the Sidama zone (Hawassa District, Gamogofa Zone (Wondogent District), Gedeo Zone (Dilla District) and Guragie Zone. The major khat producing areas of Guragie are Enemor, Ener, Meher, Aklil, Kokir, Goro, Cheha, Endibir, Gunchere and Esia.

Amhara regional state farmers are also cultivating khat and export to the Bahir Dar and Addis Ababa. About 14 administrative districts of Bahir Dar are known to cultivate the plant: Bure, Finoteselam, Dangla, Merawi, Mota, Meshenti, Hamusit, Adet, Delgi, Zenzelma, Tekle Dengay, Zeghe and Endasa. Furthermore, Southern and Northern Wollo Zones of Amhara regiosn are widely cultivating khat for local consumption.

Few areas of the Oromiya zone in Amhara region such as Kemise and Bati districts are also cultivating khat for the local markets of the zone. In Tigray regions, khat is not as such cultivated. However, currently, as it is noticed in few kiyosks of Addis Ababa as khat from Maychew which is known as Mychew khat is available during the rainy season.

To date, khat cultivation among the farms of the country are increasing in an alarming rate while items like coffee, enset and other crops are declining since khat is becoming a more significant cash crop of the regions (Karlson, 2006; Dessie *et al*., 2008). Figure 2.1 shows the rough distribution of khat in Ethiopia.

Ethiopian khat is marketed under different names (for instance; Awadai, Gelemso, Gurage, Wondo, Sebata, Belechie, Kuto, Abo Mismar, Bahir Dar, etc) depending on the locality where it is obtained. It has also been marketed as Colombia, a trade name not related to the region.

Though no taxonomic classification has been conducted in Ethiopian khat, currently, depending up on the geographical location, the varieties/types of khat are enormous in the country. Even within the same locality there are different varieties/types of khat. They differ in color, size and height of the leaves and size and height of the plant as a whole. Experienced chewers' claim that one khat type/variety differs from others in terms of its stimulation property and taste. Figure 2.2 shows two different cultivars of khat.

Al-Thobhani *et al.* (2008) conducted genotypic study on different types of khat collect from most parts of Yemen and their results indicated that samples from different geographical regions and growth habitat in Yemen showed a clear genetic differentiation among samples of different origin.

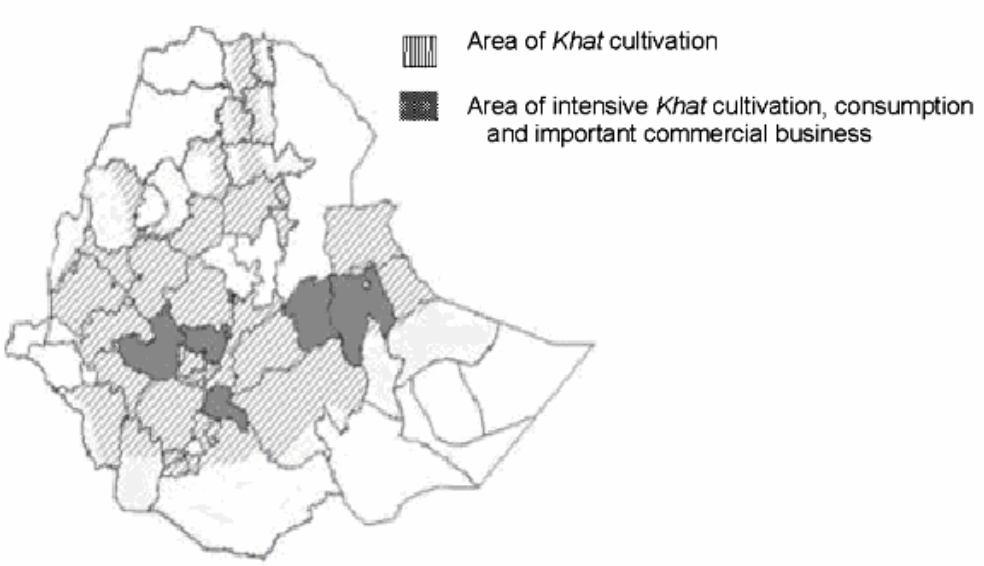


Figure 2.1 Map showing distribution of khat in Ethiopia (Lemessa, 2001)

Figure 2.2 Bundles of khat samples

2.3 Consumption and prevalence of khat chewing

The fresh young leaves and tender stems from khat trees are traditionally consumed for their stimulating properties. They taste sweet to bitter when fresh, based on the type of khat consumed. The most favored part of the leaves is the young shoots near the top of the plant. However, leaves and stems at the middle and lower sections are also used. Fresh khat leaves are typically chewed. Sometime infusion of the matured leaves is also

taken which is known as Abyssinia tea (Al-Motarreb *et al*., 2002; Keneday *et al*., 1983; Lemessa, 2001) and locally it is known as 'Hawuza'.

Khat chewing has a social and cultural tradition, and it may occur while in the company of others or alone (Balint *et al*., 2009; Hoffman *et al*., 2010). It is highly prevalent in many countries in Africa and Asia, with 75% of men using khat in some countries (Alem *et al*., 1999). About 20 years ago, it was estimated that around 10 million people commonly used khat in East Africa and Arabian Peninsula countries (Balint *et al*., 1991). In 1996, it was estimated that worldwide approximately 6 million individual portions of khat were consumed daily (Kalix, 1996). Recent reports indicate that 80–90% of East African males use khat on a daily basis and 10–60% of East African females use khat on a daily basis (Numan, 2004; Odenwald *et al*., 2005; Warfa *et al*., 2007; Al-Motarreb *et al.*, 2010).

In Ethiopia, specifically in Butajira, estimates of prevalence (Alem *et al*., 1999) approach 50%, with 17% self-described as daily users, predominantly men (a reported 5:1 male to female ratio). While early reports indicated greater prevalence in men than in women. Reda *et al*., 2012 conducted a survey among 1,890 secondary school students in Harar town in 2010 and found that prevalence of khat chewing among the sample was 24.2%. About 28.5% of females and 71.5% of males had chewed khat. 15.9% of a sample of 4001 men in Addis Ababa, Ethiopia regularly chewed khat (Tesfaye *et al*., 2008). There is evidence that khat use in Ethiopia is more prevalent in ethnic communities with a tradition of khat use but it is now becoming an every-day drug for the general population (Alem *et al*., 1999; Belew *et al*., 2000).

The number of khat users has significantly increased in Ethiopia during recent decades and the habit has become popular in all sections of Ethiopian society (Reda *et al*., 2012; Getahun *et al*., 2010). Nowadays, with the exception of children, all categories of people and ages, including college and university students are exposed to chew the plant. It has become customary to observe truck, bus or taxi drivers chewing khat while executing their daily duties. Khat used to be sold in the past in restricted areas but now is sold freely on the open market in the major cities of Ethiopia and elsewhere without any interventions (Banjaw *et al*., 2005; Dagne *et al*., 2010, Alem *et al*., 1999).

The prevalence of khat chewing in Western countries appears to be restricted to the immigrant communities from these countries where there is used (Al-Motarreb *et al*, 2010).

The habit of chewing khat has been common for many centuries. Historically, khat has been used for medicinal and recreational purposes (Kennedy *et al*., 1987; Halbach, 1972). Now, it is most valued for its stimulant effects (Al-Thobhani *et al*., 2008). It is also used for avoiding sleepiness, euphoriatic effects, to boost efficiency of work and reducing physical fatigue (Balint *et al*., 2009; Feyissa *et al*., 2008). It is also believed that processed leaves and roots have been used to treat influenza, cough, gonorrhea, asthma and other chest problems. The root is also used for stomach ache and an infusion is taken orally to treat boils (Lemessa 2001, Halbach, 1972).

2.4 Composition of khat

The chemical constitutents of khat have been studied since the late 19th century. Since then, phytochemical studies on khat revealed that various chemical species have been identified in the leaves and the tips of the tender stems of the plant. These include the alkaloids (phenylalkylamines and cathedulins), terpenoids, flavonoids, sterols, glycosides, tannins, amino acids, flavors, minerals, vitamins and fluoride (Szendrei, 1980; Halbach, 1972; Geisshüsler *et al.,* 1987; Kalix and Braenden, 1985; Hattab *et al.,* 2000; Matloob, 2003; Feyissa *et al*., 2008; Abdulsalam *et al*., 2004).

2.5 Khat alkaloids

The chemical study of khat started around 1887 when Flueckinger and Gerock, searching for caffeine as possible stimulating principle, found no traces of the chemical but discovered the alkaloid, named katin. The alkloids composition of khat was later investigated by several investigators and a number of khat alkaloids have been isolated (Szendrei, 1980; Kalix *et al.,* 1985; Kite *et al*., 2003).

The phenylalkylamines and the cathedulins are the major alkaloids (Figure 2.3). The khat phenylalkylamines comprise cathinone [S-(–)-cathinone], the two diastereoisomers: cathine [1S, 2S-(+)-norpseudoephedrine or (+)-norpseudoephedrine] and norephedrine [1R, 2S-(–)-norephedrine]. These compounds are structurally related to amphetamine. The plant contains the (–)-enantiomer of cathinone only; the (+)-enantiomer is not found (Szendrei, 1980; Kalix *et al.,* 1985; Krizevski *et al*., 2007). Thus, the naturally occurring S-(–)-cathinone has the same absolute configuration as S-(+)-amphetamine (Kalix *et al.,* 1985) (Figure 2.4).

Other phenylalkylamine alkaloids found in khat leaves are: phenylpentenylamines; merucathinone, pseudomerucathine and merucathine, which are mainly detected in khat leaves originating from Kenya (Brenneisen *et al.,* 1987). They seem to contribute less to the stimulant effects of khat (Kalix 1990) and their concentration is relatively low (Brenneisen *et al.,* 1987; Kalix 1990; Mathys *et al.,* 1993). However, no report has been made the existence of these alkaloids in khat samples from other locations (Figure 2.5)

Cathinone is mainly found in the young leaves and shoots. During maturation, cathinone is enzymatically converted to cathine and norephedrine (Krizevski *et al*., 2007; Kalix *et al.,* 1985). Drying under sun light or heat treatment of the leaves cause cathinone degradation to cathine and norephedrine also occurs during extraction of cathinone in the laboratory under alkaline condition (Szendrei, 1980; Krizevski *et al*., 2007; Dagne *et al*., 2010; Brenneisen *et al.,* 1987; Lee, 1995). Cathinone can also be oxidized to give 1-phenyl-1, 2-propandione, while the cathinone dimers, such as 3, 6-dimethyl-2, 5-diphenylpyrazine are purely artifacts of the isolation (Szendrei, 1980; Krizevski *et al*., 2007).

(−)-Cathinone is estimated to be one-third as potent as amphetamine and 10 times more potent than (+)-cathine and (−)-norephedrine (Geisshuler *et al.,* 1987)

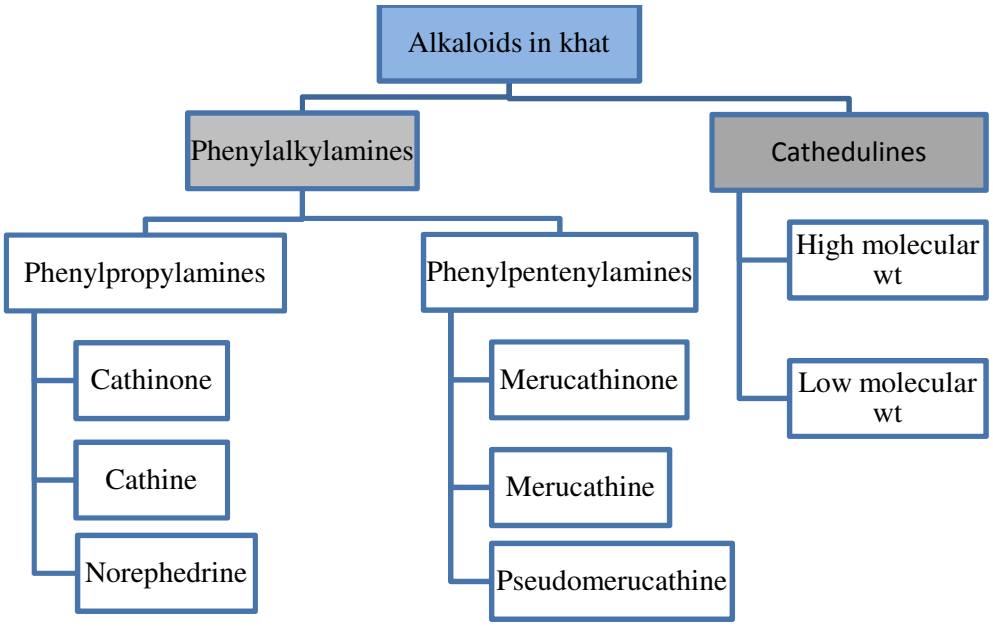


Figure 2.3 Summary of class of different alkaloids in khat leaves (Feyissa *et al*., 2008)

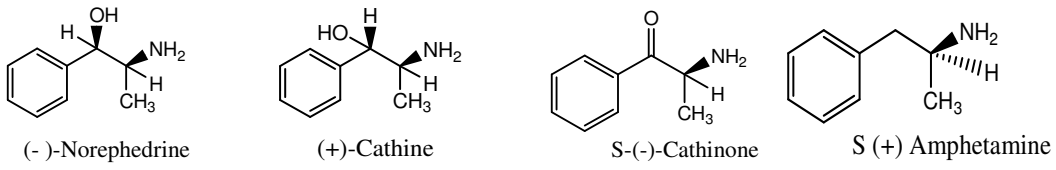


Figure 2.4 The molecular structures illustrated for the phenylpropylamines and amphetamine

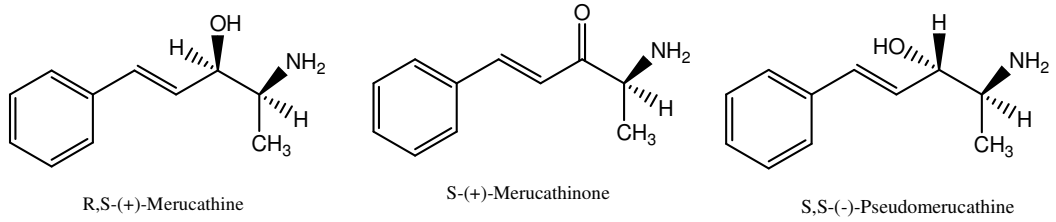


Figure 2.5 The molecular structures illustrated for the phenylpentenylamines

2.5.1 Pharmacology

The major pharmacological effects include those on the gastrointestinal system and the nervous system. The pharmacology of khat amines in the central and peripheral nervous systems is reported and the published articles have been reviewed by many scholars (Feyisa *et al*., 2008; Al-Motarreb *et al*., 2002; Al-Motarreb *et al*., 2010, Odenwald, 2007). A wide variety of *in vitro* and *in vivo* experiments demonstrate that cathinone shares the action of amphetamine on CNS as well as its sympathomimetic effects (Kalix, 1990; Odenwald, 2007). These alkaloids cause the release of catecholamines from pre-synaptic storage sites in the central and peripheral nervous system and inhibit its re-uptake and can produce depletion of central dopamine (Kalix, 1986; Schechter, 1990). In addition, these molecules may also have monoamine oxidase inhibition effects (Nencini *et al*., 1984). Peripherally, cathinone and cathine are equipotent in the release of noradrenaline at the presynaptic storage sites; thus, both have sympathicomimetic effects (Kalix, 1986). The psychotropic effects of khat start after about 1 h of chewing and they last for approximately 3 h (Kalix, 1996, Tones *et al*., 2003). Peak plasma levels of cathinone are obtained 1.5–3.5 h after the onset of chewing while it is barely detectable after 8 h. First-pass metabolism of cathinone in the liver leads to the formation of norephedrine. Only 2% of cathinone is excreted unmodified in the urine. The elimination half-life of cathine and cathinone are 5.2 ± 3.4 and 1.5 ± 0.8 h, respectively (Kalix, 1990; Tones *et al*., 2003, Balint, 2009).

The short-term physiological effects of khat reflect the sympathicomimetic and central dopaminergic activity; e.g. increased heart rate and elevated diastolic and systolic blood pressure (Toennes *et al*., 2003; Widler *et al*.,1994).mydriasis, tachy-cardia, extrasystoles, headaches, hyperthermia, increased respiration (through central stimulation, bronchodilatation and counter-regulation of hyperthermia). The immediate emotional effects in experimental human settings are euphoria (Widler *et al*., 1994, Kalix, 1990; Balint, 2009), and a subsequent depressive.

2.5.2 Toxic effect of khat alkylamines

Long-term health impact of chewing khat is not well established. Scattered reports from Yemen and elsewhere indicate that chronic khat consumption may be a leading cause of cancer, cellular toxicity and other metabolic disorders (Kassie *et al*., 2001; Al-Hebshi *et al*., 2005; Al-Ahdal *et al*., 1988). Extensive review by Al-Motarreb *et al*., 2010 and Odenwald, 2007 about khat chewing and cellular toxicity showed that some of the papers are contradicting each other and the rest are inconclusive because of limited quality data source. Thus the current understanding of many khat-related topics is still poor and that further research is urgently needed (Editorial, 2010).

Several studies have shown that the chronic use of khat may produce various harmful effects such as increased incidence of acute coronary vasospasm and myocardial infarction, esophagitis, gastritis, oral keratotic lesions and liver toxicity (Al-Halbori, 2005). Furthermore, insomnia, depression, anorexia, psychosis and impaired working memory have been reported after occasional or chronic use of khat (Balint *et al*., 2009; Colzato *et al*., 2011). In particular, khat use can exacerbate psychotic symptoms in people with pre-existing psychosis and precipitate psychotic disorders in vulnerable subjects (Yousef *et al*., 1995).

2.5.3 Analytical methods for the determination of psycho-active stimulants of khat extract

After the discovery of the three psychoactive khat alkaloids by the WHO advisory group (Szendrei, 1980), various analytical methods have been reported for identification and quantification of the alkaloids in the plant extract. TLC, HPLC-DAD, NMR, GC-FID, GC-MS and spectrophotometric techniques have been reported for quantitative determination of psychoactive components of the plant within phytochemical, forensic and law enforcement applications. Furthermore, various sample preparation protocols have also been devised and optimized prior to the instrumental analysis (Geisshuler *et al.,* 1987; Mathys *et al*., 1993; El-Domiaty *et al.* 1994; Lehmann *et al*., 1990; Lee 1995; Al–Obaid *et al.* 1998; Lebelle *et al.*, 1993; Ripani *et al.*, 1996; Krizevski *et al.*, 2007, 2008;

Dawson *et al*., 1994; Dagne *et al*., 2010). However, some of the techniques are non specific and some of them required lengthy sample preparation and clean up protocols.

Lehmann *et al*. (1990) reported the applicability of thin layer chromatography (TLC) for rapid qualitative screening test of khat alkaloids. They extracted the analytes from the finely chopped plant material with methanol under ultrasonication and the crude extract was applied for identification under UV light after it has been sprayed with ninhydrin reagent. Lee (1995) also proposed the same methodology for monitoring cathinone stability under different preservation conditions. However, the author used methanol and solid-liquid extraction (maceration) followed by extensive liquid-liquid extraction under alkaline condition. Neither of the works mentioned the possibility of differentiating the two diasteriomers (cathine and norpehdrine). Mathys *et al*., 1993) reported the applicability of TLC densitometer for quantitative determination of khat constituents after extraction of the samples in methanol using an ultrasonic assisted extraction (UAE) protocol. The authors could not separately quantified cathine and norephedrine

Due to poor accuracy and precision of TLC and time consuming nature of the scanning process, HPLC-DAD has been widely used and repeatedly reported (Geisshusler *et al*.,1987; Mathys *et al*., 1993; Al Domity, 1994; Laussmann *et al*., 2010).

Geisshusler *et al*. (1987) reported solid-liquid extraction (maceration) followed by extensive clean up protocol. The authors used 15 g of sample and 100 mL of extraction solvent. Afterwards repeated liquid-liquid extraction to remove the interferents and, the analytes were precipitated with oxalic acid and then re-disolved with appropriate solvent. Mathys *et al*., 1993 significantly reduced the sample size (4 g), solvent (50 mL) and extraction time (45 min). They applied ultrasonication and SPE extraction using C_{18} to clean up the co-extractives. However, the method still suffered from limitations as the extraction and clean up steps is carried out separately and conditioning, washing and elution steps are time consuming during SPE clean up.

El-domiaty *et al.* (1994) used about 4 g of fresh sample and extracted using 250 mL on a mechanical shaker for 10 hrs. The filtrate was repeatedly cleaned up using benzene and conducted liquid-liquid extraction under alkaline condition.

GC-FID and GC-MS have also been reported for identification and quantification of confiscated khat samples in the forensic laboratories (Krizevski *et al.*, 2007, 2008; Ripani *et al.*, 1996; Lebelle *et al.*, 1993 Gambaro *et al*., 2012)

Until recently, GC-MS method for the detection but not quantification of phenylpropyl amino alkaloids in khat plant has been reported (Krizevski *et al.*, 2007). For qualitative identification purpose, two GC-MS methods have been reported to identify the khat alkaloids from the plant extract after solid-liquid extraction and repeated liquid-liquid extraction using non-polar organic solvents and derivatizing the isolated alkaloids using (R)–(+)-α-methoxy-α-(trifluoromethyl) phenyl acetic acids (Ripani *et al.*, 1996; Lebelle *et al.*,1993).

Krizevski *et al.*, 2007 proposed a GC-MS method for quantitative determination of the alklaloids following the same extraction protocol but without derivatizing the extracts. However, the authors didn't differentiate between cathine and norephedrine. Since cathinone is sensitive to alkaline conditions (Szendei, 1980) extensive sample preparation should be avoided. Furthermore, LLE usually produces emulsion that may decrease the extraction efficiency and lengthen the time the analyst needs to complete the procedure and hence leads to analytes lose.

Because of increased confiscation of khat samples at the air ports of different European countries, forensic laboratories have proposed alternative analytical methods for the quantitative determination of the alkaloids (Chappell *et al*., 2010; Laussmann *et al*., 2010; Gambaro *et al*., 2012). Recently, Gambaro *et al.* (2012) developed GC-FID method for quantification of the alkaloids from seized khat samples at airports in northern Italy. Extraction was conducted over night using methanol followed by sequential liquid-liquid extraction. The method is extremely laborious and susceptible to cathinone degradation since cathinone is unstable in protonic solvents (Szendei, 1980; Krizevski *et al.*, 2007).

An indirect spectrophotometric method has also been reported by Al–Obaid *et al.* (1998) for quantitative determination of cathinone in khat plant extracts. The proposed method was based on treating the reductant cathinone with copper (II) neocuproine reagent followed by measuring the absorbance of copper (I) neocuproine complex at 455 nm.

This method looks simple; however it is not accurate and non specific, since the reagent stains other ketones present in khat leaves.

Dawson *et al.* (1994) Proposed nuclear magnetic resonance spectroscopy (NMR) technique for the determination of the relative amounts of the cathinone enantiomers and the relative amounts of the norephedrine/norpseudoephedrine diastereomers present in the plant at the time of analysis using air dried and freeze dried samples. However, prior to determination, isolation of the pure alkaloids from the extract is a must following several liquid-liquid extractions as per the authors claim.

The aforementioned sample preparation techniques need a large amount of sample, and high-quality organic solvents, and require intensive manipulation of the extracts. These methods are therefore expensive, both in terms of time and material consumption. The development of faster, more cost effective and environment friendly procedures is therefore a pressing demand.

2.6 Phenolic compounds

2.6.1 Types of phenolic compounds

Phenolic compounds constitute a large group of about 8000 compounds with varied structures and chemical properties (Vermerris *et al*., 2006; Sudjaroen, 2009; Crozier *et al*., 2006).

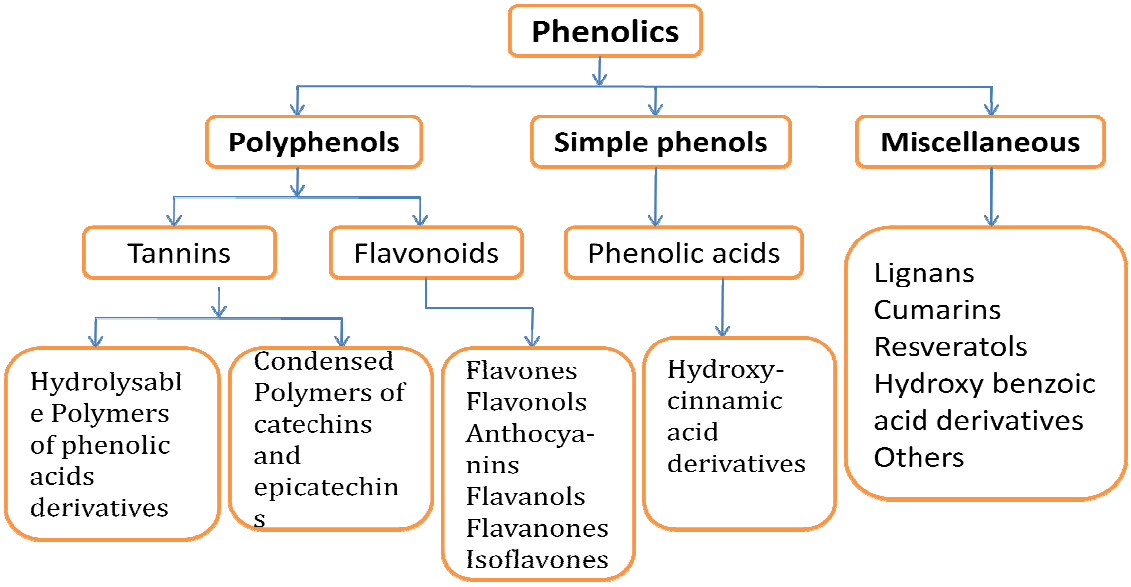
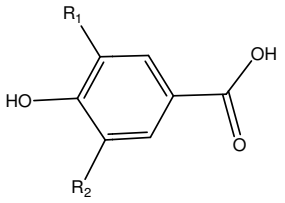


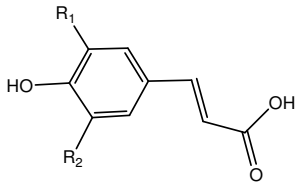
Figure 2.6 General classifications of phenolic compounds

In general, they are substances containing one or more aromatic rings with one or more hydroxyl groups and can be classified in three main categories: simple phenols, which include phenolic acids, polyphenols constituted by flavonoids and tannins; and a miscellaneous group that comprises compounds that not included in the two groups (Vermerris *et al*., 2006; Dai *et al*., 2010; Grotewold, 2006; Crozier *et al*., 2006) (Figure 2.6). Phenolic acids can be divided into two classes: derivatives of benzoic acid such as gallic acid, and derivatives of cinnamic acid such as coumaric, caffeic and ferulic acid. Caffeic acid (Figure 2.7) is the most abundant phenolic acid in many fruits and vegetables.



p-Hydroxybenzoic acid, $R_1 = R_2 = H$
Protocatechuic acid, $R_1 = OH$, $R_2 = H$
Vanillic acid, $R_1 = OCH_3$, $R_2 = H$
Syringic acid, $R_1 = R_2 = OCH_3$
Gallic acid, $R_1 = R_2 = OH$

(a)



p-Coumaric acid, $R_1 = R_2 = H$
Caffeic acid, $R_1 = OH$, $R_2 = H$
Ferulic acid, $R_1 = OCH_3$, $R_2 = H$
Sinapic acid, $R_1 = R_2 = OCH_3$

(b)

Figure 2.7 Structures of common phenolic acids: (a) benzoic acid and derivatives; (b) cinnamic acid and derivatives

Flavonoids constitute the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds (Harborne *et al*., 2000). The basic flavonoid structure is the flavan nucleus, containing 15 carbon atoms arranged in three rings (C6-C3-C6), which are labeled as A, B and C (Figure 2.8).

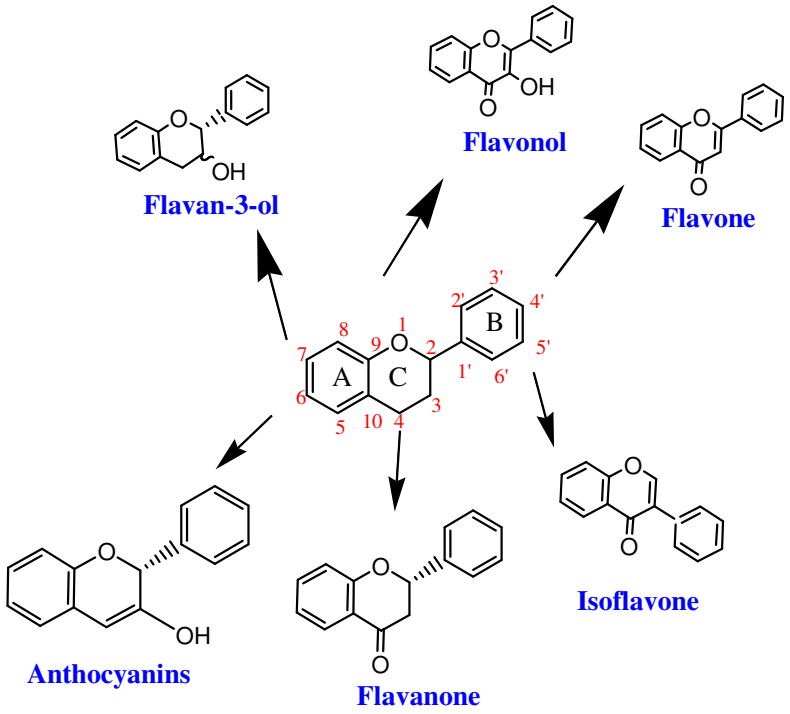


Figure 2.8 Major flavonoids structural units (Crozier *et al*., 2007)

The miscellaneous group comprises all other phenolic compounds not classified into the distinct subgroups. These include lignans, lignins, coumarins, stilbenes derivatives like resveratrol, and other phenolic compounds (Crozier *et al*., 2007).

Anthocyanins are water-soluble pigments and are responsible for providing red, blue and violet colors found in most plant species. There are six major anthocyanidins and these are cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin (Dai *et al*. 2010). On the other hand, flavonols are the most prevalent flavonoid in the plant kingdom

(Harborne *et al*., 2000). Commonly occurring flavonol aglycones include kaempferol, myricetin and quercetin.

Tannins comprise a group of polyphenols in plants with a wide diversity in structure that share the ability to bind and precipitate proteins and are usually subdivided into two groups: (1) hydrolysable tannins and (2) condensed tannins. Hydrolysable tannins are based on gallic acid, ellagic acid, protocatechuic acid polymers resulting gallotannins or ellagitannins, and condensed tannins or proanthocyanidins which are composed of flavanol polymers (Khanbabaee *et al*., 2001; Vermerris, 2006; Luthria, 2006).

2.6.2 Antioxidant and medicinal properties of phenolic compounds

In addition to their contributions to the sensory properties (astringency due to tannins), flavours to food, and color due to anthocyanins, there is significant interest in phenolic compounds due to the antioxidant and free radical scavenging abilities associated with these compounds.

Free radicals produced by radiation, chemical reactions and several redox reactions of various compounds in living tissues and cells contribute to protein oxidation, DNA damage, and lipid peroxidation (Dai *et al*., 2010; Halliwell, 1996).These oxidative stresses are common in such chronic health disorders as cancer, atherosclerosis, diabetes and liver cirrhosis (Dai *et al*., Mumper, 2010; Khanbabaee *et al*., 2001).

Generally protection against the toxicity of these free radicals is provided by antioxidant defenses including phenolic compounds, enzymes (e.g. glutathion peroxidase, catalase and superoxide dismutase), proteins (e.g. ferritin) and vitamins (A, C, E). Under a normal physiological state, research evidences point to the fact that these antioxidants have the capacity to perfectly regulate the production of reactive oxygen species (ROS). Thus antioxidant compounds can delay, inhibit, or prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress and hence reduce the risk of chronic diseases. (Rice-Evan *et al.,* 1996; Robards *et al*., 1999; Halliwell, 1996; Yang *et al*., 2001).

Recently, phenolics have been considered as powerful antioxidants and proved to be more potent antioxidants than Vitamin C and E and carotenoids (Rice-Evan *et al* 1996).

2.6.3 Mechanism of antioxidant activity of phenolic compounds

The antioxidant activity of phenolic compounds is regarded to be related to 1) scavenging free radicals, 2) chelating transition-metals involved in free-radical production and 3) inhibiting the enzymes participating in free-radical generation (Dai and Mumper, 2010; Halliwell *et al*., 1995; Halliwell, 1996).

Phenolic compounds (ArOH) act as free radical acceptors and chain breakers. They interfere with the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals $R\cdot$:

$$R\cdot + ArOH \rightarrow RH + ArO\cdot$$

The phenoxy radical intermediates ($ArO\cdot$) are relatively stable due to resonance and therefore a new chain reaction is not easily initiated. Moreover, the phenoxy radical intermediates also act as terminators of propagation route by reacting with other free radicals (Aruoma, 2002; Dai *et al.,* 2010):

$$ArO\cdot + R\cdot \rightarrow ArOR$$

In the case of phenolic compounds, the antioxidant activity depends on the numbers and positions of the hydroxyl groups in relation to the carboxyl functional group (Rice-Evans *et al*., 1996; Robards *et al*., 1999). Monohydroxy benzoic acids with the –OH moiety at the *ortho*- or *para*-position to the –COOH show no antioxidant activity, though the same is not true for the *m*-hydroxybenzoic acid (Rice-Evans *et al.*, 1996). The antioxidant activity of phenolic acids increase with increasing degree of hydroxylation, however, substitution of the hydroxyl groups at the 3- and 5-position with methoxy groups as in syringic acid reduces the activity (Rice-Evans *et al.*, 1996).

2.6.4 Determination of antioxidant activity

In vitro methods to determine free radical scavenging activity often involve the use of chemicals to generate free radicals so that the radical scavenging ability of the test antioxidant can be determined (Badarinath *et al*., 2010; Aruoma, 2002). Several methods have been developed in which the antioxidant activity is assessed by the scavenging of synthetic radicals in polar organic solvents. Common synthetic radicals used include 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-3-ethylbenzthiazolinesulphonic acid (ABTS) radicals, N,N-dimethyl-*p*-phenylene-diamine (DMPD) and 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) (Thaiponga *et al*., 2006; Badarinath *et al*., 2010; Aruoma, 2002).

One of the most frequently used methods is the DPPH assay (Williams *et al*., 2004). In this test, the scavenging of the stable DPPH radical (DPPH•) by an antioxidant (AH) is measured colorimetrically. The test antioxidant is allowed to react with DPPH• in methanol and the reduction of the radical by the AH is followed by monitoring the decrease in absorbance of the solution at 515 nm. In its radical form, DPPH• absorbs at 515 nm but upon reduction by an antioxidant, the absorption disappears (Thaiponga *et al*., 2006).

2.6.5 Methods for total phenolics assay

Because of the heterogeneity of natural phenolics in plant extract, it is much more tedious and expensive to exhaustively quantify individual phenolic compounds. Rather, determination of total phenolic content of plant extract is usually carried out using spectrophotometric methods (Padda *et al*., 2007; Singleton *et al*., 1999). For total phenolic determination, various approaches have been reported. All phenolic compounds absorb in the UV region and have maximum absorbance around 280 nm because of the presence of benzene ring in their structure. Thus direct measurements of total phenolic compounds at this wavelength have been reported. However, this method lack specificity since other non-phenolic compounds may have absorbance around this region (Herderich *et al*., 2005; Llaudy *et al*., 2004). Thus other alternatives have been reported.

A number of spectrophotometric methods for phenolic compound quantification in plant materials have been developed. Among such methods are the Folin-Denis method and Folin-Ciocalteu method. Both of the methods often employed for quantifying the total phenolic content of plant materials and beverages. They are based on the reduction of phosphomolybdic-phosphotungstic acid reagent to a blue-colored complex $[PMoW_{11}O_{40}]^{4-}$ in alkaline solution that occurs in the presence of phenolic compounds (Singleton *et al*., 1965; Singleton *et al*., 1999). Gallic acid or tannic acid is widely used as the comparison standard and values are usually compared as milligram of gallic acid equivalent per gram or liter of extract among samples.

Recently, Padda *et al*., (2007) compared Folin-Denis and Folin-Ciocalteu reagents for the quantification of phenolic compounds in sweet potato and found that the Folin–Ciocalteu reagent gave an overestimation of total phenolic acids due to the absorbance of interfering compounds (that is, reducing sugars and ascorbic acid) and hence recommended to use Folin Denis reagent to quantify total phenolic compounds in sugar rich samples.

2.6.6 Methods for quantitative determination of tannins

A number of papers have been reported for total tannin determination in plant extracts. Currently existed analytical methods for tannin analysis have been reviewed by Herderich and Smith (2005). In summary, the methods can be broadly categorized into measurements based on colorimetric and, gravimetric methods and chromatographic analysis.

Colorimetric methods are most frequently used whereby tannins are first removed from the plant extract via precipitating with proteins (like gelatin, BSA, collagen fiber, etc) and polymeric adsorbents (like PVPP, polyethylene glycol, methyl cellulose, etc) (Bajaj *et al*., 1977; Hagerman *et al*., 1978; Liao *et al*., 1995; Herderich *et al*., 2005). Direct or indirect methods are used for the analysis. In case of direct method, the tannin-protein precipitate is dissolved in alkaline media like sodium dodecyl sulfate solution and reacted with ferric chloride reagent and absorbance of the red solution is measured at 510 nm (Hagerman *et*

al., 1978). In this case, after precipitation and centrifugation, the supernatant is removed and then the precipitate should be washed two to three times to remove the carry over non-tannin phenolics on the surface of the precipitate and on the wall of the container. In doing so, it takes some time during centrifugation at each washing stage and hence it is difficult for routine analysis. Furthermore, if the tannin content is small, then some of the precipitate may be lost during washing stage unless serious precautions are taken.

Other direct methods of analysis reported so far involve measurement of absorbance at about 280 nm of the original sample and non-tannin solution after removal of tannins with protein or PVPP and the difference in absorbance is taken and this absorbance difference belongs to absorbance due to tannins (Antoine *et al*., 2004; Herderich *et al*., 2005). In such a case, all phenolic compounds may not have exactly the same maximum absorbance at 280 nm. Secondly, protein also absorb at that particular wavelength (Herderich *et al*., 2005).

In addition, Bajaj *et al*., (1977) reported indirect (by difference) method of tannin analysis in tea infusion after removal of tannins with gelatin solution. They treated the original tea infusion and non-tannin filtrate with Folin-Denis reagent and absorbance was measured at 725 nm. The difference in absorbance value before and after treatments with protein is due to the absorbance of tannins in the solution. The authors did not mention the ratio of gelatin to tannin for effective precipitation. Secondly, effect of solution pH and reaction time was not mentioned. However a number of other reports have been made on wine tannins or astringency analysis using gelatin. In most of such cases, three days of extraction time has been reported (Llaudy *et al*., 2004).

Llaudy *et al*. (2004) proposed ovalbumin for astringency evaluation in red wine. They used direct method of analysis, i.e. absorbance of the wine samples measured at 280 nm before and after removal of tannins with ovalbumin. The authors also claimed as the protein solution interferes with wine phenolics absorbance. Secondly, they did not proposed optimum tannin to protein ratio as well as optimum pH of the buffer solution for efficient precipitation since tannin-protein precipitation is pH dependent (Hagerman *et al*., 1978). Furthermore, unlike plant tannins, most tannin in red wine exist in highly polymeric form due to the fact that during wine ageing most tannin polymerize with each

other and with other phenolics compounds. Thus, optimization of specific method for each type of tannin source is of particular interest since tannin/protein interaction is specific for different tannins as well as different proteins (Asquith *et al.*, 1986).

2.6.7 Phenolic compounds in khat and their importance

Few reports have been made on antioxidant activity of khat extract (Al-Zubairi *et al*., 2008; Dudai *et al.,* 2008). Al-Zubairi *et al*. (2008) reported that short term exposure to khat crude extract by rat showed excellent antioxidant activity and they found decrease in plasma malondialdehyde concentration and increase in serum antioxidants like uric acid. The authors suggested as phenolic compounds might responsible but no explanation was given about dose of phenolic compounds administered per unit extract.

Recently Dudai *et al.,* (2008) from Yemen reported that khat has significant antioxidant activities and found to contain up to 205 mg/g of dry khat leaves using chlorogenic acid as a control. Parallel to this, they have reported that, total phenolic compounds in oven dried leaves of 13 khat varieties were about 0.1 mg/g in dry weight basis, which is about less than 0.01% of the total dry mass. This result is far below the optimum phenolic compounds reported in any green plants having such high antioxidant activity. Hence, the data looks contradictory.

Thus, we suspected that the authors might make some unit error while reporting the result and/or reagents they have used and sample preparation method employed might had a problem. It has been reported that the authors used an oven to dry the sample which has significant effect on loss of some of the phenolic compounds (Dai *et al*., 2010). They also extracted the plant using methanol for 24 hours. Research indicated that prolonged extraction time will result significant loss of certain plant phenolics. In addition to the khat cultivars they have reported, the authors analyzed other plant species grown in their research center and they reported far below results comparing with similar work on the stated species from other locations.

Because of excellent antimicrobial, antioxidant and anti-cancer activity of flavonoids, research is undergoing to identify plants with such potential secondary metabolites

content. But there is paucity of information about flavonoids distribution in khat cultivars. Luftmann *et al.* (1974) (cited in Szendrei, 1980) have isolated and characterized few flavonoids in khat which includes kaempferol, quercetin and myricetin. However, their actual concentration is not given.

Oral administration of total aqueous khat extract or of its alkaloid fraction exacerbated the oxidative stress in restrained rats due to the decreased activity of antioxidant enzymes, i.e. superoxide dismutase, catalase, glutathione-S-transferase (Al-Qirim *et al*., 2002). Similarly, khat induced an increase in reactive oxygen species and a depletion of intracellular glutathione in the cell cultures of human keratinocytes and fibroblasts, the reactions that could be opposed by addition of exogenous antioxidants (Lukandu *et al*., 2008). But on the other hand, the flavonoid fraction of the khat enhanced the activity of the antioxidant enzymes in rats and thus could provide a protection against the oxidative stress (Al-Qirim *et al*., 2002). In addition, this fraction demonstrated an anti-inflammatory activity (Al-Meshal *et al*., 1986). However, the authors followed tedious extraction (about a week) and isolation steps, where a significant loss might occur during prolonged extraction period (Al-Qirim *et al*., 2002). Thus efficient extraction method for khat secondary metabolites is crucial for further studies.

The long-term health impact of chewing khat is not well established. Scattered reports from Yemen and elsewhere indicate that chronic khat consumption may be a leading cause of cancer, cellular toxicity and other metabolic disorders (Kassie *et al*., 2001; Al-Hebshi *et al*., 2005; Al-Ahdal *et al*., 1988). Most authors tried to link the effects with the alkaloid fractions (Feyissa *et al*., 2008; Karunamoorthi *et al*., 2010), while others suspected that tannins in the plant might be responsible to the effects.

Some authors have claimed that is a claim that khat polyphenols like tannins, which are mutagenic and carcinogenic in laboratory animals (Korpassy *et al*., 1950; Kirby *et al*., 1960; Bichel *et al*., 1968) might account for the effects of khat. It has been speculated that tannins in khat cause thickening of the mucosa of the esophagus and esophagopharynx (Kennedy *et al*., 1983; Karunamoorthi *et al*., 2010) and that these changes might lead to malignancy (Craddock, 1993). Furthermore, chronic chewers often complained of symptoms suggestive of stomatitis, oesophagitis, gastritis and constipation. Though no

systematic work has been done, there seems to be an agreement among researchers as well as khat users themselves concerning the gastrointestinal problems brought about by khat-chewing. These are due probably to the astringency of khat tannins ingested (direct effect) and the sympathomimetic action of khat amines (indirect effect) (Kennedy *et al*., 1983; Karunamoorthi *et al*., 2010; Craddock, 1993; Halbach, 1972; Hassan *et al*., 2002). Thus it is important to quantitatively investigate tannin contents in different khat cultivars for further study on this aspect or to generate supportive evidence for further clinical studies. Furthermore, chronic khat chewers should be aware that khat may interact with certain drugs. Attef *et al.* (1997) showed that khat reduced the absorption of the antibiotics amoxicillin and ampicillin from the gastrointestinal tract. This has been suspected that effect is due to the binding of tannins with the antibiotics.

Apart from mentioning the existence of khat tannins, no systematic work has been reported. Al-Hebshi *et al*. (2005) reported concentration of 18 mg of tannins per gram of leophilized total solid obtained after extraction with water. The result is not quantitative, i.e. it has to be reported in terms of dry mass or fresh weight basis of the plant. Secondly, water was used as extracting solvent which is less efficient for tannins extraction from plant material. Al-Motarreb *et al*. (2002) reported tannic acid concentration of 39 different khat cultivars grown in Yemen with concentration of 3-10 g/mg. The authors did not mention about the method of analysis and whether the data are for total tannins or particular tannic acid concentration. In addition, they did not mention whether it is in dry weight basis or fresh weight basis and the unit they reported is g/mg which is odd to understand.

2.7 Fluoride

2.7.1 Importance and distribution of fluoride in the environment

Fluoride is a naturally occurring essential element found in water, air, soil and food (World Health Organization, 2002; National Research Council, 1989). Small amounts of fluoride are vital for human since it prevents tooth decay by strengthening the tooth surface and inhibiting growth of cariogenic bacteria. It also assists in repairing the early stages of tooth decay (DePaola *et al*., 1983). Periodic assessments of fluoride intake from all sources are necessary as fluoride exposures above optimal levels can have negative health outcomes (Medical Research Council, 2002; National Research Council, 1989; World Health Organization, 2002), while low fluoride exposure may result in failure to achieve the benefits of fluoride exposure (DePaola *et al*., 1983).

The main sources of fluoride exposure for the general population are fluoridated drinking water, diet, air and toothpaste (Agency for Toxic Substances & Disease Registry, 2003; National Research Council, 1989). Other potential sources of fluoride exposure include industrial emissions, e.g. from phosphate fertiliser plants, aluminium plants and coal-fired power plants (Agency for Toxic Substances & Disease Registry, 2003; Hillman *et al*., 1979),

Rocks, soil, water, air, plants, animals and foodstuffs all contain fluoride in widely varied concentrations. Fluoride is found naturally in low concentration in drinking water and foods.

Seawater typically contains about 1 mg/L while rivers and lakes generally exhibit concentrations of less than 0.5 mg/L. In ground waters, however, low or high concentrations of fluoride can occur, depending on the nature of the rocks and occurrence of fluoride-bearing minerals. Concentrations in water are limited by fluoride solubility, so that in the presence of 40 mg/L calcium, it should be limited to 3.1 mg/L (Hem, 1989). It is the absence of calcium in solution, which allows higher concentrations to be stable (Edmunds *et al*., 1996). High fluoride concentrations may therefore be expected in groundwater from calcium-poor aquifers and in areas where fluoride-bearing minerals are

common. Fluoride concentrations may also increase in groundwater in which cation exchange of sodium for calcium occurs (Edmunds *et al*., 1996).

The common fluoride bearing minerals found in soil are fuorospar (CaF_2), sellaite (MgF_2), cryolite (Na_3AlF_6) and chiolite ($Na_5Al_3F_{14}$). The mobility of fluoride in soil is determined by the amount of clay minerals present, the soil pH, the adsorption of positively charged complexes, and the concentrations of Ca, Fe, Al and P in soil (Rao *et al*., 2003).

Although the primary etiological factor for causing fluorosis is mainly due to consumption of water containing large amounts of fluoride, evidence suggest that certain food items and beverages can add on to the body burden of fluoride (Bamidele *et al*., 1982; Pires *et al*., 1996; Kubakaddi *et al*., 2005). Factors that contribute to the fluoride concentration of food include the location where the food was grown and the use of fluoride containing fertilisers and pesticides (Agency for Toxic Substances & Disease Registry, 2003). Fluoride intake from dietary sources is generally higher in areas with fluoridated water supplies or where there are naturally occurring elevated concentrations of fluoride in the environment including drinking-water (Ophaug *et al.*, 1985; World Health Organization, 2002).

Excessive fluoride may lead to fluorosis, manifested as dental fluorosis and skeletal fluorosis. As for several of the essential elements, there is a suggested range of safe and adequate intake. For fluoride, safe intake is said to be 1.5–4.0 mg/day for adults and less for children (National Research Council, 1989). Fluorosis is prevalent in Ethiopia for the past many years and the effect of fluoride on human health has been clearly understood by now (Kloos *et al*., 1999; Nemade *et al*., 2002; Ayoob *et al*., 2006). The effect of fluoride on human health other than dental and skeletal fluorosis is still under study (Connett *et al*., 2010).

2.7.2 Fluoride in khat and tooth staining due to khat chewing

Few reports pointed out that regular uses of khat causes tooth discoloration, gum disease and oral cancer (Hill *et al.,* 1987; Hattab *et al*., 1999; Hailu *et al.* 2006).

Hill *et al.* (1987) conducted case studies on chronic Yemeni khat chewers and they found positive correlation of khat chewing and tooth staining. They reported as khat leaves accumulated 360 ppm of fluoride and could be one cause of staining. However, they did not mention method of sampling, extraction and analysis.

Surprisingly contradicting data have been reported from the same country by Hattab *et al*. (2000) on khat fluoride following systematic procedure. But they extracted water soluble fluoride within 30 second using sonicator which is too short to leach fluoride from the leaves. They reported as Yemeni khat found to posses negligible amounts of F^- in the khat leaves, < 0.18 µg F^-/g fresh weight basis, 0.93 µg total F^-/g in dried leaf and 2.1 µg total F^-/g in ash. But, they did not evaluated effect of leaf maturity on the distribution of fluoride.

Furthermore, Hailu *et al*. (2006) conducted a case study on East African chronic khat chewer and found intrinsic and extrinsic stain on the surface of the teeth. The authors concluded that various chemical components in the leaf and some other multiple external sources such as sugar and drinks taken during chewing process cause external and possible internal teeth stains. The cause of internal staining may be from swallowing of the khat juice due to the fluoride content of the khat leaves (Hailu *et al*., 2006). Thus, khat fluoride and tooth discoloration due to khat chewing is controversial and inconclusive and there is paucity of information about fluoride accumulation in khat.

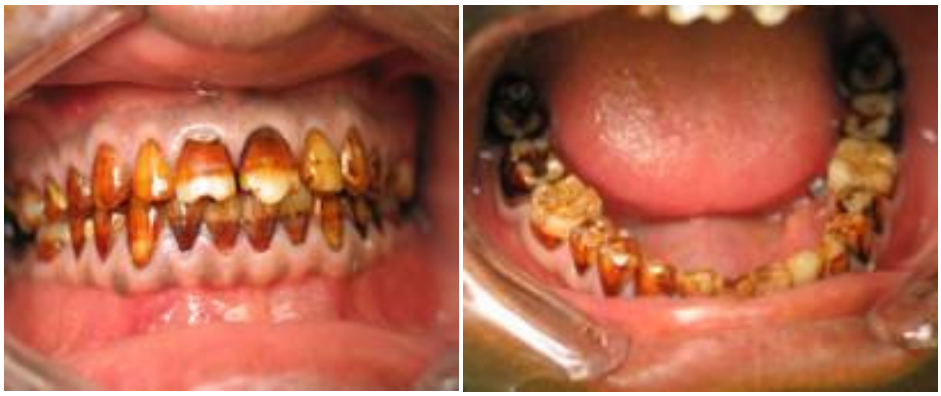


Figure 2.9 Stained tooth of case studied an East African chronic khat chewer (Hailu *et al*., 2006)

It is known that accumulation of fluoride in the plant varies from place to place depending on its availability in the soil and water for irrigation (Häni, 1978; Elrashidi *et al*., 1985, Singh *et al*., 1995; Fung *et al*., 1999). Furthermore, the nature of the soil and its pH affects the bioavailability of fluoride in the different parts of the plant (Fung *et al*. 1999; Miller *et al*., 1999). In the past, khat was grown in natural soil but nowadays fertilizers are used to boost production. It is also known that plants can take up more fluoride when the mineral fertilized soil is used (Hillman *et al*., 1979; Lemessa, 2001).

Some parts of Ethiopia are in the rift valley region where fluoride predominantly exists in the soil and water (Malde *et al.* 1997; Kloos *et al*., 1999). Some of these areas are best known in khat production. Thus, khat grown in these areas may conceivably accumulate fluoride and thereby contribute to the daily fluoride intake. Therefore, khat cultivars grown in the rift valley and non-rift valley regions need to be systematically assessed for their accumulation of fluoride in different position of the leaves so as to resolve the controversies and to evaluate the contribution of khat to total fluoride intake of the chewers per day.

2.7.3 Methods for fluoride determination

To date various analytical methods have been reported for the quantification of fluoride in biological and environmental samples. Campbell (1987) reviewed most of the sample preparation and detection techniques developed for identification and quantification of fluoride in different matrices. The most widely used method of fluoride quantification has involved potentiometery, spectrophotometry, gas chromatography (GC), ion chromatography, atomic and molecular spectroscopy and others (Stevens *et al*., 1995; Campbell, 1987; Barghouthi *et al*., 2012). However (GC), spectrophotometry, atomic and molecular spectroscopy methods either lack sensitivity, time consuming and/or not so tolerant to interferences.

Because of its excellent performance, speed, and general convenience, potentiometery (specifically fluoride selective electrode) has become an important method for determining fluoride in a wide variety of biological, environmental and industrial samples (Campbell, 1987).

Ion selective potentiometirc methods are simple to perform and have good precision and sensitivity. Recoveries are usually >90%, but this is dependent on the type of sample and the sample preparation required. The method detects only free fluoride ions in solution. Because of the inherent restriction of this technique, several approaches have been recommended to prepare the sample for analysis. The most common methods for decomposition of biological materials used are open ashing, fusion, oxygen combustion and acid digestion (WHO, 1984; Campbell, 1987, Haldinmann *et al*., 1993). Each is probably relevant for a particular type of material but it would be unwise to consider that each is in anyway universal in application (Campbell, 1987)

Fusion (especially alkali fusion) is a powerful technique both for organic matrices and those with a high silica and alumina content (e.g. rock, dust, slags, ashes) having relatively high contents of trace elements.

Acid digestion and alkali fusion are most frequently used methods to librates fluoride from plant material. Stevens *et al.* (1995) compared the efficiency of the two methods

using a range of plant materials and certified reference materials. Acid digestion procedures tested failed to obtain certified value for fluoride in certified reference material (SRM). This was due to failure of acids to liberate fluoride bound strongly within silicate minerals found in the plant materials. The liberated fluoride as hydrogen fluoride (HF) could also volatilize during the acid digestion. Acid digestion is therefore, not recommended for determination of total fluoride (Stevens *et al.,* 1995).

2.8 Mineral nutrients

2.8.1 Existence and importance of selected mineral nutrients in plant and soil

The human body requires a number of minerals in order to maintain good health. A number of minerals essential to human nutrition are accumulated in different parts of plants: minerals essential for growth from the environment and metals such as Cd, Co, and Ag which are of no known direct benefit to the plant (Tan, 1996; Grusak *et al*., 1999). It has been reported that traces of Cd and Pb can be detected in all plants and foodstuffs (Kabata-Pendias *et al*., 2001). Thus plants are intermediate reservoirs through which trace elements from soil, and partly from water and air, move to man and animal.

The plant nutrients may be divided into macronutrients and micronutrients. Macronutrients are found and needed in plants in relatively higher amounts than micronutrients. Following this classification based on the element content in plant material, the following elements may be defined as macronutrients: C, H, O, N, P, S, K, Ca, Mg, Na, Si). The micronutrients are: Fe, Mn, Cu, Zn, Mo, B, Cl. This division of the plant nutrients into macro- and micronutrients is somewhat arbitrary. The Fe or Mn content of plant tissues for example is sometimes nearly as high as the content of S or Mg. The content of the micronutrients is often in excess of physiological requirements, which is true for Mn for example (Franzle, 2010; Grusak *et al*., 1999; Akinrinde, 2004).

A micronutrient is an element that plants must have to complete their life cycles but need only in a small amount. These elements have often been called *trace elements* or *minor elements,* but micronutrient is the preferable term (Akinrinde, 2004; Franzle, 2010). The

terms *micronutrient* and *trace element* must not be construed to imply that these nutrients are somehow less important than the macronutrients. To the contrary, the effects of micronutrient deficiency can be very severe in terms of stunted growth, low yield, and even plant death. For most essential trace elements a high intake causes toxicity (Akinrinde, 2004). Heavy metals have come to the forefront as dangerous substances and are considered as serious chemical health hazards for human and animals (Loftleidir *et al.,* 2005; Kabata-Pendias *et al.,* 2001; Ajasa *et al.,* 2004).

The main factor controlling the mineral content of plant material is the specific, genetically fixed nutrient uptake potential for the different mineral nutrients. This accounts for the fact that the N and K content of green plant material is about 10 times higher than that of P and Mg which in turn is about 100 - 1000 times higher than the content of the micronutrients. This general pattern occurs in all species of higher plants. The second factor controlling the mineral content of plant material is the availability of plant nutrients in the nutrient medium (Iskandar *et al.,* 2001; Voutsa *et al*., 1996; Loftleidir *et al.,* 2005: Kabata-Pendias, 2004, Anke, 2004; Begerow *et al*., 2004; Nouri *et al*., 2009). To all of these, can be added other sources generated by agricultural technologies such as: irrigation with wastewater, the administration of organic and mineral fertilizers with a load of heavy metals, or application of pesticides, which contain in their structure such chemical elements (Prabu, 2009; Hart *et al*., 2005; Nwachukwu, 2008; Ramadan *et al.,* 2007).

The nature of soil is considered one of the most important factors that determine heavy metal content of food plants, probably because this is the binding and retention site for the toxicants (Kabata-Pendias, 2004; Nouri *et al*., 2009; Anke, 2004; Itanna, 2002; Iskandar *et al.,* 2001).

The mineral content of plants is also very much dependent on age. Young plants and young plant tissues have high contents of N, K and P, whereas in older plants and more mature plant parts, higher contents of Ca, Mn, Fe and B are often observed (Mengel *et al.,* 1978). Mineral content in the plant not only depends on nutrient availability in the

soil, but it is also affected by other factors such as the kind of plant organ or tissue, the age of the plant and the supply of the plant with other plant nutrients mentioned above.

The plants rate of absorption of nutrients involves processes going on in both the plant's root and the soil (Akinrinde, 2004) Soil properties are greatly influenced by its pH. Soil pH values can differ widely from values of about 3 to as high as 10. High H^+ concentration favour the weathering of minerals resulting in a release of various ions such as K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, and Al^{3+} (Kabata-Pendias, 2004; Akinrinde, 2004, Iskandar *et al.,* 2001).

2.8.2 Levels of metals in the khat plant

There is an extensive literature for khat, providing information about its history, chemistry and pharmacology, and exploring the social, economic, medical, psychological, and oral aspects of its use (Kalix, 1990; Griffiths *et al*., 1997; Abdulwaheb, 2007; Toennes *et al*., 2003; Al-Motarreb *et al*., 2002; Lemessa, 2001; Al-Hebshi *et al.,* 2005). Despite this extensive literature, studies that have investigated its mineral nutrients (both essential and non-essential) are much less than one may expect. Furthermore, the correlation between minerals in the soil and in the different khat cultivars is still not studied.

Thus, khat is becoming more popular all over the country and other parts of the world, having detail documents focusing on essential and non-essential minerals is very important to evaluate the total mineral intake of the individual who is using this plant regularly. Furthermore, analyzing the soil where khat is largely grown is very important to correlate accumulation of a particular metal in the plant and in the soil. This intern has an advantage to control the soil parameters and locate source of pollution based on the available data so as to take action.

Except the work reported by Matloob, (2003) about the concentration of Zn, Cu, Cd and Pb in khat sample collected from Yemen, no work has been done on composition of minerals in khat cultivars. Thus, there is still paucity of information on the extent of essential and non-essential metals in khat and soil where the plant is cultivated as well as

their toxicological implications to the chewers. It has to be noted that khat is subject to a wide range of insect pests, diseases, weeds, and animals that damage its leaves, newly growing shoots, stems, and roots. As a result, uses of synthetic insecticides are becoming increasingly common (Lemessa, 2001). In addition to pesticides application, uses of fertilizers and irrigation with contaminated sewage are the common practice among khat cultivars which are expected to be major source of contamination.

3. DEVELOPMENT OF SAMPLE PREPARATION TECHNIQUES FOR THE DETERMINATION OF KHAT ALKALOIDS

3.1 Background

The motive behind sample preparation can be multi-fold: to increase the efficiency of an assay procedure, to eliminate or reduce potential interferences, to enhance the sensitivity of the analytical procedure by increasing the concentration of the analyte in the assay mixture, and sometimes to convert the analyte of interest to a more suitable form that can be easily separated, detected, and/or quantified (Dietz *et al*., 2007; Tang *et al*., 2009; Luthria *et al*., 2006).

Major advances have been made over the last decade in the area of bioactive and controlled substances analyses in biological and pharmaceutical samples for biomedical and forensic science application. The major work was mainly in the development of analytical instruments. Despite the fact that these instruments are so sophisticated, they cannot handle biological samples directly and hence sample preparation is still a vital part of the analytical process (Luthria *et al*., 2006; Wille *et al.,* 2007; Robards, 2003; Smith *et al*., 2007; Thieme *et al.,* 2003). An effective sample preparation protocol is essential to achieve reliable results and maintain instrument performance. Analysis of "clean samples" also reduces the time to sample processing and in turn the cost of the assay (Kinsella *et al*., 2009).

Sample preparation impacts nearly all the assayed steps and is critical for unequivocal identification, confirmation and quantification of analytes. Generally, a clean sample results in improved separation and detection, while a poorly treated sample may invalidate the whole assay (Tang *et al*., 2009; Luthria *et al*., 2006).

Sample preparation protocols reported for khat alkaloids analysis has been reviewed in chapter 2 (section 2.5). Some of the methods reported require multistage isolation and clean up steps which are prone to analyte loss, are laborious and time consuming. Whereas, some of the methods are non selective towards the analytes of interest and need

a large amount of sample, high-quality organic solvents, and require intensive manipulation of the extracts.

As a result, there is a great need for change in analytical sample preparation procedures. This need led to the development of alternative efficient and modern extraction methods which are fast, consume less solvents and are cost effective, environmentally friendly and allow selective extraction of the desired alkaloids so as to facilitate the currently expanding health aspect studies (both *in vivo* and *in vitro* studies) as well as to monitor the free circulation of the vegetable by the forensic regulatory bodies.

This chapter thus presents the sample preparation techniques that have been developed for the quantitative analysis of the psychoactive phenylpropylamino alkaloids from khat (*Catha Edulis* Forsk) leaves. They include matrix solid phase dispersion (MSPD), molecularly imprinted solid phase extraction (MISPE) and quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction based methods. For each method, separate background, experimental section, results, discussion and conclusion has been given in the following subsequent sections.

3.2 MATRIX SOLID PHASE DISPERSION

3.2.1 Background

Matrix solid phase dispersion (MSPD) was developed in 1989 (Barker *et al*., 1989) and, during the recent years, this method has found again its important place among the preparation techniques applied in the analysis of plant and food samples.

Matrix solid phase dispersion (MSPD) has found particular application as an analytical tool for the preparation, extraction and fractionation of solid, semi-solid and/or highly viscous biological samples. Its simplicity and flexibility have been cited as contributing to it being chosen over more classical methods for these purposes (Barker, 2000).

3.2.2 Basic procedure

The basic set-up for MSPD has been described in detail (Barker *et al*., 2000). MSPD is based on the solid phase dispersion of the sample matrix for the subsequent isolation of various analytes. In the first step, samples are ground with a solid sorbent in a mortar using a pestle in order to disrupt the structure of the raw material, and achieve its homogeneous distribution around the sorbent particles. The blend is then transferred to a column or cartridge, from which analytes possessing various chemical properties can be isolated by the elution profile of solvents (or their mixtures) with different elution powers and polarities. The main advantage is the fact that this technique allows to perform several steps in the sample preparation simultaneously (Barker, 2007; Barker *et al*., 1989).

Grinding or blending with a mortar and pestle destroys the sample architecture through applied shearing forces. The sorbent material, for example C_{18}, serves to assist in the sample disruption process by solubilizing integral lipids, seemingly unfolding and disrupting the cell membranes and internal substructures themselves, thus acting like a solvent or detergent that dissolves and disperses the sample components. This greatly enlarges the surface area for the extraction and the sample components are distributed over the surface according to their relative polarities (Barker, 2000; Kristenson *et al*., 2006).

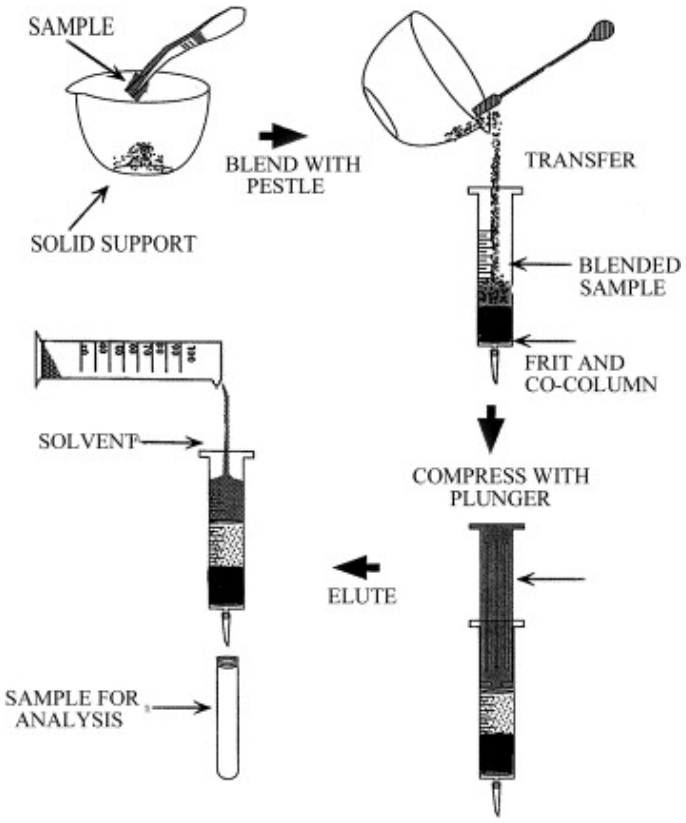


Figure 3.2.1 Steps in a typical MSPD extraction

Regarding the elution process, both retentive and non retentive mode can be applicable depending on the analytes of interest, elution solvent type and the solid sorbent material. In case of retentive mode of MSPD, the target analytes are retained on the column and interfering compounds are eluted in a washing step while, next, the target analytes are eluted by a different solvent. But in the case of non retentive mode of MSPD, interfering matrix components are selectively retained on the column and the target analytes directly eluted. Then additional clean-up is performed or the sample is directly analyzed (Barker, 2000; Kristenson *et al*., 2006). The scheme of the MSPD process is illustrated in Figure 3.2.1.

3.2.3 The parameters of MSPD extraction

There are many factors that affect the MSPD procedure. As it has been reviewed by Barker (2000), Barker (2007), Kristenson *et al*. (2006), Zhao *et al*. (1999), Chu *et al*. (2005), García-López *et al*. (2008) factors like (1) the nature of the solid support (silica versus polymeric, pore size, endcapping), (2) the nature of the bonded phase (normal-phase versus reversed-phase, total carbon content), (3) pretreatment or modification of the sample (pH adjustments, etc.), (4) choice of elution solvents and the sequence of their application (5), the nature of the sample matrix, (6) the elution volume and (7) the sample to solid support ratio should be taken into account while developing a method for a particular analysis.

The interactions between the individual components and the analyte of interest in MSPD involve the analyte with the solid support, the analyte with the bonded phase, the analyte with the dispersed matrix, the matrix with the solid support, the matrix with the bonded phase; all the above components interact with the elution solvents, and these dynamic interactions act simultaneously (Kristenson *et al*., 2006; García-López *et al*., (2008).

The selectivity of MSPD procedure depends on the sorbent/solvent combination used. Most methods reported to date use reversed-phase materials, such as C_8- and C_{18}-bonded silica as the solid support. Primary and secondary amines (PSA) have also found to be effective for fat containing matrices. The bonded alkyl chains contribute to dissolve their components, providing relatively clean extracts from fatty matrices. Normal-phase, non-bonded sorbents (Florisil, alumina, and silica) have also been optimized and applied for numerous analytes and matrices (Barker, 2000; Barker 2007, Kristenson *et al*., 2006; Zhao *et al*., 1999; Chu *et al*., 2005, García-López *et al*., 2008).

Particles less than 20 μm of the sorbent material can prolong the procedure time and they decrease the flow rate through the MSPD column. The particle size of 40–60 μm is optimal but sorbents with the size of about 100 μm can also be used (Kristenson *et al*., 2006).

The generic sample/sorbent ratio is approximately 1:4 but it can be modified according to the chemical properties of the analytes and the consistency of the sample matrix. Similar to SPE assay, the conditioning is very important. The choice of the washing and eluting solvents is also very important. If additional cleaning step is necessary, it is possible to use a MSPD column with another sorbent (Florisil, Silica, Alumina) at its bottom (co-column) (Kristenson *et al*., 2006).

Many studies have demonstrated the feasibility of matrix solid-phase dispersion (MSPD) that meets the above-listed requirements, for the isolation of biologically active compounds (herbicides, pesticides and other pollutants) from various matrices and few bioactive natural products from medicinal plants (Ziakova *et al*., 2003; Fernandes *et al*., 2007; Xiang *et al*., 2009; Wang *et al*., 2011; Teixeira *et al*., 2005; Kristenson *et al*., 2006; Kristenson *et al*., 2006; García-López *et al*., 2008) and to the best of our knowledge, no report has been made for khat alkaloids extraction.

3.2.4 Objectives of the study

The main objectives of this work were (i) to develop MSPD-HPLC-DAD (DAD, diode array detection) procedure for the analysis of khat alkaloids in various khat cultivars/varieties and compare with the conventional methods (ultrasonication followed by SPE clean up and (ii) to study the variation of alkaloids in khat of different geographical origin.

3.2.5 Experimental

3.2.5.1 Apparatus, chemicals and reagents

All the reagents used were of analytical or HPLC grade. Methanol and acetonitrile (Merck KGaA, Darmstadt, Germany), orthophosphoric acid, hydrochloric acid, diethyl ether and NaOH (Merck Chemicals, Gauteng, South Africa), tripropylamine (Fluka, Switzerland), (–)-norephedrine [(1*R*) (2*S*)-norephedrine and (+) ephedrine hydrochloride were purchased from Sigma Aldrich. (–)-Cathinone oxalate was isolated from the fresh

leaves of the plant. Primary and Secondary amine (PSA) QuEChERS bulk sorbent, C_{18} EC QuEChERS bulk sorbent, Silica SAX QuEChERS bulk sorbent and Isolute C18 (EC) SPE columns were obtained from Agilent Technologies (USA). The reagent water used was obtained from MilliQ system (Millipore, Milford, Mass, USA). The mobile phase was filtered through a Whatman membrane filter (47 mm diameter and 2 µm pore size) while all plant extracts were filtered through Acrodisc syringe filter (PVDF membrane with 0.45 µm pore size).

Standard solutions

Standard stock solutions of cathinone oxalate (1 mg/mL), norphedrine (1 mg/mL) and (+) ephedrine in MilliQ water were prepared and stored at 4 ^{o}C. Calibration standard solutions were prepared by appropriate dilution of aliquots of the stock solutions containing 100 µg/mL internal standard (IS). The resulting solution was evaporated on a rotary evaporator under vacuum at a temperature of 40 ^{o}C to dryness and reconstituted with the mobile phase.

3.2.5.2 Equipment

The analysis was performed on an Agilent 1200 Series HPLC (Agilent Technologies Inc. Santa Rosa, CA, USA) equipped with a binary pump and a DAD set at 200 nm. Separation of the compounds was achieved on an Agilent ZORBAX SB-Phenyl column (4.6 x 250 mm, 5 micron). The data was processed by the Agilent ChemStation for LC/MS 2D system software. A reverse-phase method developed by Mathys *et al.,* (1993) was employed with some modification (using different column, mobile phase composition, and amine modifier) to separate and quantify the alkaloids. The mobile phase consisted of aqueous buffer of pH 2.65 (8.5 g/L orthophosphoric acid and 0.3 mL/L tripropylamine) (solvent B) and 5% acetonitrile in water (solvent A) at a flow of 1.5 mL/min in a gradient profile as was follows: 0-10 min (77% B in A, isocratic); 10-11 min (77-30% B in A, linear gradient); 11-18 min (30% B in A, isocratic). The mobile

phase was filtered under vacuum through a 0.45 µm-nylon membrane filter and degassed by sonication.

3.2.5.3 Plant material collection

All the khat samples were collected from different regions of Ethiopia and processed in the laboratory of Addis Ababa University, Department of Chemistry, Ethiopia. Seventeen khat samples were collected from different regions of the country. Namely: Oromiya region, Southern Nation Nationality Peoples' Region (SNNPR) and Amhara region. These regions are best known for khat cultivation for local consumption and for the market of the capital city Addis Ababa, Ethiopia and to the neighboring countries. Specific areas of the sampling sites with respective trade name of khat cultivar analyzed and geographical coordinates are given in Table 3.2.1. Some of the samples (such as Awadai, Kerti, Hirna and Gelemso type khat samples) were collected from kiosks in the Addis Ababa town, Ethiopia.

Table 3.2.1 Geographical details of the sampling sites of the khat cultivars

Sampling area	Region/province	Trade name of khat variety	Latitude	Longitude	Altitude (m)
Anferara/Kibremengist	Oromiya region	Anferara/Dole	$5^{o}53'N$	$38^{o}59'E$	1705
Awaday	Oromiya region	Awaday	$9^{o}21'N$	$42^{o}2'E$	2036
Bahir Dar	Amhara region	Bahir Dar	$11^{o}35'N$	$37^{o}23'E$	1791
Beleche	SNNPR	Beleche	$7^{oo}5'N$	$38^{o}37'E$	1757
Hageremaryam	Oromiya region	Berdaye	$5^{o}38'N$	$38^{o}14'E$	1889
Chengie	SNNPR	Chengie	$7^{o}03'N$	$38^{o}28'E$	1921
Gelemso	Oromiya region	Gelemso	$8^{o}49'N$	$40^{o}31'E$	1697
Debremarkos	Amhara region	Colombia	$10^{o}20'N$	$37^{o}43'E$	2463
Aleta wondo/Gerbicho	SNNPR	Gerbicho/Tula	$6^{o}36'N$	$63^{o}25'E$	1921

Welkite	SNNPR	Guragie	8°17'N	37°47'E	1863
Hirna	Oromiya region	Hirna	9°13'N	41°06'E	1793
Kerti	Oromiya region	Kerti	NF	NF	NF
Mokonisa	SNNPR	Mokonisa	6°15'N	38°14'E	2006
Sebeta	Oromiya region	Sebeta	8°55'N	38°37'E	2323
Suke	SNNPR	Suke	6°28'N	38°45'E	1776
Basha /Wondogenet	SNNPR	Wondogenet/Basha	7°1'N	38°35'E	1723
Sike	SNNPR	Sike	NF	NF	NF
Damile	SNNPR	Damile	NF	NF	NF
Bole	SNNPR	Bole	NF	NF	NF

NF: Not Found

Some of the sampling sites share comparable climatic conditions. For example, Kerti, Hirna and Awaday type khat cultivars are found in the Eastern part of the country. Similarly, Beleche and Wondogenet/Basha type khat cultivars are grown in the Southern part of the country, in Wondogenet district, where comparable climatic conditions may be shared but differ in soil characteristics and composition (see Chapter 6, here in). All the samples were brought from the sampling sites in an ice box and kept in a deep freeze (-20 ^{o}C). The chewable parts of all the samples were taken and freeze dried within 5 h of arrival in the laboratory, blended in an electrical blender and passed through a 0.5 mm sieve. The ground sample was packed into polyethylene bags and taken to Chemistry Department of Rhodes University, South Africa for analysis.

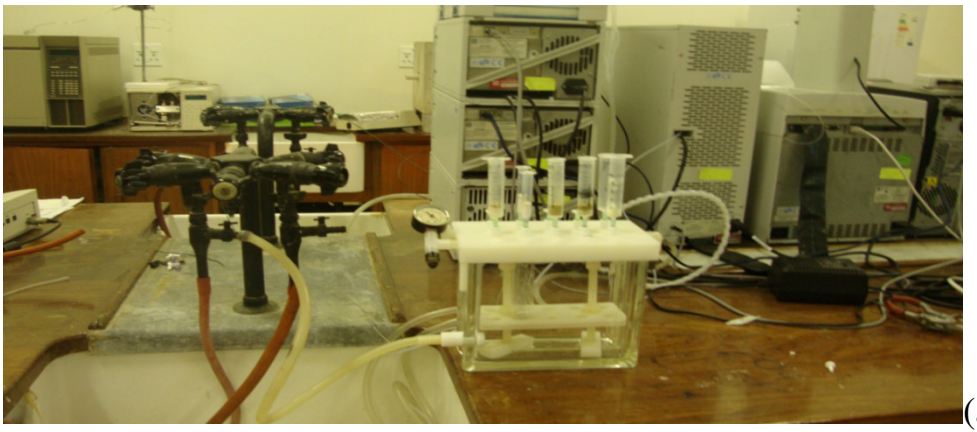
3.2.5.4 Matrix solid phase dispersion extraction of the plant materials

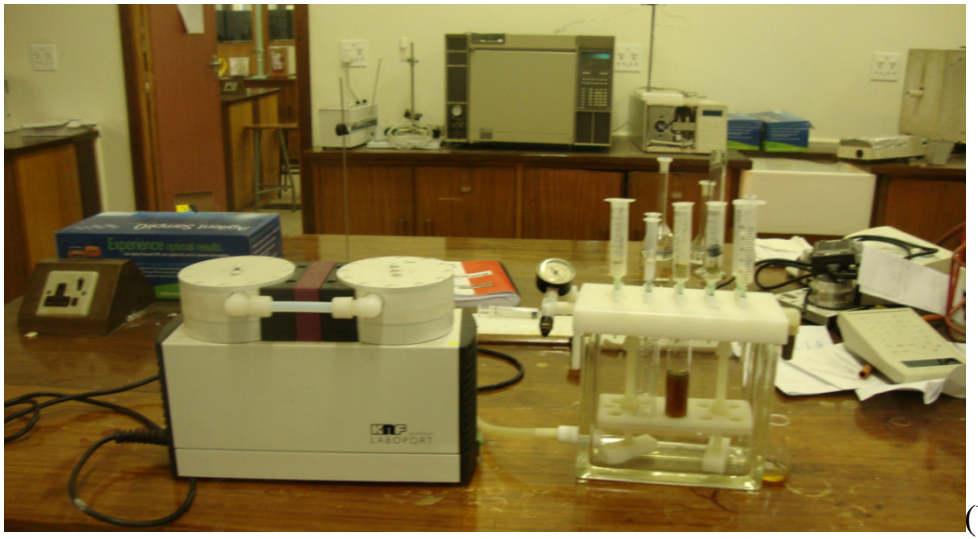
A 0.25 g aliquot of the sample was placed in a mortar and mixed with 0.75 g of the sorbent and 100 µL (5 mg/mL) of (+)–ephedrine hydrochloride (IS). The mixture was then homogenized in the agate mortar using an agate pestle to obtain a homogenous mixture. The blend was then transferred into a 10 mL syringe with a paper frit at the bottom. The sample was then covered with another paper frit and the flow of the solution

through the syringe was driven by the pressure from vacuum manifold pump (Figure 3.2.2) or can be compressed manually using a syringe plunger. The alkaloids were eluted directly with the elution mixture and the residue, after evaporation to dryness, was dissolved in the mobile phase, filtered through a PVDF membrane and injected into the HPLC system.

3.2.5.5 Ultrasonic assisted extraction followed by SPE (UAE/SPE)

For the UAE/SPE experiments, the procedure developed by Mathys *et al*. (1993) was used. Briefly, 0.25 g sample was mixed with 500 µg of ephedrine hydrochloride (IS) and extracted 3 times with a total of 50 mL of 0.1 M HCl in ultrasonic bath for 45 min. The combined filtrate was evaporated to dryness at 40 ^{o}C using a vacuum rotary evaporator. The residue was dissolved in the mobile phase (acetonitrile-water 5:95, containing 8.5 g/L orthophosphoric acid (85%) and 0.3 mL/L tri-propylamine) and passed through a pre-conditioned SPE cartridge with 3 mL methanol and 3 mL of (acetonitrile-water 5:95, containing 8.5 g/L orthophosphoric acid (85%) and 0.3 mL/L tri-propylamine). Then, cartridge was eluted with a portion of 1 mL followed by 3 mL of acetonitrile-water 5:95; containing 8.5 g/L orthophosphoric acid (85%) and 0.3 mL/L tri-propylamine.


(a)


(b)


(c)

Figure 3.2.2 Setup of vacuum manifold a) connected to a tap water and b) connected to a vacuum pump; c) extract of different cultivars of khat

3.2.5.6 Reproducibility and recovery

The reproducibility of the analytical methods and the repeatability of the extraction procedure were assessed by evaluating the peak area ratio variation of the three alkaloids present in the extracts. Two replicates were performed for each extraction assay and two replicate LC–DAD analyses were performed on each filtrate. The recovery of the MSPD using the C_{18} QuEChERS bulk sorbent extraction method was assessed by measuring the recovery of 0.25 g of a dried Kerti type khat sample containing 100 µL of 5 mg/mL of ephedrine hydrochloride (IS) and a standard stock solutions of 180 µL of 1 mg/mL cathinone oxalate (equal to 150 µg cathinone) and 100 µL of 1 mg/mL norephedrine after blending the mixture with 0.75 g of C_{18} QuEChERS bulk sorbent. Parallel to this, an un-spiked sample was also analyzed and recovery was calculated by taking the difference in concentration before and after spiking and dividing it by the spiked amount.

3.2.5.7 Statistical analysis using one-way analysis of variance (ANOVA)

Significant differences among the 17 khat cultivars for each of the compounds were determined by ANOVA using SPSS Program (version 20.0).

3.2.6 Results and discussion

3.2.6.1 Method development

As it has been reported elsewhere, efficiency of MSPD extractions depend on the quantity of sample, type and quantity of dispersing phase, as well as the nature and volume of the eluting solvent. In this study, sorbents with different physical and chemical properties in their binary combinations were evaluated. Furthermore, various elution media, and optimal elution volumes were determined.

Selection of elution solvent

The nature of the elution solvent is an important parameter since the target analytes should be efficiently desorbed while the remaining matrix components should be retained in the column. Taking into account the polarity of the alkaloids and previously reported solvents for the extraction of these metabolites, the following solvents were tested: methanol, water, 0.1 M HCl in water, 0.1 M HCl in methanol and methanol: water (80:20). Hence, initial trials were conducted applying the most usual sample/sorbent material ratio (1:4) (Capriotti *et al*., 2010). Different volumes of elution agents (5-20 mL) were used in all experiments. For each parameters, area ratio of the analyte to the IS was calculated and results are shown in Figures 3.2.3a-c.

As it can be observed from the Figures, all the tested solvents considerably extracted the alkaloids. The highest yields of the alkaloids analyzed were achieved with aqueous 0.1 M HCl under identical conditions followed by acidified methanol and aqueous methanol.

The effect of elution solvent volumes of different solvents on the extraction yield of the alkaloids was evaluated. The data presented in Figures 3.2.3a-c showed that, increasing the elution solvent volume resulted in improvement in extraction efficiency for all the studied solvents. However, the quantities extracted with 10 mL of 0.1 M HCl was not significantly different from those extracted with larger volumes. Thus, 10 mL of 0.1 M HCl was regarded as optimum extraction solvent. However, the remaining solvents were significantly dependent on solvent volume for better extraction yield.

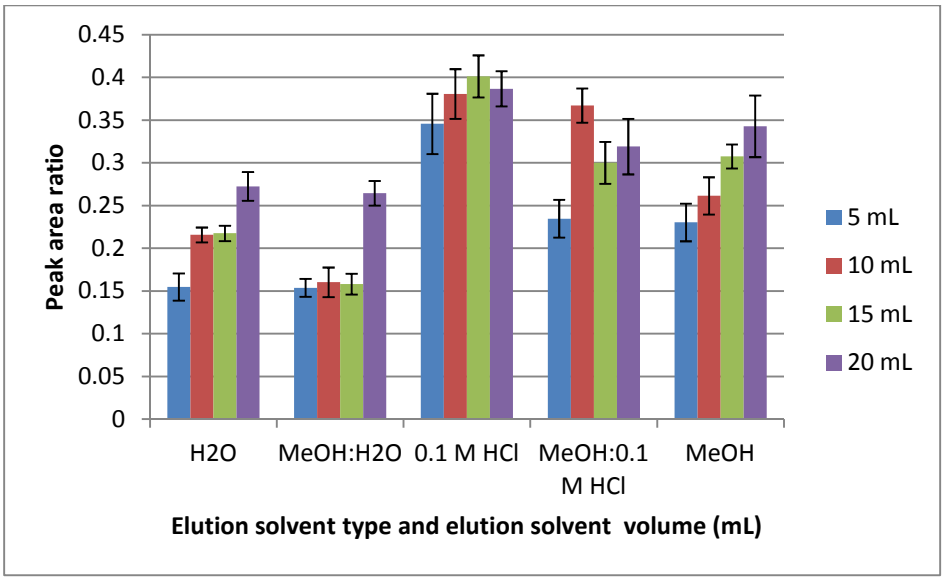


Figure 3.2.3a Effect of elution solvent and solvent volume on the quantitative extraction of norephedrine

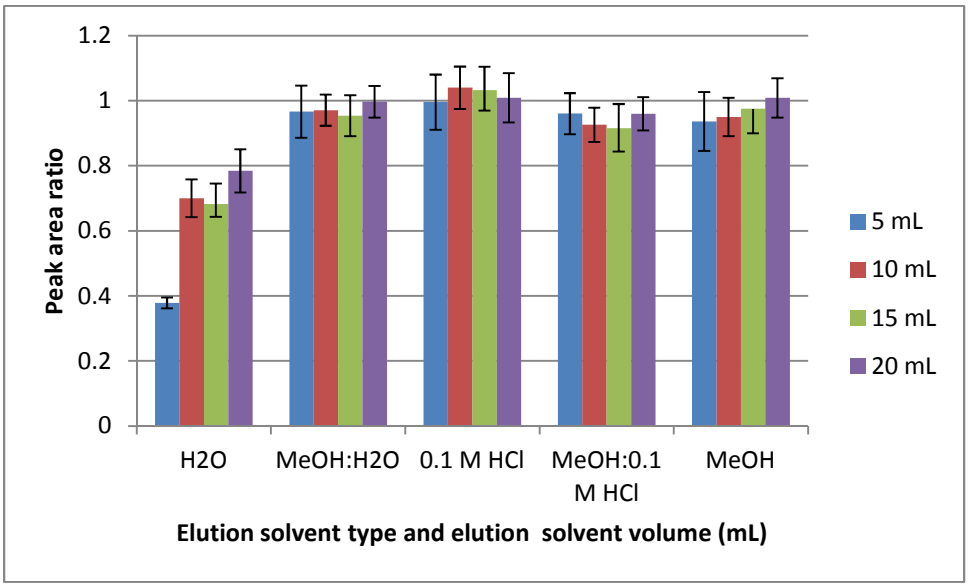


Figure 3.2.3b Effect of elution solvent and solvent volume on the quantitative extraction of norpseudoephedrine

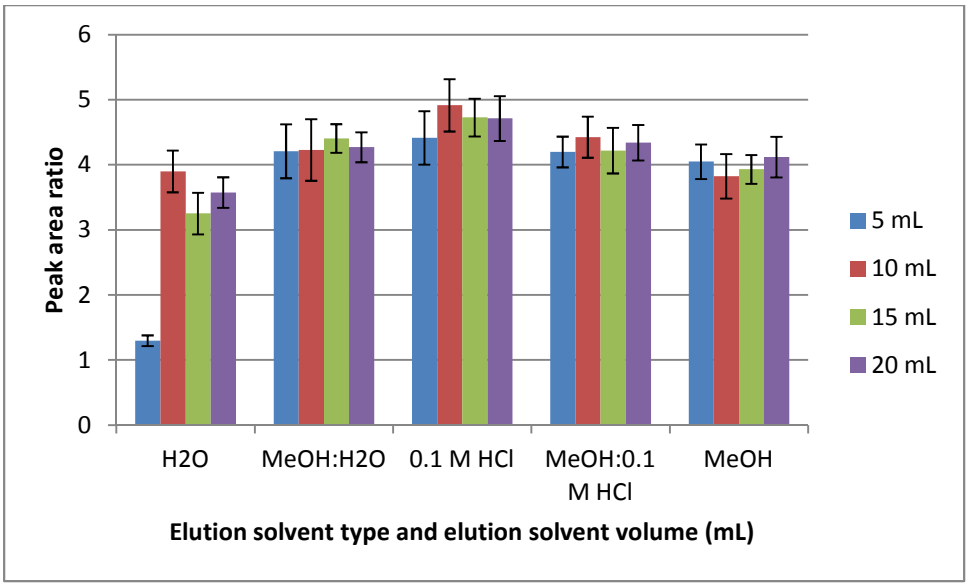


Figure 3.2.3c Effect of elution solvent and solvent volume on the quantitative extraction of cathinone

Most of the reported studies on MSPD have used non-polar solvents to remove pigments and fat soluble compounds (Ziakova *et al*., 2003; Fernandes *et al*., 2007). Thus, effect of pretreatment of the blended material with hexane showed no difference to the quality of the chromatogram (results not shown).

Selection of sorbent and sample to sorbent ratio

In the MSPD procedure, it is important to select a suitable sorbent because not only does it act as an adsorption separation material but it also acts as a blending solid support to disrupt and disperse the herb (Wang *et al*., 2011; Shi *et al*., 2010). In this study, C_{18}, PSA, silica and their binary 1:1 weight ratio mixtures were evaluated using 10 mL of 0.1 M HCl as elution solvent. Results are shown in Figure 3.2.4. It was found that the type of sorbent used had some influence on the extraction yield. The C_{18} and silica sorbents exhibited better performance in terms of extraction yield but the other QuEChERS bulk sorbents PSA and binary 1:1 compositions of C_{18}, PSA and silica sorbents provided a relatively lower extraction yield as compared to C_{18} and silica sorbents. Furthermore,

PSA and silica required relatively higher pressure during manual/hand plugging of the syringe plug in cases when the vacuum manifold was not available to derive the elution solvent though the blended and packed sample in the syringe. Thus, C_{18} was selected as optimum dispersant for the analysis.

It has also been reported that the ratio of sample to dispersion sorbent is dependent on sample type and significantly affects the extraction efficiency and hence must be examined as a major variable during method development. Both low and high ratios have been applied successfully (García-López *et al*., 2008). In this study, different amounts of C_{18} were evaluated to optimize the ratio of sample to dispersion sorbent (1:1, 1:2, 1: 3, 1:4 and 1:8 weight ratios). Results obtained are shown in Figure 3.2.5.

Results of the analysis indicated that (Figure 3.2.5), the yield of the three alkaloids increased with sorbent ratio (up to 1:3) but no significant difference in analytes yield were found with further increase in the C_{18} sorbent ratio. In order to reduce the quantities of dispersion sorbent and solvent, a ratio of 1:3 of sample to dispersion sorbent was deemed adequate.

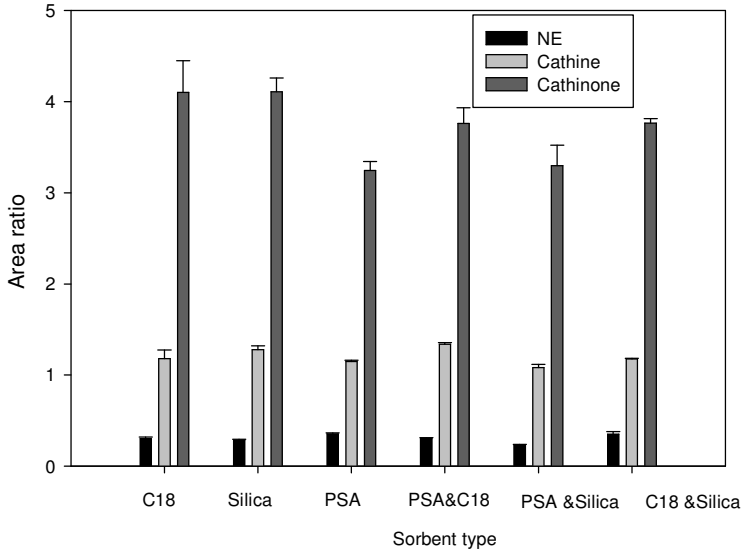


Figure 3.2.4 Effect of sorbent types on the extraction of norephedrine, cathine and cathinone

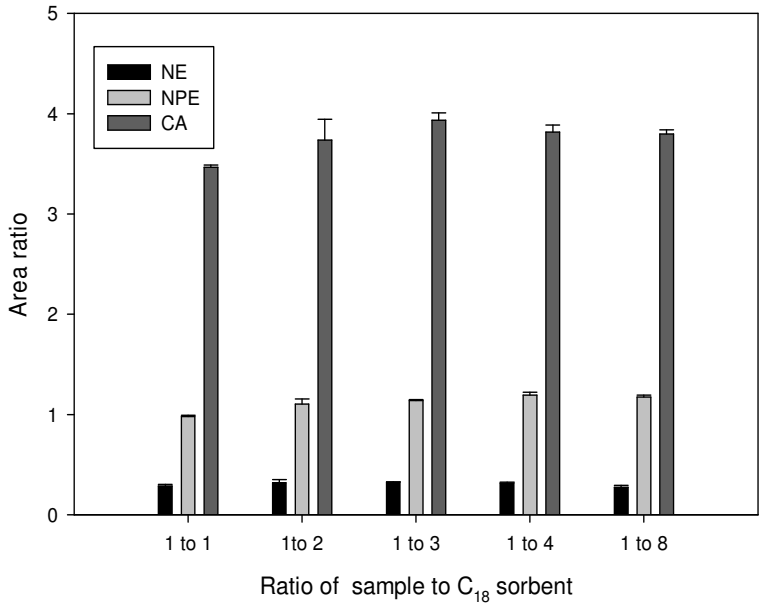


Figure 3.2.5 Effect of the ratio of sample to sorbent on the extraction yield of alkaloids

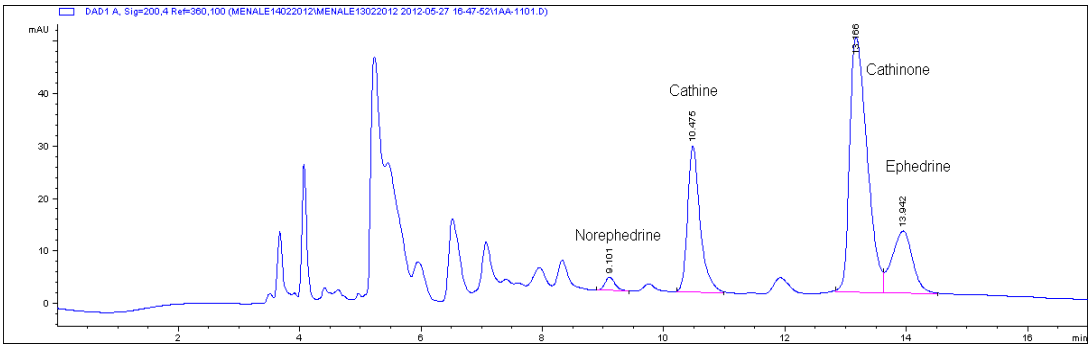
Evaluation of the analytical method

Once the MSPD conditions for the extraction were optimized, known concentrations of the alkaloids were spiked to the khat sample and extraction was conducted. Figure 3.2.6 shows the chromatograms of the MSPD extracts of (a) unspiked khat sample, (b) the same khat spiked with norephedrine and cathinone and (c) standards. Table 1 shows the recoveries together with the precision results. As can be seen, adopting the optimal conditions of the MSPD extraction in a khat samples, all three compounds were extracted very efficaciously and showed good recoveries (89-92%). The limit of detection (LOD) of the method was calculated using the calibration curve parameters (The LOD was established using LOD = 3.3 x (s/S), where s is the standard deviation of the intercept and S is the slope of the curve). The LOD obtained for cathinone and norephedrine were 26 µg/g and 34 µg/g, respectively (Table 3.2.2). Due to lack of pure cathine standard, its recovery was not performed and its concentration in the sample was determined using the calibration curve parameters of norephedrine due to the fact that cathine and norephedrine are diateriomers and expected to have comparable molar absorptivity.

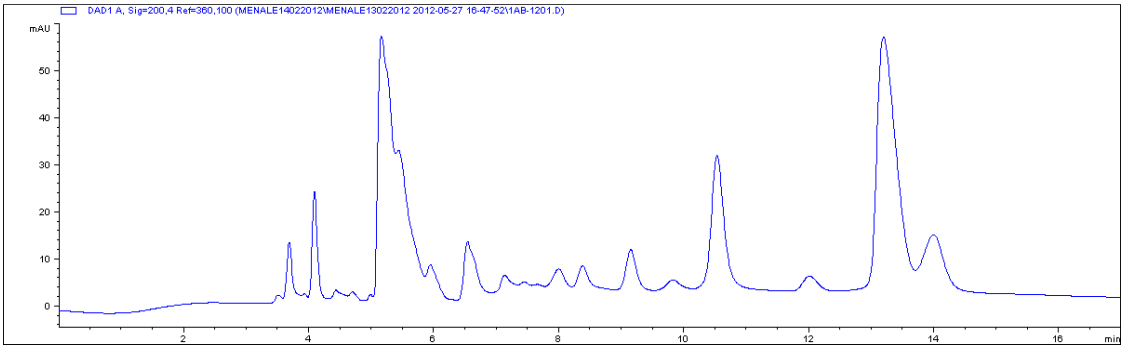
Table 3.2.2 Reproducibility, recovery and limit of detection of khat sample spiked with the two khat alkaloids

Compound	[a]Initial conc. (µg/g)	Amount spiked (µg/g)	[a]Amount obtained (µg/g)	Recovery	Reproducibility, %RSD (n = 4)	LOD (µg/g)
Cathinone	3377	600	3909	89	2.6	26
Norephedrine	201.3	400	565	92	6.2	34

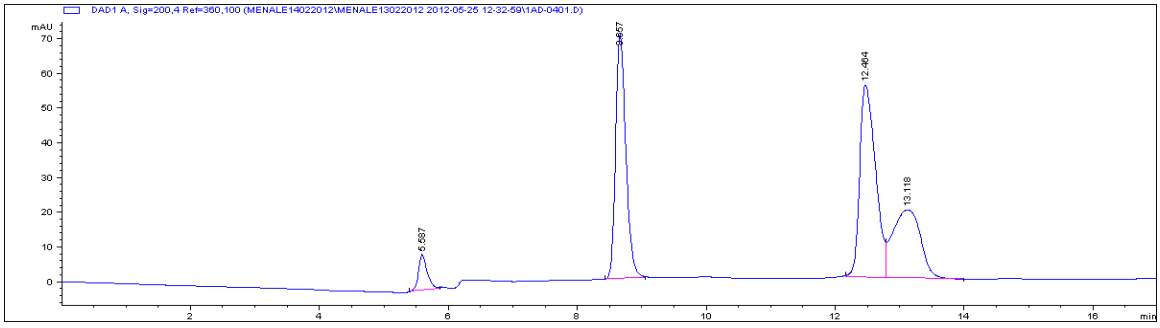
[a]Average of four measurements.



(a)



(b)



(c) Standard (left to right: oxalate, norephedrine, cathinone and ephedrine)

Figure 3.2.6 Chromatograms of unspiked (a) and spiked (b) khat sample and (c) standard

3.2.6.2 Application to khat cultivars

Finally, the optimized MSPD method was applied to various khat cultivars from different geographical locations in Ethiopia to identify and quantify the khat amines. The main purpose was to check the applicability of the method developed to different cultivars/varieties of khat. Furthermore, it was aimed to document the khat amine

concentrations in different varieties of khat for future concentration based pharmacological studies. To achieve the objective, it was necessary to study the potential of using the method for routine monitoring of the concentration of khat amines in seventeen major khat cultivars widely consumed in the country and exported to various East African and Middle East countries.

The concentrations of the analytes extracted from these samples are reported in Table 3.2.3 and the percentage of each analyte with respect to the total alkaloid contents is also given in the Table. It can be seen that amounts of the alkaloids varied greatly with the cultivar. Cathinone (2354–4316 µg/g), cathine (408–2235 µg/g) and norephedrine (113–375 µg/g) were detected in the investigated khat cultivars.

The values were extremely low in Gerbicho/Tula type khat (with total alkaloids content of 3092 µg/g) and very high in Awadai type khat (with total alkaloids content of 6143 µg/g) (Table 3.2.3). Some of these results are in agreement with the findings by Geisshusler and Brenneisen, (1987) but much higher alkaloids concentration were obtained from the present study compared with the results reported in khat samples from Israel by Krizevski *et al*. (2007 and 2008).

In all the studied samples, the concentration of cathinone was much higher (> 60 %) followed by cathine (7 to 38%) and norephedrine (<10%) of the total alkaloids composition indicating that the young leaves/shoots of khat were rich in cathinone. But upon drying or maturation, part of the cathinone can be converted into cathine, norephedrine and other metabolites (Szendrei, 1980) and that is why results reported on confiscated khat samples in the forensic laboratories showed higher concentrations of cathine and norephedrine compared with cathinone (Gambaro *et al*., 2012; Chappell *et al.,* 2010).

Except for few khat cultivars, ANOVA results showed significant differences between the alkaloids profiles among samples of different varieties, in terms of cathinone, cathine and norephedrine ($p < 0.05$). ANOVA also showed the influence of geographical origin on the three alkaloids ($p < 0.05$).

Those khat varieties regarded as best qualities by the chewers; namely, Awadai, Bahir Dar, Colombia, Kerti and Sebeta type khat accumulated higher concentrations of the total alkaloids in the edible portion of the plant. Thus, the khat amine contents of the plant can be taken as one of the quality markers of a khat sample.

Wide variation of the alkaloids composition in the studied samples collected from different geographical locations can be explained in terms of variations of the environmental factors and soil parameters (see section 5.2). However, it was anticipated that khat samples collected from the same province could accumulate statistically comparable concentration of the alkaloids. For example, Basha and Beleche khat cultivars are grown in Wondo-Genet province. Similarly, Mokonisa and Suke type khat cultivars are grown in Sidama province while Awaday, Hirna and Kerti type khat cultivars are from Hararge province. However, recognizable variations in the alkaloids composition were noticed among samples collected from the same province. Nature and properties of the soil might be the major factor for the variation in addition to the possibility of the existence of genotypic variability of the samples studied. So far, there is no reported study about the genetic variability/similarity between cultivars of Ethiopian khat. However, one reported article on various khat types from Yemen has shown the existence of wide genotypic variability among some of khat samples (Al-Thobhani *et al*., 2008).

3.2.6.3 Comparison of MSPD extraction with UAE/SPE

The MSPD extraction was compared with ultrasonic assisted extraction followed by solid phase extraction and results are shown in Figure 3.2.7. From the Figure, it can be seen that the methods were successfully extracted the alkaloids. MSPD yielded comparable results with UAE/SPE but with reduced sample preparation steps and ease of handling the process. Furthemore, slightly lower concentration of cathinone but within the acceptable range have been observed. In fact, it was anticipated that UAE/SPE protocol could yield better result than MSPD as ultrasonication is much more efficient extraction technique. The SPE clean-up protocol reported by Mathys *et al*. (1993) is quite different

from the normal trend of SPE clean up. Thus, further parameter optimization is a pressing demand to exploit the potential of the ultrasonication and SPE clean up.

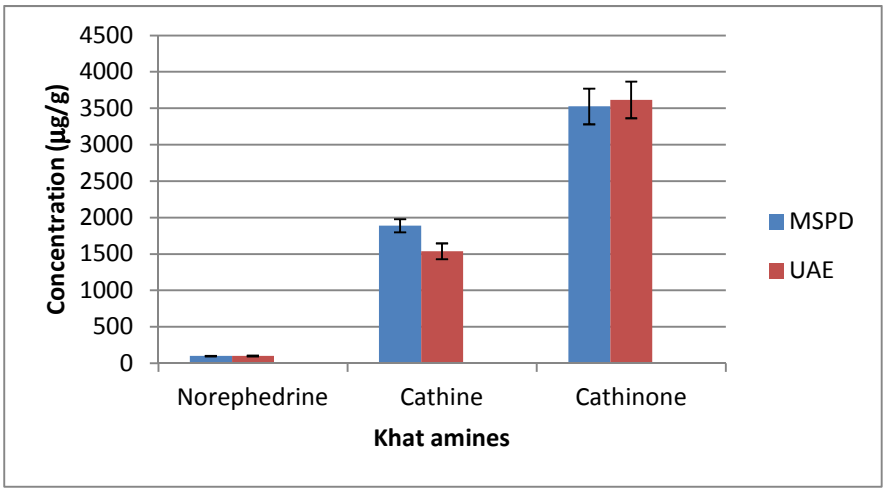


Figure 3.2.7 Comparison of MSPD extraction with ultrasonication/SPE extraction

Table 3.2.3 Khat alkaloids composition (mean ± SD µg/g dry weight basis, n = 4) of various khat types collected from different geographical locations in Ethiopia

Khat type	Norephedrine	Cathine	Cathinone	Total alkaloids
Anferara	241 ± 21	679 ± 39	3232 ± 244	4152
Awadai	174 ± 13	1652 ± 81	4316 ± 131	6142
Bahir Dar	490 ± 28	830 ± 86	3161 ±120	4481
Belechie	113 ± 9	1796 ± 120	4018 ± 368	5927
Berday	376 ± 29	804 ± 51	3639 ± 214	4819
Chengie	241 ± 19	748 ± 62	3583 ± 130	4572
Gelemso	324 ± 25	740 ± 73	3287 ± 327	4351
Colombia	188 ± 10	794 ± 72	4122 ± 358	5104
Gerbicho/Tula	243 ± 27	494 ± 21	2354 ± 155	3092
Guragie	297 ± 25	923 ± 65	2791 ± 91	4012
Hirna	272 ± 29	1497 ± 166	3228 ± 136	4997
Kerti	208 ± 20	2235 ± 44	3392 ± 82	5835
Mokonisa	287 ± 25	484 ± 12	3792 ± 122	4563
Sebeta	150 ± 17	1697 ± 65	2356 ± 164	3253
Suke	361 ± 13	687 ± 87	2530 ± 121	3578
Green belechie	344 ± 25	408 ± 11	4861 ±243	5613
Wondogenet	144 ± 8	519 ± 26	3544 ± 74	4207

3.2.7 Conclusions

In this study, an efficient, fast and easy method was successfully developed and applied for the extraction of psychoactive phenylpropylamino alkaloids in khat (*Catha edulis* Forsk) leaves. The procedure was found to be simple, rapid and requires only small sample sizes and small solvent volumes, integrating sample clean-up and without dedicated instrumentation. The results have demonstrated that the precisions of the proposed method are satisfactory for the extraction of the alkaloids. Thus, it can be used as a routine technique in any forensic as well as pharmacological research laboratories and it has been demonstrated to be a suitable preparation technique, a simple alternative to LLE and SPE, for the isolation of khat amines from plant material.

Khat amines distributions in 17 different varieties of Ethiopian khat grown in different geographical locations were studied and ANOVA results showed as the alkaloids compositions varied significantly ($p < 0.05$) with geographical origin.

3.3 EFFECT OF VARIOUS SAMPLE PRESERVATION TECHNIQUES ON THE COMPOSITION OF KHAT ALKALOIDS

3.3.1 Background

The general problem in the study of natural products in the plant is that their nature and amount are dependent on the various factors, which must be controlled as far as possible. Some of these factors are: stress; the metabolic state of the plant may change when it is stressed in any manner. This can be the problem before as well as after harvesting a plant part for analysis. As cells die (the senescent process), the cellular integrity is lost and as a result the enzymes come in contact with substrates to which they are not normally exposed in living cells. In addition, it also increases the redox process, racemization, dimerization etc which is a problem with some of bioactive compounds like phenolic compounds and alkaloids, e.g, cathinone (Rickman *et al*., 2007; Szendrei, 1980). In order to avoid these changes, the metabolic activities of the cells need to be curbed

immediately after harvesting. Thus, the fresh material should be kept on ice and transported under dark conditions and various drying techniques at different temperature can be applied depending on the nature of the analytes and the nature of sample (Müller *et al.,* 2006; Satong-aun *et al*., 2011; St. George *et al*., 2004; Abascal, *et al*., 2005).

Furthermore, for routine experiments, it is difficult to obtain fresh samples all the time and hence drying is the most common method of plant material preservation. Drying represents 30% to 50% of the total costs in medicinal plant production (Müller *et al.,* 2006).

In the contrary, drying processes, including freeze-drying can cause undesirable effects on the constituent profiles of plant samples, therefore, caution should be taken when planning and designing research studies on the medicinal properties of the plants (Abascal *et al*., 2005).

Some reports indicated that the conversion of cathinone to cathine occurs in the young shoots after harvesting, and believed to occur rapidly upon drying and continue during storage while other report indicated that room temperature drying could not cause appreciable change on the concentration of cathinone (Chappell *et al*., 2010; Szendrei, 1980; Lee, 1995; Dagne *et al*., 2010; Brenneisen *et al.,* 1987).

Thus, until recently, it was believed that dried khat plant exclusively contains cathine due to conversion of cathinone upon drying/storage (Lee, 1995; Dagne *et al*., 2010). As a result almost all pharmacological studies and chemical analysis of khat have been conducted using the fresh leaves of the plant. This is due to the fact that there was strong belief that cathinone undergoes significant (if not complete) decomposition upon drying and storage.

Currently, cathinone is categorized as aschedule I controlled substance under federal regulations of the United States of America. However, dried leaves have given less attention among the regulatory authorities since they are believed to contain only cathine which is a less potent and a non-controlled chemical (Chappell *et al*., 2010). Even in Australia, available evidence suggests that the vast majority of khat import licenses are

granted for dried khat suggesting that the majority of khat being imported is low in the most active constituents of cathinone (Fitzgerald, 2012).

Recently, Dagne *et al.* (2010) investigated the distribution of cathine and cathinone in different part of the plant and also studied the variation of the alkaloids upon sun drying of the leaves. They reported that the fresh young leaves of khat contained entirely cathinone but it could not be detected in sun dried young leaves, while the cathine level was 1.5% in the sun dried samples. As a result, khat users prefer fresh material over the dried one, since drying the samples affect the main stimulant of the plant.

Furthermore, using seized khat samples in domestic crime laboratories of USA, Chappell *et al*. (2010) conducted an investigation to identify the stability of cathinone and its kinetic decomposition upon drying and storage. Three drying conditions were considered during their study: drying under ambient conditions (approximately 20 ^{o}C) with air circulation inside a fume hood, heating within a convection oven (55 ^{o}C) and microwave oven drying (100 ^{o}C). As per the report, the authors found high concentrations of cathinone in the ambient-dried sample (16.9%) compared to the heated preparations (convection oven, 11.7%; microwave oven, 10.8%) and un-dried sample (13%). However, the authors have claimed that the time between harvesting the samples and analysis was not known and part of the leaves were wilted and showed color change. Thus, it is not possible to conclude the effect of drying on cathinone composition since more than 75% of cathinone may have been converted before processing.

Lee *et al*. (1995) conducted qualitative study of cathinone composition of khat under different conditions (air drying and freezing at –2 ^{o}C). They demonstrated that by air drying the young khat shoots at ambient temperature, cathinone may be detected in khat samples that have been harvested for more than 10 days. Refrigeration for two weeks and freezing for one month of the khat samples also yield identifiable levels of cathinone. However, quantitative data was not reported.

Dawson *et al.* (1994) provided information on the relative amounts of the cathinone enantiomers and the relative amounts of the norephedrine/norpseudoephedrine diastereomers present in the plant at the time of analysis using air dried and freeze dried

samples. Except the qualitative information about racemization of the alkaloids to the corresponding R- and S- configurations under different conditions, no quantitative data was reported about the actual concentration of cathinone, cathine and norephedrine under different drying conditions.

As a result, there is confusion among researchers working on khat related projects about khat preservation without significant loss of the alkaloids. Though it is a general thought that freezing and freeze drying protocols are the best options to preserve biological samples for long periods of time, their actual effect on the particular samples like khat need to be investigated (FAO/IAEA, 2000).

3.3.2 Objectives of the study

The objective of this work was to investigate the effect of different sample preservative conditions (air drying, freeze drying, oven drying, sun drying and freezing) so as to recommend a plausible preservation condition for khat sample preservation without significant change in the alkaloids content.

3.3.3 Experimental

3.3.3.1 Apparatus, chemicals and reagents

All the reagents used were of analytical or HPLC grade reagents. Acetonitrile (Merck KGaA, Darmstadt, Germany), orthophosphoric acid, hydrochloric acid, (Merck Chemicals, Gauteng, South Africa), tripropylamine (Fluka, Switzerland), (–)-norephedrine, (+) cathine hydrochloride and (+) ephedrine hydrochloride were purchased from Sigma Aldrich. (–)-Cathinone oxalate was isolated from the fresh leaves of the plant. C_{18} EC QuEChERS bulk sorbent, were obtained from Agilent Technologies (USA). The water used was from MilliQ system (Millipore Milford, Mass, USA). The mobile phase was filtered through a Whatman membrane filter (47 mm diameter and 2 µm pore size) while all plant extracts were filtered through Acrodisc syringe filter (PVDF membrane with 0.45 µm pore size).

3.3.3.2 Equipment

The analysis was performed on an Agilent 1200 Series HPLC, Agilent Technologies Inc. (Santa Rosa, CA, USA) equipped with a binary pump and a DAD set at 200 nm. Separation of the compounds was achieved on an Agilent ZORBAX SB-Phenyl column (4.6 x 250 mm, 5 micron), Agilent technologies, USA. The data was processed by the Agilent ChemStation for LC/MS 2D system software. A reverse-phase method developed by Mathys *et al.,* 1993 was employed to separate and quantitate the alkaloids. The mobile phase consisted of aqueous buffer of pH 2.65 (8.5 g/L orthophosphoric acid and 0.3 mL/L tripropylamine) and 5% acetonitrile in water at a flow of 1.5 mL/min in a gradient mode. ^{1}H and ^{13}C NMR spectroscopy (Bruker Avance 400 NMR spectrometer at 400 MHz frequency) was used to confirm the isolated oxalate salts,

3.3.3.3 Plant sample collection

First round sample collection for cathinone and cathine isolation

Samples were collected from Sebeta, Ethiopia in November, 2011 and transported to the Chemistry Department of the Addis Ababa University, Ethiopia in an ice box. As soon as brought to the laboratory, twigs and young leaves (near the tip of the shoot) were taken from part of the sample and immediately extracted as mentioned below so as to isolate cathinone. The remaining portion of the sample was taken and dried under strong sun light for 15 days. It should be noted that only the young shoot (but not the lower sections) in which only cathinone is expected to exist was taken for the analysis. At the end of the day, the dried khat sample was ground and extracted as mentioned below for cathine isolation. Confirmation of the isolates was done in the Chemistry Department, Addis Ababa University using ^{1}H and ^{13}C NMR spectroscopy. Further confirmation was also done by HPLC-DAD at Rhodes University.

Second round sample collection for investigation of the effect of sample preservation

Samples were collected around the end of December from the Bahir Dar area, Ethiopia, more specifically from Zegie and Zenzelima. Comparing with the nature of the plants, samples from Zegie were from a large tree, while khat samples collected from Zenzelima were very small, just bush/shrub type. Samples were collected in the afternoon (around 5 PM) and transported to Addis Ababa overnight in the ice box on the same date. As per the information gathered from experienced chewers, khat from tree type is more potent than khat from shrub like plant. Furthermore, it is also believed that khat from tree type remains fresh for longer time, without wilting, than leaves from shrub type khat.

As soon as it arrived in the laboratory of the Department of Chemistry, Addis Ababa University, the edible parts of the plant were taken and portion of the samples were kept in a deep freeze while the rest were subjected to drying under different conditions (air drying, freeze drying, oven drying at 60 ^{o}C and sun drying) until a constant weight was obtained. This time, not only the young shoot but also the lower sections but edible part of the leaves was taken for processing. The dried and ground samples were packed into polyethylene bag and kept in moisture free and light protected environment. The refrigerated samples in ice box and the dried samples were taken to Rhodes University, South Africa for chemical analysis. All the samples were stored for about four months before analysis.

3.3.3.4 Isolation of cathinone

The procedure reported by Dagne *et al*. (2010) was used to isolate the salt of cathinone from the fresh leaves of the plant. Namely, 500 g of the sample was crushed under liquid nitrogen and subsequently extracted in 0.1 M HCl for 2 h by placing on a magnetic stirrer. The extract was filtered by using suction filtration and the filtrate was subjected to liquid-liquid extraction (x3) using Et_2O and (x1) using benzene to remove pigments and other fat soluble metabolites. The acidic aqueous solution was alkalinized to pH 10 with 10% NaOH and extracted with Et_2O and extracted with Et_2O (x2). Oxalic acid (1% in

Et_2O) was added drop-wise to the Et_2O extract and left to stand for 20 h in the refrigerator (4 ^oC) to yield cathinone oxalate (150 mg) as a white precipitate.

3.3.3.5 Isolation of cathine

As suggested by Dagne *et al*. (2010), an attempt was to isolate cathine oxalate, following the above procedure except dried and powdered samples were used in place of the fresh leaves.

3.3.3.6 Quantifying khat alkaloids in the samples processed under different conditions

Khat alkaloids were extracted from all the dried samples (air drying, sun drying, air drying and oven drying) using matrix solid phase dispersion (MSPD) (section 3.2) while the alkaloids were extracted from the refrigerated sample using the method reported by Mathys *et al*. (1993).

Extraction of khat alkaloids from dried samples. A 0.25 g aliquot of the sample (sun dried, oven dried, freeze dried or air dried) was placed in a mortar and mixed with 0.75 g of sorbent and 100 µL of (+)–ephedrine hydrochloride (IS). The mixture was then homogenized in the agate mortar using an agate pestle to obtain a homogenous mixture. The blend was then transferred into a 10 mL syringe with a paper frit at the bottom. The sample was covered with another paper frit and the flow of the solution through the syringe was driven by the pressure of vacuum manifold pump or can be compressed manually using the syringe plunger. The alkaloids were eluted directly with 10 mL of 0.1 M HCl and the residue after evaporation of the extract to dryness was dissolved in the mobile phase. Eluents containing the analytes were filtered through a PVDF membrane and injected into the HPLC system.

Extraction of khat alkaloids from fresh samples. 1.04 g of fresh khat sample was mixed with 500 µg of ephedrine hydrochloride (IS) and crushed in the presence of liquid nitrogen. The powered sample was extracted 3 times with a total of 50 mL 0.1 M HCl in

ultrasonic bath for 45 min. The combined filtrate was evaporated to dryness at 40 ^{o}C using vacuum rotary evaporator. The residue was dissolved in the mobile phase and passed through a pre-conditioned SPE cartridge. Then, the cartridge was eluted with the mobile phase.

3.3.4 Result and discussion

3.3.4.1 Isolation of cathinone and cathine

Preliminary investigation was made by taking all the edible portion of the leaves (the upper and lower but the soft part of the leaves). However, traces of cathine were detected (result not shown). Then, only twigs and young leaves (near the tip of the shoot) were taken for the analysis. Pure cathinone oxalate was isolated and characterized by ^{1}H NMR and ^{13}C NMR (Figures 3.3.1a-c). The multiplicity and chemical shifts in the ^{1}H NMR, ^{13}C NMR spectra as well as orientation of the DEPT (Distortionless Enhancement by Polarisation Transfer) spectra conform to what was expected theoretically and to the reported data by Dagne *et al*. (2010). Table 3.3.1 shows the interpretation of the ^{1}H NMR and ^{13}C NMR data. Results of the analysis showed that young leaves (near the tip of the plant) contain entirely of cathinone but not the other alkaloids (cathine and norephedrine). This result is corroborating with the earlier report by Dagne *et al*. (2010). However, the lower sections but edible portion of the leaves may contain cathine in addition to the major alkaloids (cathinone).

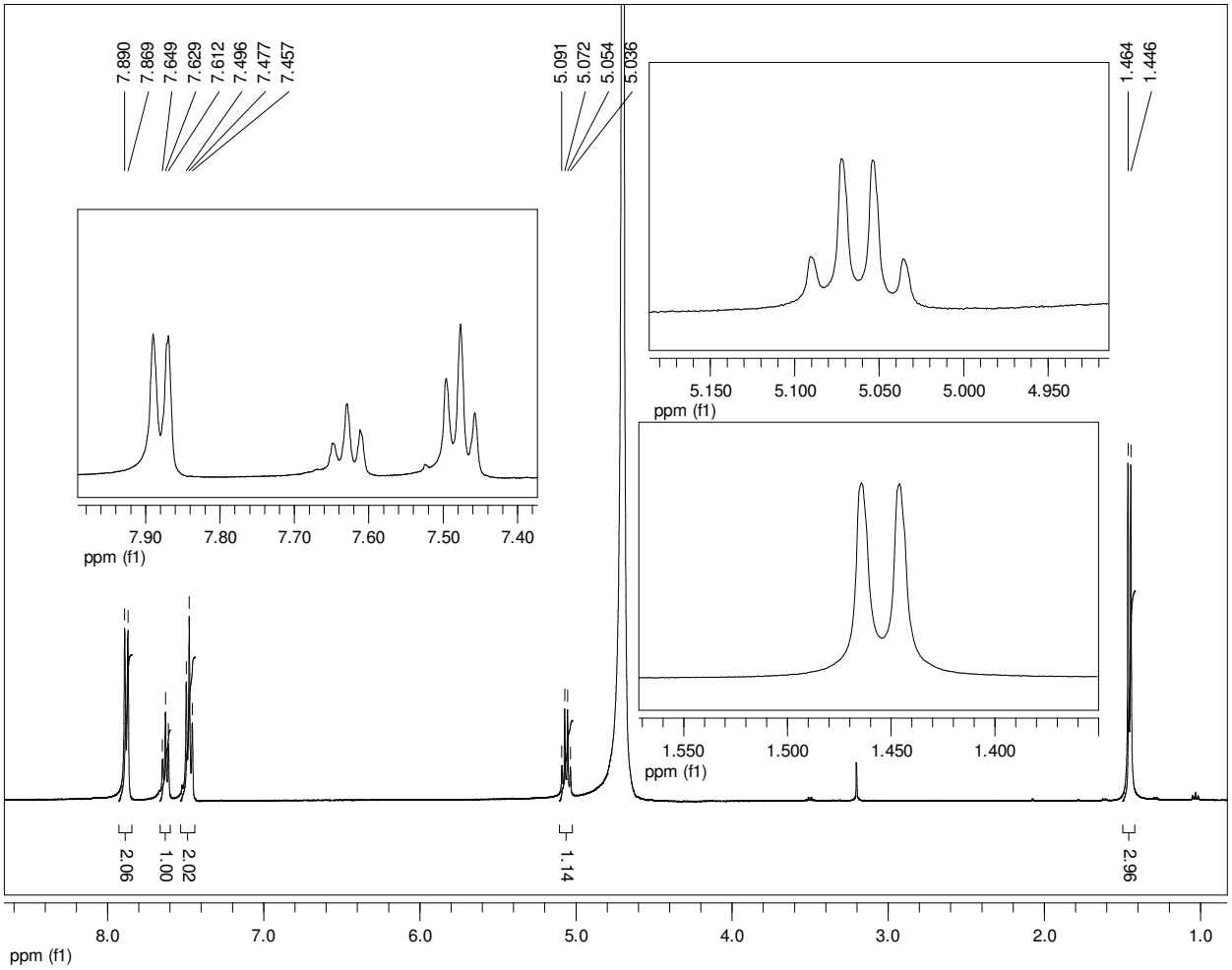


Figure 3.3.1a ^{1}H NMR spectrum of cathinone oxalate run in D_2O

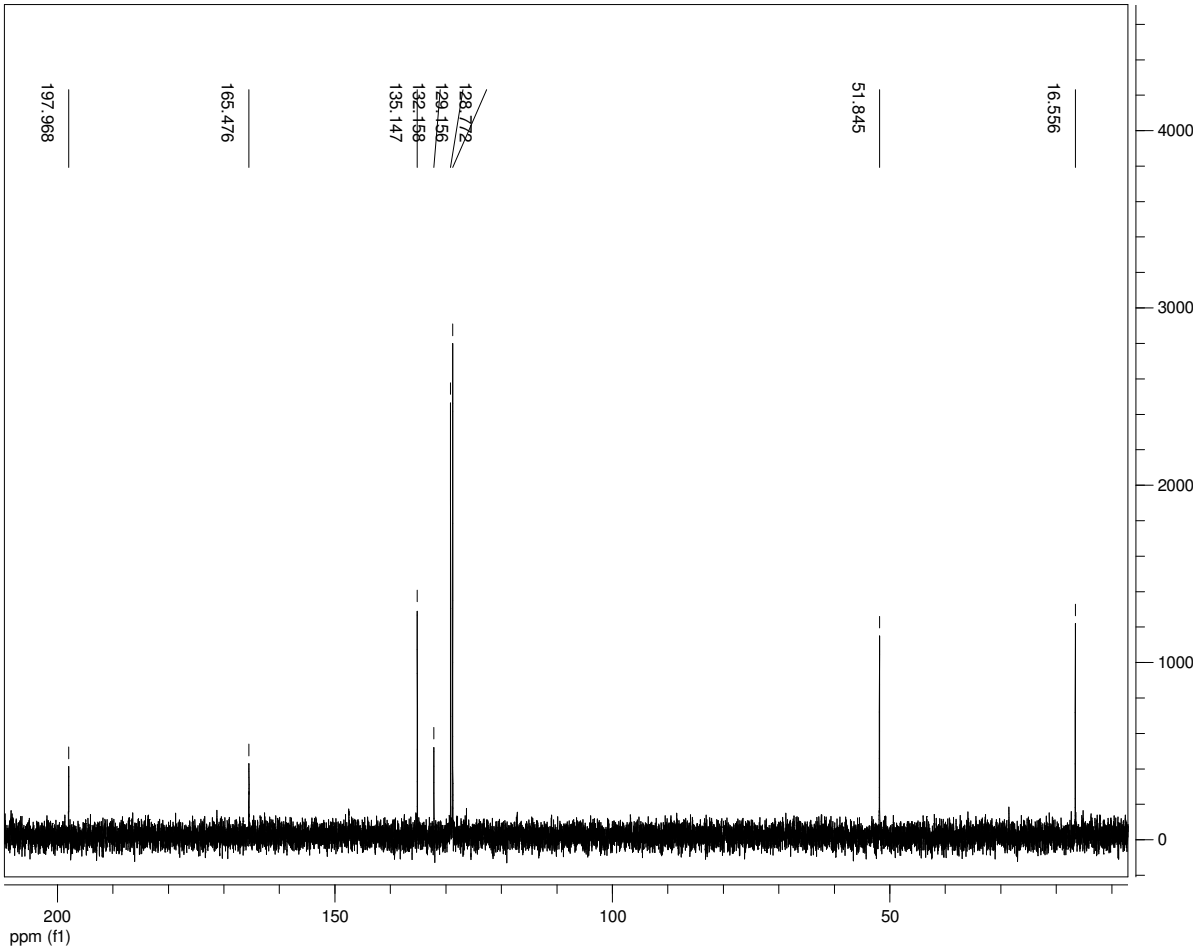


Figure 3.3.1b ^{13}C NMR spectrum of cathinone oxalate

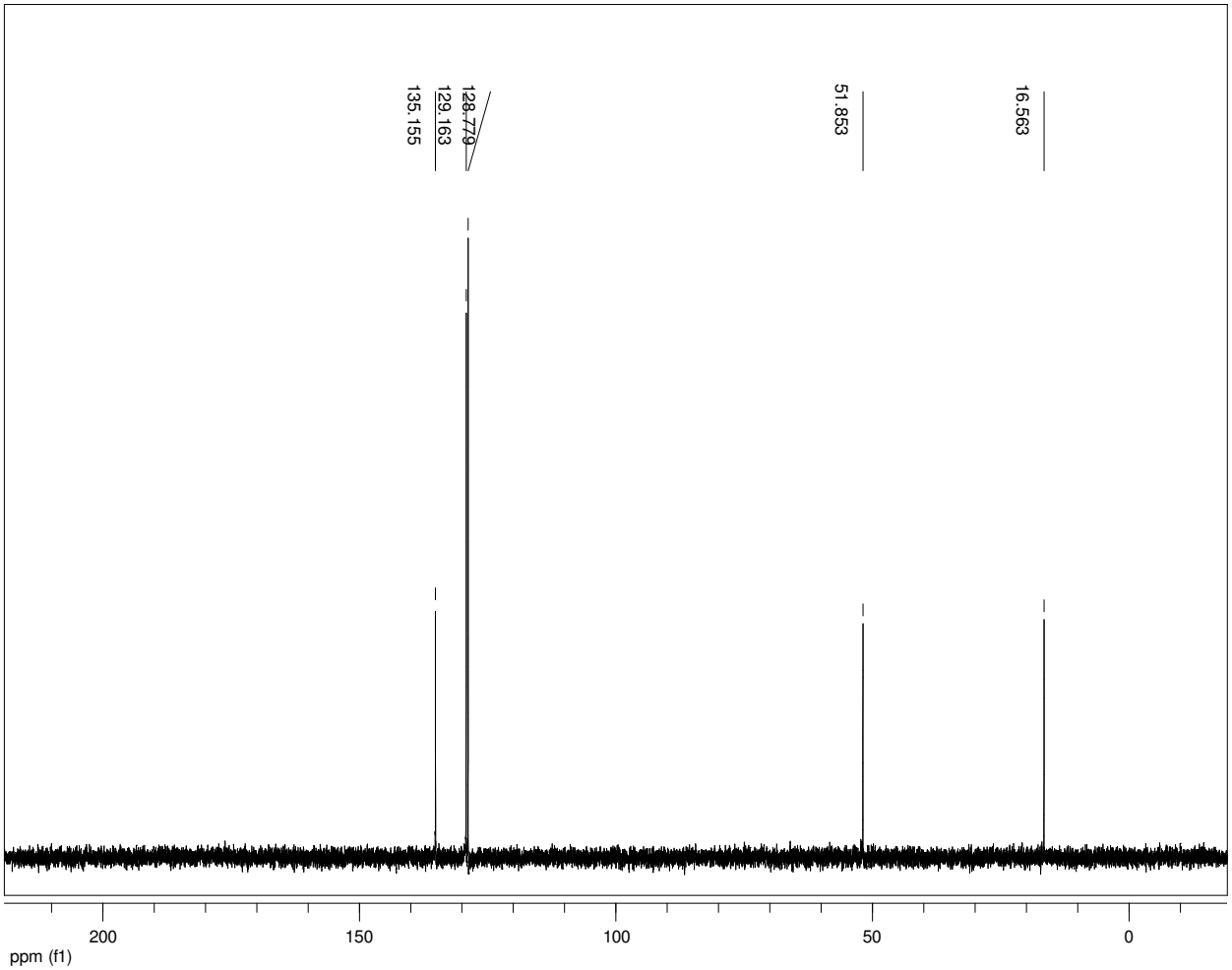


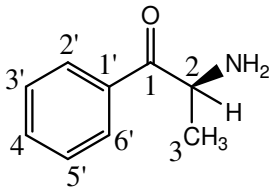
Figure 3.3.1c DEPT ^{13}C NMR spectrum of cathinone oxalate

Table 3.3.1a Interpretation of ^{1}H NRR data for cathinone

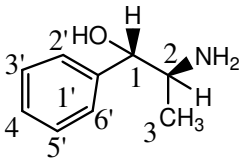
Chemical shift (ppm)	Multiplicity	Assignment	Literature chemical shift	Reference
7.88	Doublet	2H	7.89	Dagne *et al*., 2010
7.63	Triplet	1H	7.67	
7.47	Triplet	2H	7.48	
5.00	Quartet	1H	5.06	
1.46	Doublet	3H	1.46	

Table 3.3.1b Interpretation of ^{13}C NMR data for cathinone

Chemical shift (ppm)	Multiplicity	Assignment	Literature chemical shift	Reference
197.9	Singlet	C-1	198.1	Dagne *et al*., 2010
135.1	Singlet	C-4’	135.2	
132.2	Singlet	C-1’	132.3	
129.2	Singlet	C-2’, 6’	129.3	
128.8	Singlet	C-3’,5’	128.9	
51.8	Singlet	C-2	51.9	
16.6	Singlet	C-3	16.6	



S-(-)-Cathinone



(+)-Cathine

Figure 3.3.2a and Figure 3.3.2b show the ^{1}H NMR and ^{13}C NMR spectra, respectively, for oxalate salt of sun dried khat sample. Looking at Figure 3.3.2a and b, ^{1}H NMR signal at δ 7.23 (m, 5H), 4.46 (d, 1H), 3.4 (m, 1H) and 0.96 (d, 3H) and ^{13}C NMR (D_2O): δ 74.7 (C-1), 51.8 (C-2), 14.5 (C-3), 128.9 (C-1’), 128.8 (C-2, 6’), 126.7(C-3’, 5’) and 128.7 (C-4’) corresponds to protons of cathine/norephedrine (Dagne *et al*., 2010). While from ^{1}H NMR, the signal at δ = 7.83, 7.58, 7.42, 5 and 1.46 belongs to protons of cathinone (see the above table). Similarly, from ^{13}C NMR, the signal δ (198.1) is a characteristic peak for carbonyl carbon of cathinone. Therefore, cathinone was identified as amajor components in a sun dried sample. Thus it is wrong to assume that sun drying of khat samples completely decompose cathinone to cathine and norephedrine as stated elsewhere (Dagne *et al*., 2010).

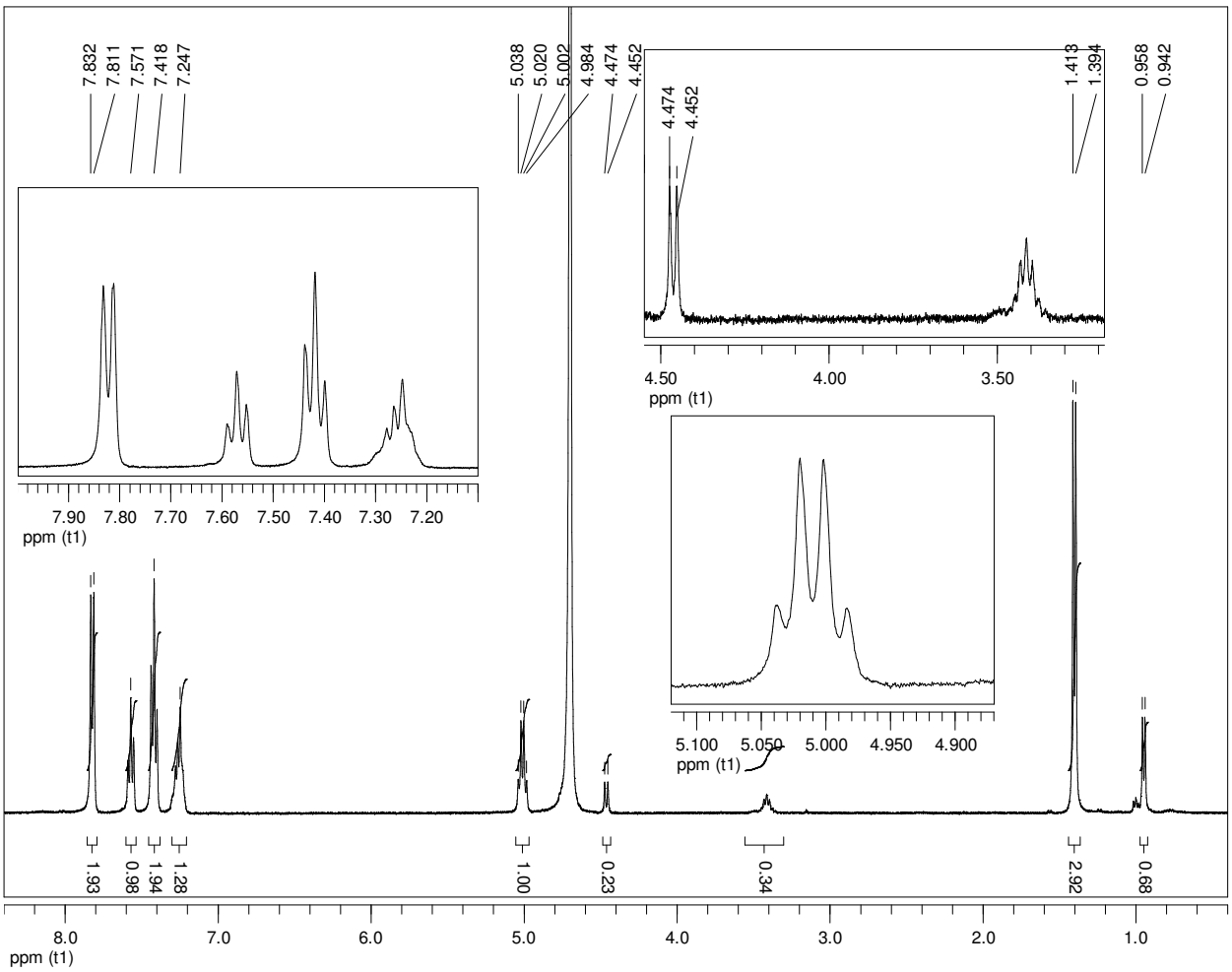


Figure 3.3.2a 1H NMR spectrum of cathine oxalate and cathinone oxalate run in D_2O

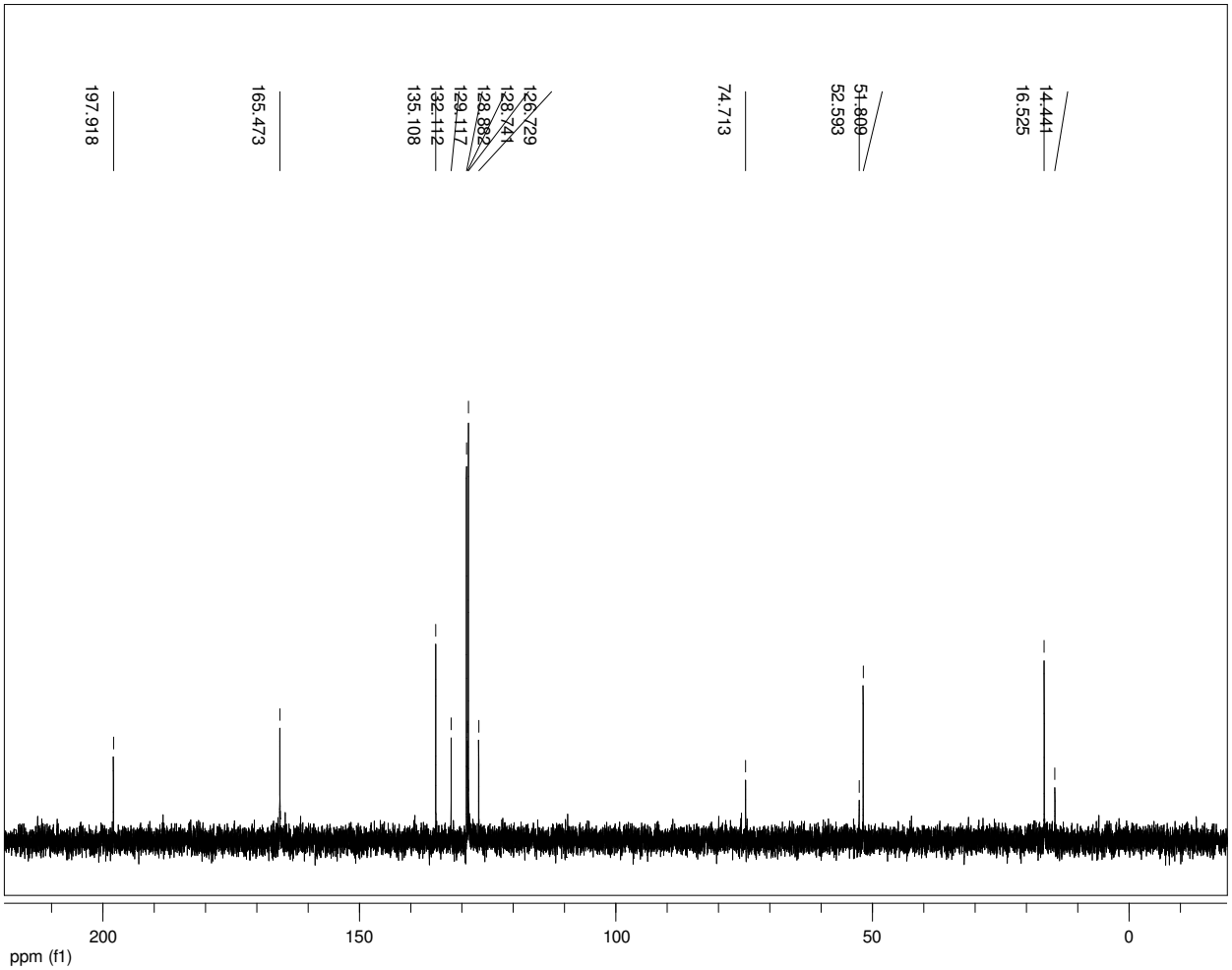
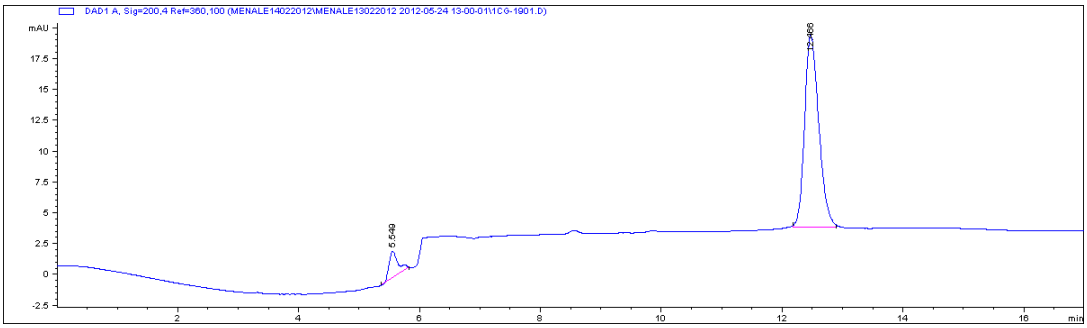
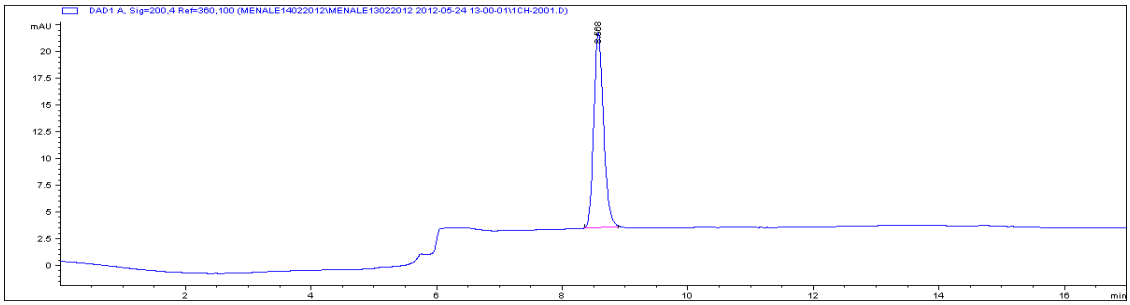


Figure 3.3.2b ^{13}C NMR spectrum of cathine oxalate and cathinone oxalate

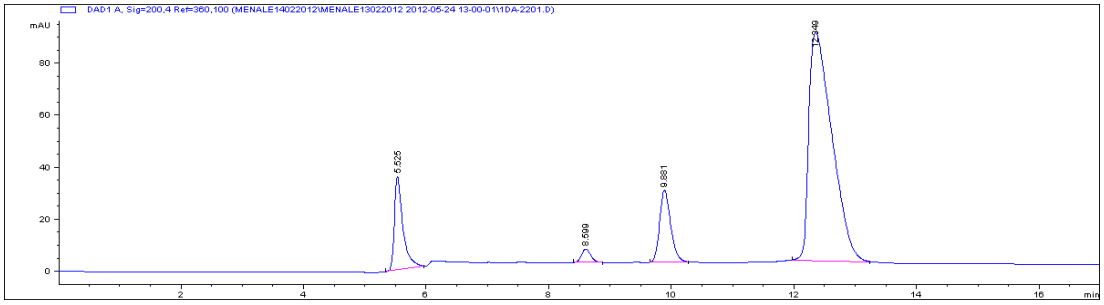
The chromatogram of comercially available, norphedrine, isolated cathinone oxalate, mixtures of alkaloids obtained from the dried young shoot of khat and the spiked samples is shown in Figures 3.3.3 a-d.



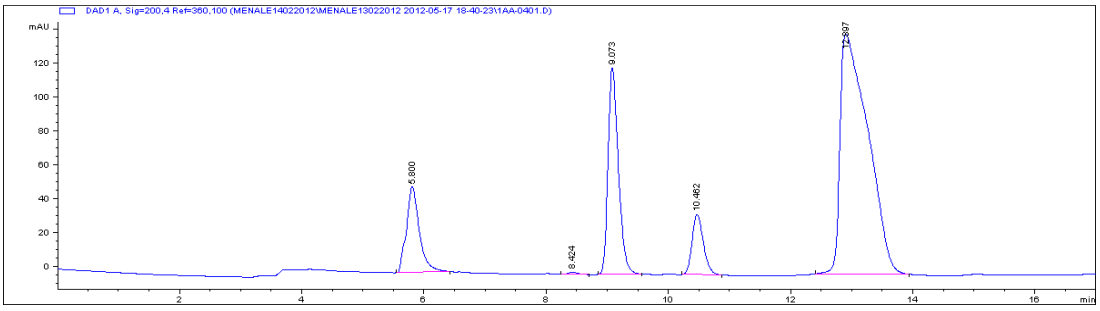
a) Chromatogram of isolated cathinone oxalate (left to right: oxalate, cathinone)



b) Chromatogram of comercially available norphedrine



c) Isolates of khat alkaloids in the form of oxalate salt obtained from sun dried young shoot (left to right; oxalate, norephedrine, cathine and cathinone)



d) Spiked norphedrine and cathinone oxalate to isolates of khatamine oxalates

Figure 3.3.3a-d Chromatograms of khat alkaloids

3.3.4.2 Quantification of the khat alkaloids in differently processed khat samples

Previous studies on khat illustrated the importance of using freshly harvested young shoots and leaves such that cathinone contained within the plant, could be suitably isolated and identified. Upon drying and storage of the cut plant material, cathinone is readily converts to the reduced product, cathine, which necessitates rapid extraction and chemical analysis for cathinone identification (Szendrei, 1980; Brenneisen *et al*., 1987; Lee, 1995; Dagne *et al*., 2010). In this study, five preservative methods have been compared and results of the analysis are shown in Table 3.3.2.

Looking at the table, all the preservative methods were found to retard the complete conversion of cathinone. Refrigeration and freeze drying protocols significantly retarded the conversion of cathinone to cathine and norephedrine up on storage for at least three months. However, oven drying and sun drying protocols were susceptible to cathinone degradation to the corresponding alcohols (norephedrine and cathine).

Comparing the preservation protocols with each other, the percentage of cathinone after deep freezing, freeze drying, room temperature drying, sun drying and oven drying was 77%, 73%, 57%, 42% and 37%, respectively.

The proportion of cathinone among the alkaloids was highest in the deep freezed sample (77%) and freeze dried sample (73%) compared to the air dried and heated preparations (sun drying and oven drying). These data corroborate previous reports as drying under ambient temperature or higher temperature, as well as sun drying significantly reduces cathinone (Szendrei, 1980; Brenneisen *et al.*, 1987; Lee, 1995; Chappell *et al.*,, 2010; Dagne *et al*., 2010). However, it has been confirmed that sun drying or drying at about 60 ^{o}C could not completely degrade the cathinone to cathine and norephedrine unlike the previous reports in this regard (Dagne *et al*., 2010).

A similar trend was observed with the proportion of cathine and norephedrine, where lower concentrations were noticed in the freeze dried and deep freeze samples compared with oven dried and sun dried counter parts. This observation further confirmed the

assertion that cathinone decomposes to cathine and norephedrine upon drying (Lee, 1995; Chappell *et al.,* 2010).

Even though air drying protocol could result in a lower concentration of cathinone (57%) compared with freeze drying and deep freezing, it is a far better way to preserve cathinone compared with sun drying and oven drying.

Chappell *et al. (*2010) examined the effect of air drying, oven drying and microwave oven drying conditions and then compared their results with the un-dried sample. They found the percentage of cathinone to be (11-16%) and 13% in the dried and un-dried samples respectively, which is far lower than the present study. Furthermore, the authors concluded that air drying of khat samples significantly retarded cathinone conversion. However, the present study signified that air drying the samples could significantly degrade cathinone compared with freeze drying and refrigeration of the leaves. This might be due to the fact that the sample considered by them originally had started wilting before being treated with the specified conditions, whereby majority of the cathinone had undergone reduction to the cathine and norephedrine. Hence, their data could not lead us to precisely conclude about effect of drying on cathinone preservation.

Table 3.3.2 Psychoactive phenylpropylamino alkaloids composition of khat and its dried preparations

Preparation conditions	Mean khat concentration (x ± SD) in µg/g		
	Percentage sum of the alkaloids		
	Norephedrine	Cathine	Cathinone
Room-temp. dried	778 ± 17	2208 ± 244	3913 ± 255
	11%	32%	57%
Freeze dried	594 ± 59	1266 ± 49	4701 ± 243
	9%	19%	73%
Sun dried	1279 ± 88	2641 ± 71	2763 ± 184
	19%	39%	42%
Oven dry	1375 ± 73	2604 ± 59	2196 ± 50
	22%	42%	36%
Deep freezed	628 ± 82	975 ± 69	5426 ± 412
	9%	14%	77%

The distribution of khat amines in two types of khat differing by size of the plant was investigated. The results of the analysis are given in Table 3.3.3. As can be seen from the table, khat leaves collected from tree type khat contained a significantly higher concentration of the alkaloids compared to the shrub type khat. This result is in good agreement with experienced chewers' preference towards khat bundles harvested from tree plant than shrub khat.

Table 3.3.3 Khat alkaloids content in tree type and shrub type plant after freeze drying the samples (mean ± SD, µg/g, n = 4).

Sample type	Norphedrine	Cathine	Cathinone
Tree type	594 ± 59	1266 ± 49	4701 ± 243
Shrub/bush type	490 ± 28	830 ± 86	3161 ±120

3.3.5 Conclusion

This study provides conclusive results about khat alkaloids composition variation when different sample pretreatment is considered and stored for about four months. The study demonstrates that by freezing or drying under different conditions, cathinone could be detected in khat samples. Storing the samples after freeze drying or deep freezing in the refrigerator can preserve more than 73% of cathinone unlike other pretreatment techniques like air drying, sun drying and oven drying at about 60 ^{o}C temperature. Even if significant decomposition of cathinone could be observed under these conditions, it is wrong to assume that sun drying the leaves can result complete conversion of cathinone to cathine and norephedrine. Results of the present study confirmed that it is possible to preserve khat samples for longer period after freeze drying or deep freezing without significant loss of cathinone. Isolation of the pure salt of cathinone from freshly picked twigs and young shoot near the tips of khat plant proved the assertion that these parts of the plant contain exclusively cathinone while cathine and norphedrine are also produced in the plant during maturation, drying after harvest or during storage and extraction. Results of this study further indicated that the composition of the alkaloids in khat leaves harvested from khat tree was significantly higher than leaves from khat shrub.

3.4 MOLECULARLY IMPRINTED POLYMER FOR SOLID PHASE EXTRACTION OF KHAT ALKALOIDS FROM KHAT PLANT EXTRACT

3.4.1 Background

Molecularly imprinted polymers (MIPs) are synthetic polymeric materials with specific binding sites at molecular level designed to selectively recognize a target molecule during rebinding. The technique of molecular imprinting consists of the self-assembly of a functional monomer and a template molecule in solution, followed by the co-polymerization of the functional monomer and an excess of an appropriate cross linking monomer (Lanza *et al*., 2001; Dong *et al*., 2005).

After the synthesis, the template is removed by an extraction process, leaving behind imprinted binding sites (cavities) within the polymer network that are tailored in size, shape and chemical functionality to the template. A schematic diagram of the polymerisation is shown in Figure 3.4.1. Under appropriate conditions, these cavities are able to rebind with the template molecule or structurally related compounds in a strong and selective manner when they are reintroduced to the polymer (Yan *et al.,* 2006).

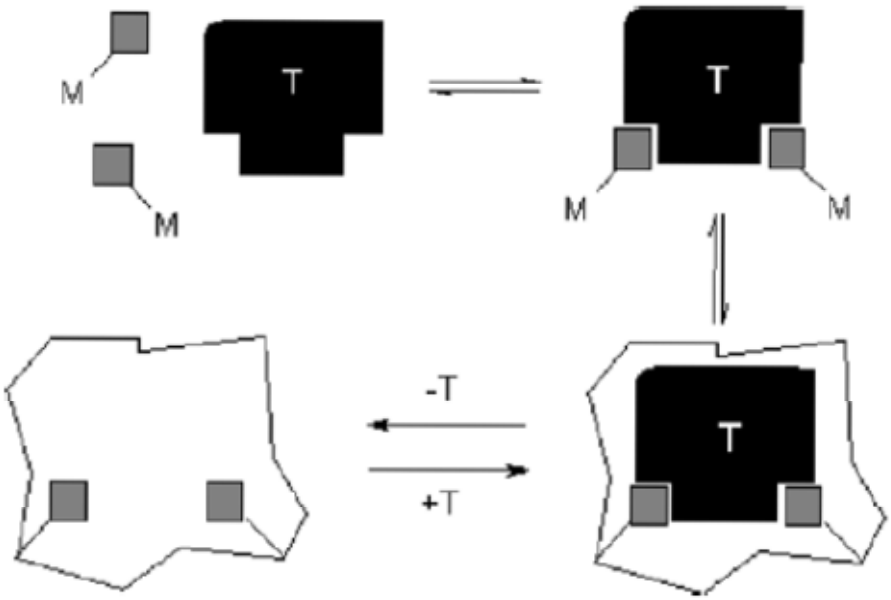


Figure 3.4.1 Principle of molecular imprinting. M = functional monomer; T = template (Lanza *et al.,* 2001)

MIPs are highly cross-linked polymers that have pre-determined selectivity for a single analyte or a group of structurally related analytes. The materials are physically stable, resistant to mechanical stress, high pressure and elevated temperatures. These characteristics make a MIP an ideal analytical material (Pichon *et al.,* 2008).

3.4.1.1 Approaches to MIP Synthesis

The key to synthesizing a successful imprinted polymer is achieving reaction conditions that favor the arrangement of a functional monomer around the template during polymerization. The method by which this monomer-template complex is maintained

during the synthesis distinguishes the two major types of molecular imprinting that have been developed; covalent and non-covalent molecular imprinting (Spivak, 2005; Yan *et al.,* 2006).

In covalent molecular imprinting, the functional monomer is coupled to the template molecule by a reversible covalent bond during polymerization. After the polymer is formed, the imprint template is extracted by chemical cleaved of the covalent bonds, usually by acid hydrolysis. These covalent bonds must easily be cleaved. Template molecules without suitable functional groups have to be derivatized for a linkage. The resulting sites are therefore obtained with an induced process of pre-organization. During rebinding, the target molecule is again covalently linked to the matrix within the binding sites. The advantage of using reversible covalent binding is that the functional groups of the polymer matrix will only be within the cavity and arrange nearly perfect around the template after the template is removed and the non-specific binding effects. The disadvantage is that a small number of different template (diols, ketones, aldehides, carboxylic acides and primary amines)-monomer complexes can be created, limiting the number of molecules that can be targeted by MIPs. The bulk of covalent imprinting strategies involve condensation reactions requiring addition or loss of a water molecule during the cleavage or rebinding process respectively (Arshady *et al*., 1981; Mayes *et al.,* 2005; Yan *et al.,* 2006; Mayes *et al.,* 2005; Kim *et al*., 2001; O' Brein, 2005).

The alternate, and more recent approach, uses non-covalent interactions during the synthesis of MIPs. In non-covalent imprinting, the complex of template and functional monomer is formed in situ by non-covalent interactions, such as hydrogen bonding, electrostatic forces, van der Waals forces, or hydrophobic interactions. Templates are removed by disrupting the interactions and extracting the template using appropriate solvents. Rebinding of analytes by the polymer also occurs through the same non-covalent interactions (Takeda *et al.,* 2005; Yan *et al.,* 2006; Lanza *et al.,* 2001; O'Mahony *et al*., 2005).

This technique is advantageous in terms of easy preparation of the template/monomer complex, easy removal of the templates from the polymers and fast binding of templates to MIPs. Non-covalent imprinting is the most widely used for the preparation of MIPs. In

this technique, optimization is needed and the polymerization conditions must carefully be chosen. As a result, MIPs utilizing the non-covalent approach are more widely used due to the ability to imprint a wide range of compounds. The disadvantage of this approach is that functional groups of the polymer matrix are not always located within the cavities, leading to higher non-specific binding (Lanza *et al.,* 2001; Mayes *et al.,* 2005; Yan *et al.,* 2006).

Compared to the covalent imprinting method, the major advantages of noncovalent imprinting are the versatility in the range of size, shape and functionality of the template molecule, and the time-saving synthesis. When working with non covalent imprinting, it is preferable to use lower polymerization temperatures; higher temperatures will result in weaker monomer/template complexes. In both cases, the procedure of imprinting depends highly on successful extraction of the templates (Yan *et al.,* 2006; Mayes *et al.,* 2005).

3.4.1.2 Factors affecting the imprinting process

The methodology of molecular imprinting is quite straight forward. However, the rational design of MIPs is very complicated because of the number of experimental variables. For example, the template, functional monomer, ratio of functional monomer to template, cross linker, ratio of functional monomer to cross-linker, solvent, initiator, temperature and method of polymerization. Thus, investigation and optimization of various parameters is essential in order to make progress in maximizing recognition effects (Lukbe *et al*., 2000; Yan *et al.,* 2006; Mayes *et al*., 2005 O'Mahony *et al*., 2005; Spivak, 2005).

Template (print molecule)

The strength of binding affinity of the MIPs to analytes depends on the number and type of interaction sites, the template shape and the imprint cavity rigidity. Templates offering multiple interactions sites for the functional monomer are likely to yield binding cavities with higher specificity and affinity for the template (O'Mahony *et al*., 2005; Spivak,

2005; Lubke *et al*., 2000; Yan *et al.,* 2006). For practical applications, the analyte of interest is generally used as the print molecule for preparing the imprinted polymer. The chemical structure of the template is used as the starting point for selecting functional monomer candidates especially if the non-covalent approach is followed (O'Mahony *et al*., 2005; O'Brien, 2005; Mayes *et al.,* 2005).

Although the choice of the template is almost, always the target analyte, where the molecule is very expensive to buy, not readily available, very toxic or not soluble, a structural analogue template can be employed to deal with these challenges (Theodoridis *et al*., 2003; Mayes *et al.,* 2005). The analogue must resemble the print molecule in terms of shape, size and functionalities and should give rise to imprints that have the ability to bind the target analyte. In addition to the type of template, the ratio of the template to the functional monomer has been known to play a key role in the selectivity and sensitivity in the imprinted polymers. The optimum ratio has to be determined for each individual template (Mayes *et al.,* 2005).

Monomer

The functional monomer used for producing a useful MIP must strongly interact with the template to achieve a high yield of imprinted binding sites and allow the maximum number of complementary interactions to be developed in the polymer matrix. In general, analytes containing basic functional groups are best imprinted with monomers containing acidic functional groups and vice versa. Some common monomers used are shown in Figure 3.4.2 Methacrylic acid is one of the most widely used monomer. It interacts ionically with the amine functional group and via hydrogen bonding with a variety of polar functional groups such as alcohols, carboxylic acids, carbamates and carboxylic esters (Mayes *et al.,* 2005; Mullett, 2000; Kim *et al.,* 2003). Template-functional monomer ratios of ≥1:4 are common for non-covalent imprinting (Spivak, 2005; Mayes *et al.,* 2005).

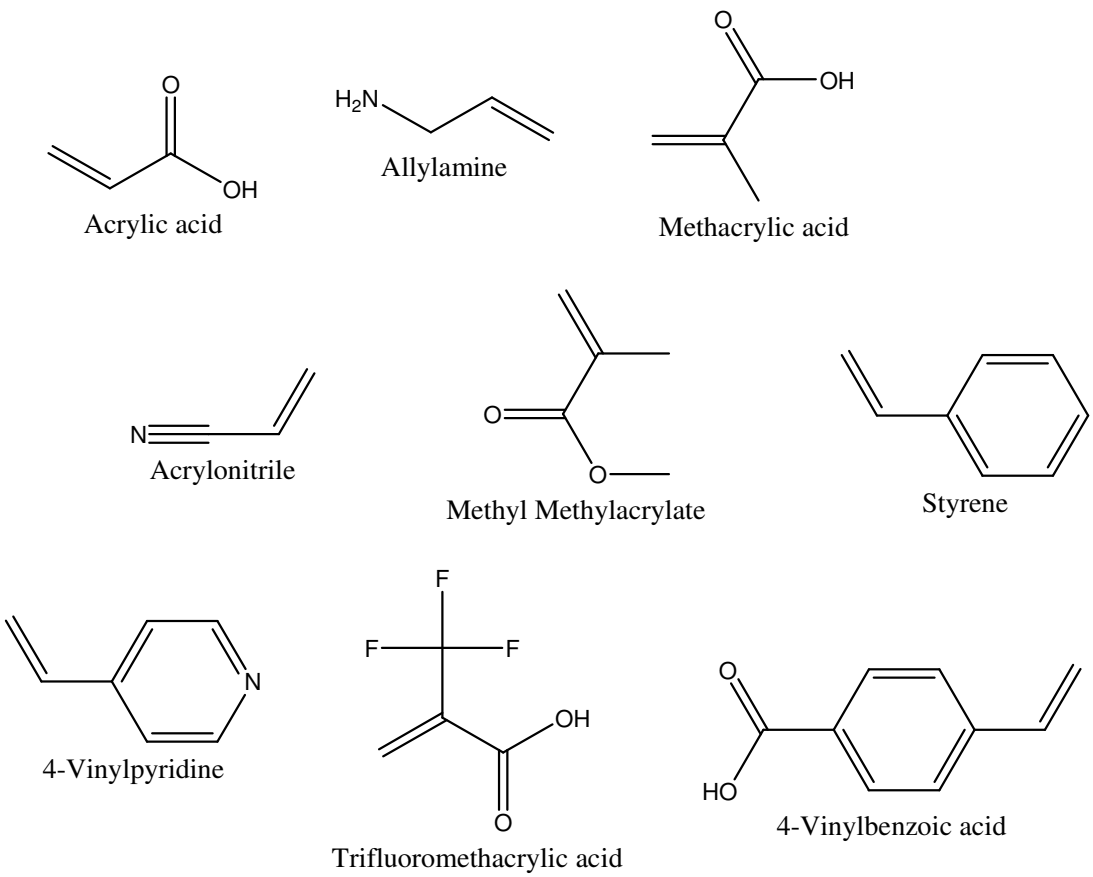
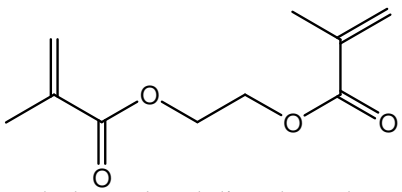


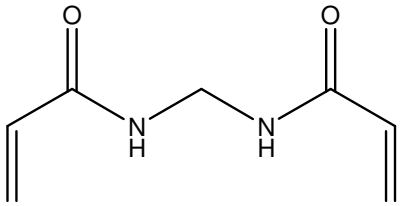
Figure 3.4.2 Some of the common monomers for MIP production

Cross-linker

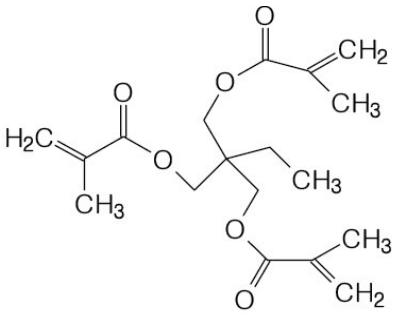
In order to achieve a high specificity and selectivity for the polymer, a high degree of cross-linking is used in the imprinting process. Generally, the cross-linkers fulfill three major functions, including control the morphology of the polymer matrix, stabilize the imprinted binding site in the desired polymer structure and impart stability to the polymer matrix (Yan *et al.,* 2006; Simon, 2005).



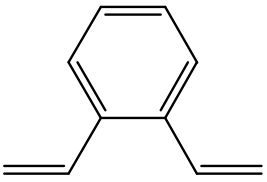
Ethylene glycol dimethacrylate



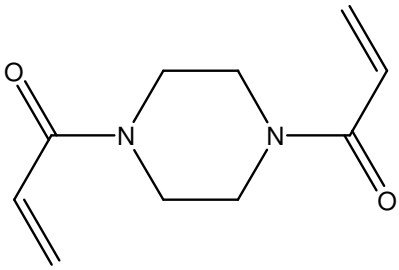
N,N-methylene bisacrylamide



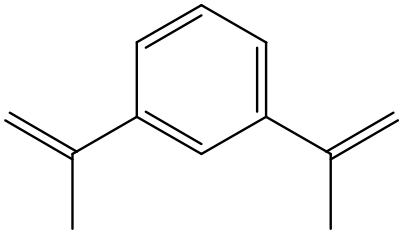
Trimethylopropane
Trimethacrylate



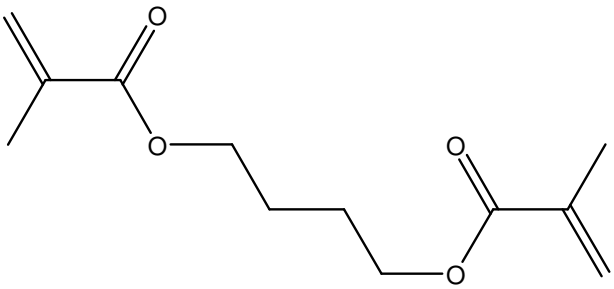
Divinylbenzene



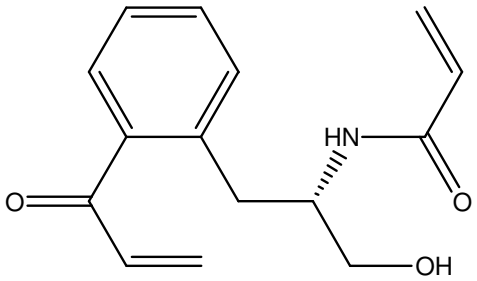
1,4-Diacryloyl piperazine



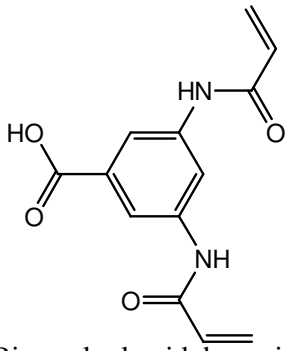
1,3-Diisopropenyl benzene



Tetramethylene dimethacrylate



N,O-Bisacryloyl-phenylalaninol



3,5-Bisacryloylamidobenzoic acid

Figure 3.4.3 Some of the common cross linkers for MIP production

The cross-linker is the component in excess in most polymerisation protocols, and being a di- or tri- functional monomer, it ensures rigidity and robustness of the final material. Being the component in excess it also determines the hydrophobic/hydrophilic character of the polymer as well as its swelling properties. The cross-linker can also play an important role in the porosity of the MIP (Walsh, 2010). Standard formulations such as 1:4:20 of template, monomer and cross-linker have been proposed to give a simple, fast and rational way of obtaining MIPs with improved molecular recognition sites and in which polymers with cross-linker ratios in excess of 80% are often used (Simon, 2005; Mullett, 2000; Cormack *et al.,* 2004; Dong *et al*., 2005; Walsh, 2010; Mayes *et al.,* 2005). Nowadays, many cross-linkers have been tested and reported for several areas of applications. Some of them are listed under Figure 3.4.2 and of which ethylene glycol dimethacrylate (EGDMA) has been used widely as a cross-linker in non-covalent molecular imprinting technique.

Porogen

In the traditional imprinting protocol, the materials are produced by solution polymerisation, meaning that a solvent (porogen) is present. The choice of porogenic solvent is critical in most molecular imprinting procedures. It is also necessary, not only in order to generate a homogenous pre-polymerisation mixture where the template-monomer equilibrium can take place, but also to assist in the production of a porous polymer network, accessible for both the removal of the template as well as the re-binding of the analytes of interest. The porogen solvent is a chemically inert agent which promotes channel formation in the highly cross-linked polymer. The solvent used in the polymer formation should be as non-polar as possible in order to maximize the strength of hydrogen-bonding and ionic interactions between the template molecular and the monomer. Porogenic solvents with low solubility phase separate early and tend to form larger pores and materials with lower surface areas (Yan *et al.,* 2006; Simon, 2005; Walsh, 2010).

However, in recent year, sufficiently strong template: monomer interactions have been observed in rather polar solvents (e.g. methanol/water). In such case, usually hydrophobic

forces are being used to drive the complexation. Few papers have been published using polar porogenic solvents for polymers involving hydrophilic force for complexation. (Yan *et al.,* 2006; He *et al*., 2001; Yang *et al*., 2008; Bravo *et al*., 2009; Mayes *et al.,* 2005).

Initiator and polymerization conditions

Since most molecular imprinting protocols are based on free-radical polymerisations, a free-radical *initiator* is added. The selection is based upon the choice of thermal or photochemical polymerization. However, photochemical polymerization is the method of choice as higher temperature affects the morphology and hence selectivity of the polymer since most thermal polymerization is effective at a temperature above 60 ^{o}C (Cormack *et al.,* 2004; Yan *et al.,* 2006; He *et al*., 2001; Yang *et al*., 2008; Dong *et al*., 2005). The most commonly used initiators are: Azobisisobutyronitrile, dimethylacetal of benzyl, benzoylperoxide, azobisdimethylvaleronitrile, and 4,4'-azo(4-cyanovaleric acid).

3.4.2 Application of molecularly imprinted polymers

During the last few decades, a great deal of research has been dedicated in understanding of the mechanism of formation of MIP. However, majority of researchers were exploring the potential fields of applications of MIPs. These can be categorized in four main areas; namely, chromatography, including solid phase extractions (SPE), immunoassay type of applications, catalysis and sensing (Cormack *et al.,* 2004; Yan *et al.,* 2006; Simon, 2005; Walsh, 2010; Lanza *et al.,* 2001).

Recently, MIPs have appeared as new selective sorbents for SPE of organic compounds in complex materials. Some of the areas of applications of molecularly imprinted solid phse extraction (MISPE) have been reviewed (Qiao *et al*., 2006; Pichon, 2007). MISPE is based on conventional SPE where the processes of conditioning, loading, clean-up and elution are typically applied. MISPE has found to have the ability of selectively isolating specific compounds or their structural analogs from complex matrices. The application of these synthetic polymers as sorbents allows not only preconcentration and cleaning of the

sample but also selective extraction of the target analyte, which is important, particularly when the sample is complex and impurities can interfere with quantification. It is important to underline the fact that MIPs are not intrinsically selective. Therefore, optimization of the extraction steps is required, especially the washing and elution steps, in terms of pH, ionic strength and solvent composition.

3.4.3 Synthesis of molecularly imprinted polymers based on a khat alkaloid's template

There is an on-going challenge for rapid and efficient determination of compounds by low cost analytical methods in the biomedical, environmental, forensic and drug development fields.

Due to the complexity of the khat composition, tedious procedures involving several liquid–liquid extractions (LLE) were repeatedly performed for the determination of the khat alkaloid in the plant extract (Ripani *et al*., 1996; Krizevski *et al*., 2007 and 2008; Gambaro *et al*., 2012; Chappell *et al*., 2010; Laussmann *et al*., 2010). Clean up was conducted using organic solvents under alkaline conditions. Since cathinone is sensitive to alkaline conditions (Szendrei, 1980) extensive sample preparation should be avoided. Furthermore, LLE usually produces emulsion that may decrease the extraction efficiency and lengthen the time the analyst needs to complete the procedure and hence leads to analyte loss.

In addition to LLE, solid phase extraction (SPE) using C18 has been reported. This technique is more rapid, simple, economical and environment-friendly than the traditional liquid-liquid extraction. The materials used in SPE are usually based on the non-specific binding of the targets, which often suffer from some shortcomings, such as low specificity and selectivity (Lanza *et al*., 2001).

Recently, solid-phase extraction involving molecular imprinted polymers (MISPE) have proved to be successful applications such as the separation, extraction and detection of bioactive compounds and naturally occurring as well as synthetic controlled drugs and

their metabolites and therefore can be useful in the field of forensic toxicology and biomedical sectors.

In the past, no attempt was made to use MIP materials as sorbents for the extraction of psychoactive khat alkaloids prior to further analysis of cathinone and its metabolites in the plant extract for biomedical and forensic science application. In this chapter, the synthesis of MIP materials for (-)-norephedrine as template and its group selective application in a novel sample preparation method based on MISPE for the analysis of cathinone, cathine and norephedrine from khat plant extract is described.

3.4.4 Objectives of the study

The objectives of this study are

1. To synthesize MIPs using free base (-) norephedrine as templates,
2. To optimize and evaluate the use of MISPE columns prepared for khat alkaloids extraction (cathinone, cathine and norephedrine)

3.4.5 Experimental

3.4.5.1 Chemicals and reagents

All reagents used were analytical or HPLC grade reagents. (-) Norephedrine, methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA) (Sigma Aldrich, Germany), 1,1'-Azobis(Cyclohexanecarbonitrile) (Sigma Aldrich, USA), methanol and acetonitrile (Merck KGaA, Darmstadt, Germany), orthophosphoric acid, chloroform and acetic acid (Merck Chemicals, Gauteng, South Africa), tripropylamine (Fluka, Switzerland). Cathinone was isolated from the young shoot of the plant in the department of chemistry of Addis Ababa University, Ethiopia. The mobile phase was filtered through a Whatman membrane filter (47 mm diameter and 2 µm pore size) while all samples were filtered through Acrodisc syringe filter (PVDF membrane with 0.45 µm pore size) prior to HPLC analysis.

3.4.5.2 Apparatus and equipments

Analysis of the extract was performed on an Agilent 1200 Series HPLC, Agilent Technologies Inc. (Santa Rosa, CA, USA) equipped with a binary pump and a diode array detector (DAD) set at 200 nm. Separation of the compounds was achieved on an Agilent ZORBAX SB-Phenyl column (4.6 x 250 mm, 5 micron), Agilent Technologies Inc. (Santa Rosa, CA, USA. The data was processed by Agilent ChemStation for LC/MS 2D system software. A reversed-phase method developed by Mathys and Brenneisen (Mathys *et al.,* 1993) was employed to separate and quantitate the alkaloids. The mobile phase consisted of aqueous buffer of pH 2.65 (8.5 g/L orthophosphoric acid and 0.3 mL/L tripropylamine) and 5% acetonitrile in water at a flow of 1.5 mL/min in a gradient mode. UV-Vis Spectroscopy (A lamda 25 Perkin-Elmer spectrophotometer, Santa Clara, CA, USA) was used to study the binding capacity of the polymers. A mercury lamp was used to carry out the polymerization. Scanning electron microscopy (SEM) images were acquired by a TS5136ML Digital Vega Microscope from Tescan (Brno, Czech Republic). FTIR (400-4000 cm^{-1}) spectra were recorded on a Perkin Elmer Spectrum 100 spectrometer (Massachusetts, USA) equipped with a universal ATR sampling accessory.

Plant sample collection

Khat samples of different varieties were collected from Ethiopia and processed in the laboratory of the Department of Chemistry, Addis Ababa University, Ethiopia. The samples were brought from the sampling sites in an ice box and kept in a deep freezer. The chewable parts of the sample were taken and freeze dried as soon as they brought to the laboratory. The dried samples were ground using electrical grinder and passed through 0.5mm sieve. The ground samples were packed into polyethylene bag and taken to the Department of Chemistry, Rhodes University, , South Africa for the analysis.

3.4.5.3 Preparation of the molecularly imprinted polymer (MIP) and non-imprinted polymer (NIP) with bulk polymerization

Polymers were prepared using a method described previously (Lasakova *et al*., 2009; Dong *et al*., 2005). All polymerization were performed under equivalent conditions and composition of the mixture was kept constant throughout the experiments, except for varying the nature of porogenic solvents. The polymerization reaction was carried out in sealed vials. Four different polymers were prepared as follows: norephedrine (0.45 g, 3.0 mmol) was dissolved with each of 15 mL of chloroform, acetonirile, methanol and acetonitrile-methanol (8:1 v/v) and then 1.02 mL (12 mmol) MAA was added in to the tube, which was ultrasonicated for 5 min. EGDMA (11.31 mL, 60 mmol) and 1, 1'-azobis (cyclohexanecarbonitrile) (200 mg) were added to the mixture. The solution was sonicated for 5 min and sparged with nitrogen gas to remove oxygen and properly sealed. The polymerization was initiated with 365 nm UV light at ambient temperature. After 24 h, the bulk polymer was ground using a blender (Fritsch, Pulverisette, Germany) and particles were sieved using 45µm pore size sieve.

Fine particles were removed by repeated sedimentations in acetone. (−)-Norephedrine and un-reacted reagents in the polymers were removed by Soxhlet extraction with 20% HAC in methanol for 24 h. Then, it was packed in to 200 mL bond elute cartridge and rinsed with 10% acetic acid in MeOH until no template was detected by HPLC. Finally, the polymer was rinsed with methanol and dried in the oven at 50 ^{o}C. As a control, non-imprinted polymer (NIP) was also synthesized with the same method in the absence of template molecule.

3.4.5.4 Selection of loading solvent, rebinding study of MIPs and Scatchard analysis

Methanol and water are good solvents for the extraction of khat alkaloids from the plant materials (Krizevski et *al*., 2007; Gambaro *et al*., 2012). They were compared as solvents for analyte loading and retention on the MIP and NIP by measuring the analyte concentration in the supernatant solution after an equilibrium adsorption experiment and

calculating the binding capacity of the polymers. In order to investigate the binding capacity of the MIPs synthesized, batch experiments were conducted in aqueous and methanolic solution. A portion of 50 mg of polymer particles was added into 25 mL conical flask containing 5 mL norephedrine solutions of various concentrations (0.1-20 mM). The mixture was shaken on a horizontal shaker for 24 h at 25 °C, and filtrated through Acrodisc syringe filter (with 0.45 µm pore size) after it has been centrifuged. At the same time, blank sample was also prepared containing all the components except the analyte of interest. The absorbance of the free norephedrine in the supernatant was measured with a UV-Vis spectrophotometer at about 210 nm. The amount of norephedrine bound to the MIP was calculated by subtracting the concentration of free norephedrine from the initial concentrations. The same experiment was performed using the non-imprinted particles. Meanwhile, the maximum binding sites (Q_{max}) was estimated by processing with the Scatchard equation for one particular polymer having higher binding capacity and then used for real sample analysis.

To calculate Q_{max}, a schard plot was made using Q/C_{free} vs Q. Then, from the plot, Q_{max} can be calculated following the formula:

$$Y_{intercept} = Q_{max} / K_d \tag{3.3.1}$$

$$X_{intercept} = Q_{max} \tag{3.3.2}$$

$$Slope = -1/ K_d \tag{3.3.3}$$

$$Q = \left(\frac{C_{initial} - C_{final}}{m} \right) \times V \tag{3.3.4}$$

where Q (mg/g) is the amount of analyte adosorbed per gram of sorbent, C_{free} (mg/L) is the free (unbound equilibrium concentration) of the analyte, $C_{initial}$ (mg/L) is the initial concentration of analytes studied, Q_{max} is the maximum binding quantity (capacity) of the polymer, K_d is the dissociation constant of the polymer, V (L) is the volume of the solution used for adsorption experiment, and m (g) is the mass of the sorben material (polymer)

3.4.5.5 Khat sample preparation

0.5 g of dried khat sample was soaked into 20 mL of deionized water for 15 min and sonicated into ultrasonic water bath for 30 min. After centrifugation the supernatant was used for the analysis.

3.4.5.6 SPE cartridge preparation and optimization of the MISPE conditions

500 mg of MIP or NIP particles were slurry packed with acetonitrile into empty SPE cartridges with two polyethylene frits at each end. The flow of the solution through the SPE sorbent beds was driven by the pressure of vacuum manifold. Before use, the cartridge was conditioned with 3 mL of water and subsequently the sample was loaded before it dried.

In order to selectively retain and then desorb the analytes from aqueous solution, conditions (such as washing and eluting solvents) need to be carefully optimized. Therefore, loading and elution solvents were optimized using a standard norephedrine dissolved in aqueous solution and then the resulting conditions were applied for further parameter optimization (loading capacity of the column, washing solvent and volume of elution solvents) using the plant extract. Two loading solvents (methanol and water) and washing solutions with different amounts of polar solvents (water, acetonitrile (ACN), 50% ACN in water, 75% ACN in water and methanol) were tested while mixture of methanol and acetic acid (HAc) were tested for elution capability.

Selection of elution solvent

In order to btain a satisfactory recovery, the typical elution solvents like 0.5% HAc, 1% HAc, 5% HAc and 10% HAc in methanol were evaluated. 1 mL of water containing (−)-norephedrine (0.25 $mgmL^{-1}$) was loaded on the cartridge. Aliquots of 1.0 mL of elution solvent were then applied. The fractions collected after the loading and elution steps were evaporated using a rotavapor at 40 ^{o}C and reconstituted with the mobile phase prior to analysis by HPLC.

MIP cartridge washing

To investigate the effect of different washing solutions, deionized water, methanol, ACN and water containing 50 and 75% v/v of ACN were investigated after loading 1 mL of the khat extract to the preconditioned column. The cartridge was washed with 1 mL aliquots of the washing solvents until the nonspecifically adsorbed analytes are removed from the surface of the sorbent. Then, it was eluted with 5 mL of 5% HAC in methanol. Each of the loading eluent, washing eluent and elution eluents were evaporated and reconstituted with the mobile phase and analysed by HPLC.

Loading capacity of the sorbent

500 mg of dry polymer was slurry packed into 3 mL cartridge (MIP or NIP). Prior to applying the sample, the polymer was pre-equilibrated with 3 mL of water. Aliquots (1.0 mL) of green or redish khat extract (25 mg/mL) were successively applied to the preconditioned column until either of the alkaloids was detected in the eluent collected from the loaded extract. Each time, the collected fraction was evaporated and reconstituted with 1 mL of the mobile phase. After maximum loading of the extract, the cartridge was washed with 25 mL of 50% aqueous ACN and then eluted with 10 mL of 5% HAC in methanol. The collected eluent from sufficiently loaded cartridge was evaporated and reconstituted with the mobile phase.

3.4.5.7 MISPE and khat sample analysis

MISPE sorbents were conditioned with water (3 mL) and then 1 mL of the crude khat extracts were loaded onto the MISPE cartridges by allowing them to drip through under vacuum flow. The columns were then washed with 7 mL of 50% ACN in water. The alkaloids were eluted using 5 mL of 5% HAc in methanol. The eluant was evaporated to dryness under vacuum at 40 °C and the residue was reconstituted in 1 mL of the mobile phase. A 5 μL aliquot of sample was then analyzed by HPLC-DAD.

3.4.5.8 Reproducibility and recovery

A 1 mL of khat extract containing 50 µg and 100 µg of cathinone and norephedrine, respectively, was passed through a preconditioned MIP cartridge. The cartridge was washed and eluted as described above.

3.4.6 Results and discussion

3.4.6.1 Characterization

The synthesized MIPs and NIPs using chloroform as a porogenic solvent were characterized by FT-IR and scanning electron microscopy (SEM). Results are shown in Figures 3.4.4 and 3.4.5, respectively. The FT-IR spectra of unwashed MIP, washed MIP, and NIP displayed similar characteristic peaks, indicating similarity in the back bone structure of different polymers. In the IR spectra, the absorbance's pertaining to norephedrine (spectrum d), stretching of the O—H and N—H group of the template (3260 cm^{-1}, 3340 cm^{-1}), stretching of aromatic C=C (1602 cm^{-1}), stretching of aromatic C—H (1212 cm^{-1}) and bending of aromatic C—H (790 cm^{-1}) are not observed in the MIP spectrum, and hence the polymerization was complete (Figure 3.4.4).

Morphology of MIP and NIP particles determined by a SEM is shown in Figure 3.4.5. The image of both MIP and NIP had an irregular, rough morphology (having many small cavities). There are remarkable differences seen in the morphology of the polymers and a more porous surface is observed for the MIP.

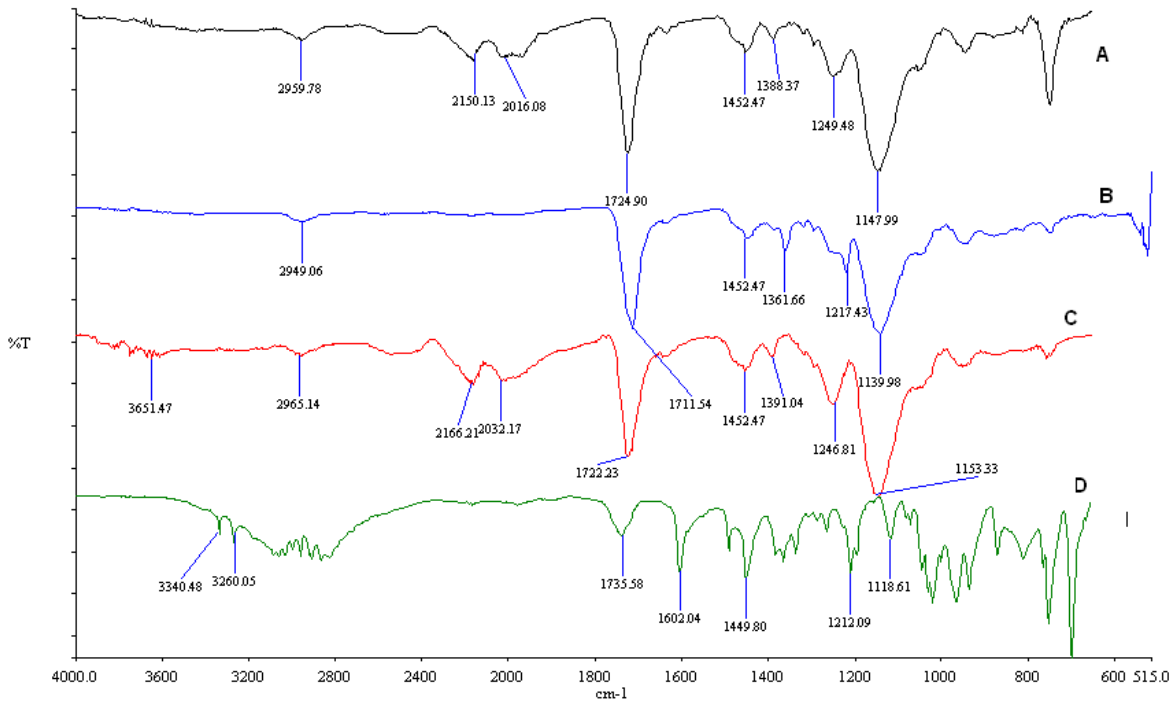
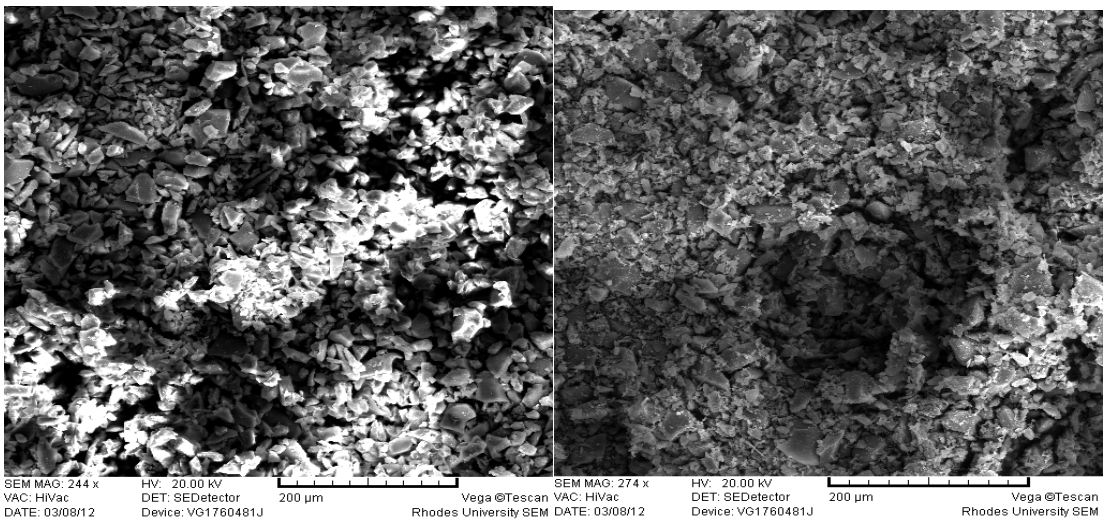


Figure 3.4.4 FT-IR spectra of un-leached MIP (A), leached MIP (b), un washed NIP (C) and template (D)



A b

Figure 3.4.5 SEM micrograph (x 200) of (a) washed MIP and (b) NIP using chloroform solvent and bulk polymerization process

3.4.6.2 Optimization of MIP formulation using various porogenic solvents and effect of loading solvents on the binding capacity of the polymer

Effect of progenic solvent and loading solvent

Different (-)-norephedrine–MIP and NIP compositions were tested by studying the influence of different porogen on the properties of the MIP. Solvents play an important role in the formation of a porous structure of MIPs and influence final characteristics of the obtained materials in terms of affinity and selectivity for the target analyte (Lopez *et al*., 2012). As it has been reported elsewhere, porosity and surface area are determined by the type of solvent used in the polymerization (Yan *et al*., 2006).

In this study, the binding capacity of MIPs and NIPs synthesized using chloroform, ACN, methanol and mixture of ACN/methanol (8:1) from methanolic and aqueous solution have been investigated and results are shown in Figure 3.4.6 and 3.4.7, MIP from chloroform followed by MIP from ACN have much better binding capacity for norephedrine from aqueous and methanolic solution than MIP synthesized using other solvents. While the binding capacity of MIP and NIP synthesized using methanol as a solvent is significantly lower than any of the tested porogenic solvents, which signifies that methanol is not a good solvent for the particular MIP preparation. Therefore, chloroform was selected as porogen for the analysis. This result is also corroborating with previous reports (Lopez *et al*., 2012; Yan *et al*., 2006).

The efficiency of methanol and water as a loading solvent were compared and the results are shown in Figure 3.4.6 and Figure 3.4.7. As can be seen from the figures, the binding capacity of MIPs for norephedrine from aqueous solution is significantly higher when compared with methanol as a loading solvent. This might be due to the methanol having competed with the analyte towards for active sites. Furthermore, no selectivity was seen between MIP and NIP when the analyte was loaded in a methanol solution which indicate that the interaction is non-specific. Therefore, water was selected as a loading solvent for the study.

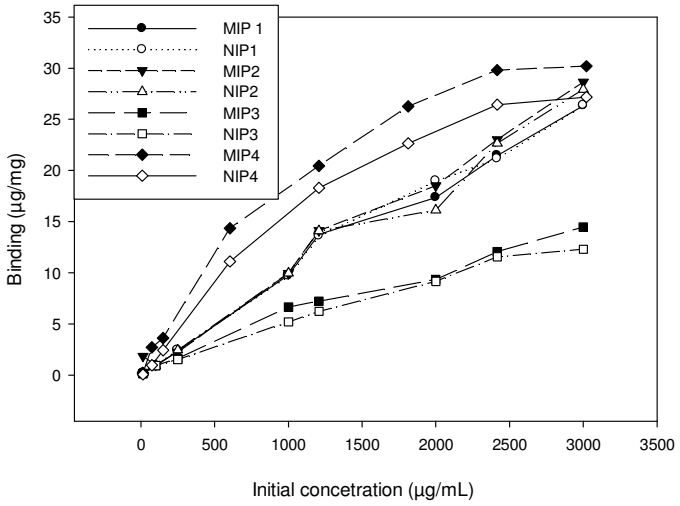


Figure 3.4.6 Binding isotherm of (-) norephedrine from methanolic solution on MIP and NIP synthesized using porogenic solvents of $CHCl_3$ (1), ACN (2), MeOH (3) and ACN/MeOH (4)

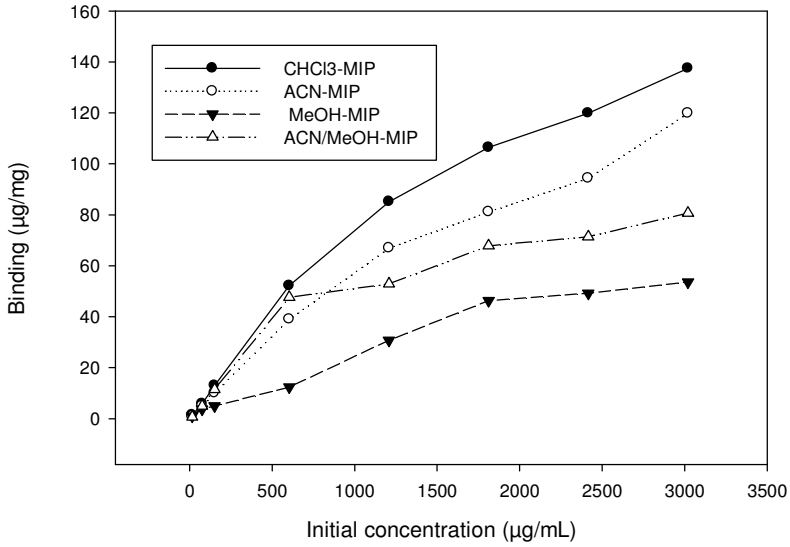


Figure 3.4.7 Binding isotherm of (-) norephedrine from aqueous solution on MIP synthesized using porogenic solvents of $CHCl_3$, ACN, ACN/MeOH and MeOH

Rebinding study of MIPs and Scatchard analysis

After the MIP and NIP had been synthesized using chloroform as a porogenic solvent, equilibrium adsorption experiment was performed with MIP and NIP particles so as to evaluate the binding affinity of the MIP and NIP from an aqueous environment. It has been shown that MIP has higher binding capacity than NIP beyond (500 µg/mL solutions), which is due to the presence of specific binding sites in the MIP (Figure 3.4.8). As can be seen from the figure, the amount of NE adsorbed increased as the initial concentration of the NE solution increased. The static adsorption capacities (apparent maximum binding sites) of the MIP and the NIP were calculated as 128.6µg/mg and 84µg/mg, respectively. In the MIP, there were specific and nonspecific binding sites, but in the NIP, there were only nonspecific binding sites. From the experimental results, it seems that norephedrine adsorbed to the nonspecific sites before it adsorbed to the specific sites. After most nonspecific sites had been occupied, specific sites began to be occupied. This is the reason why, at low concentrations, the adsorption capacities for the MIP and the NIP are similar. But at high concentration of norephedrine, when the nonspecific sites were almost all occupied in the MIP, the specific sites containing a cavity tailor-made for norephedrine could interact with norephedrine efficiently and the adsorption of the MIP was more than that of the NIP (Guo *et al*., 2008).

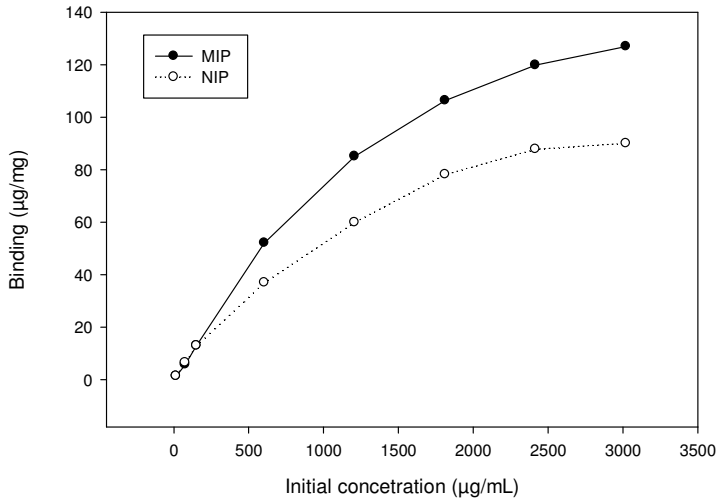


Figure 3.4.8 Binding isotherm of (-) norephedrine from aqueous solution on MIP and NIP using $CHCl_3$ as porogenic solvent

3.4.6.3 Evaluation of the elution solvent

The optimization of the elution step was performed using methanol containing different concentrations of acetic acid. Figure 3.4.9 shows the percentage of (-) norephedrine recovered after each volume of elution solvent passed through the column. From the graph, it can be depicted that good recoveries were obtained for all the eluents with increase in elution solvent volume but the volume of the elution solution required for the complete elution of the (−)-norephedrine decreased when the concentration of acetic acid increased. Highest recovery (more than 90%) was obtained when using 4 mL of 5% and 10% acetic acid solution as eluting solvent. Thus, for the sake of completeness, 5 mL of 5% acetic acid in methanol was taken as elution solvent for the MISPE method.

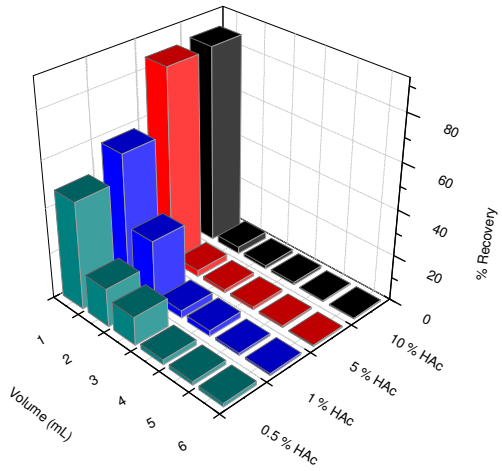


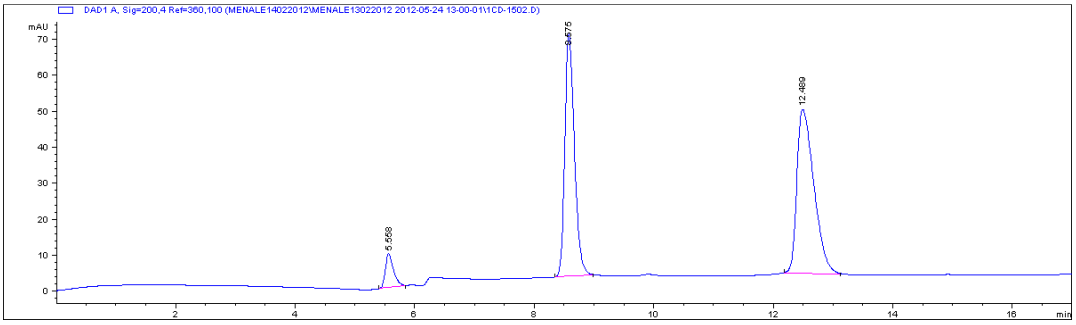
Figure 3.4.9 Eluting ability of elution solutions with different acetic acid (HAc) concentrations for (−)-norephedrine

3.4.6.4 MIP cartridge washing

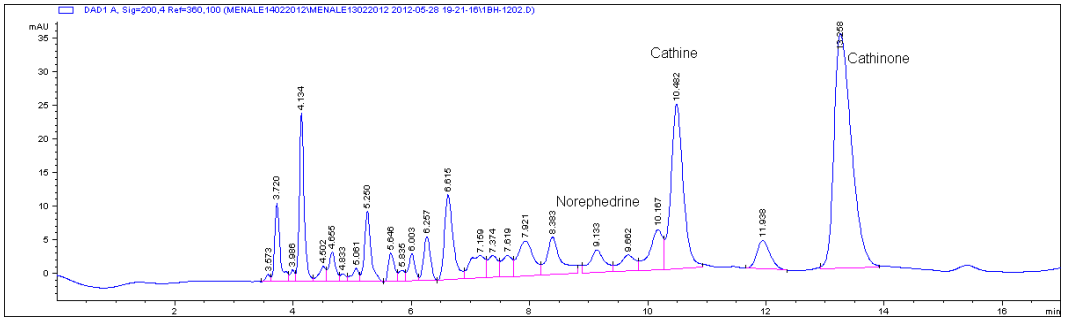
After direct injection of the khat extract without the MISPE pretreatment, very intensive and a broad peaks, resulting from overlapping peaks from the many compounds in khat absorbing in the UV range, made an interpretation very difficult (Figure 3.4.10). This is due to the fact that khat extract contains many components including simple phenolic acids, tannins, flavonoids, sugar and other primary and secondary metabolites (Szendrei 1980). Hence, the washing step was the most crucial procedure to maximize the specific interactions between the analytes and binding sites, and to simultaneously decrease nonspecific interactions and to discard matrix component in the polymers. Therefore, deionized water, methanol, ACN and water containing 50 and 75% v/v of ACN were investigated after loading 1 mL of khat extract. The cleaning-up abilities of the four solvents were compared by examining the HPLC profile of the extracts after the washing steps. It has been noted that pure water and pure acetonitrile significantly remove the matrix components when about 5 to 7 mL of the washing solvent was applied. Mixing acetonitrile with water (50% and 75%) completely removes matrix components and leaving the three structurally related psychoactive alkaloids retained on the sorbent material (no analytes were washed out in both cases). It was found that, one matrix components was also strongly adsorbed onto the polymer with the analyte of interest. However, because of strong interaction between the alkaloids and the sorbent material, the tested solvents were unable to remove the nonspecifically bound analytes from the polymers and hence the washing solvents have resulted in poor selectivity between the MIP and NIP.

Methanol was also evaluated and it was anticipated that nonspecifically bound analytes on the NIP were eluted quantitatively with increasing volume of methanol (up to 5 mL). Concerning the MIP, stronger binding of the alkaloids was expected on the imprinted active sites. However washing the cartridge with upto 5 mL of methanol eluted a significant portion (up to a third of) the alkaloids, revealing the heterogeneity of the binding sites. But rinsing the cartridge with 1 mL of methanol after washing it with 7 mL of 50% water in acetonitrile resulted in the largest difference in recovery between MIP

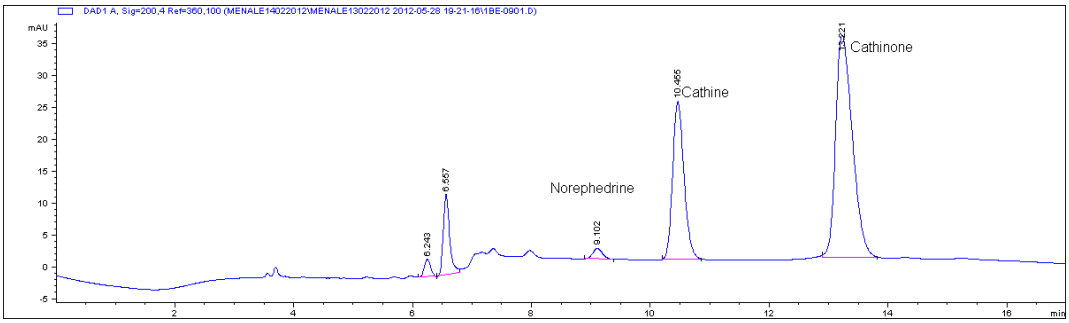
and NIP as well as lower recovery (MIP (80.2%) and NIP (60.5%) as compared to methanol untreated extract. Finally, 7 mL of 50% water in acetonitrile was taken as elution solvent even though the selectivity between MIP and NIP was poor.



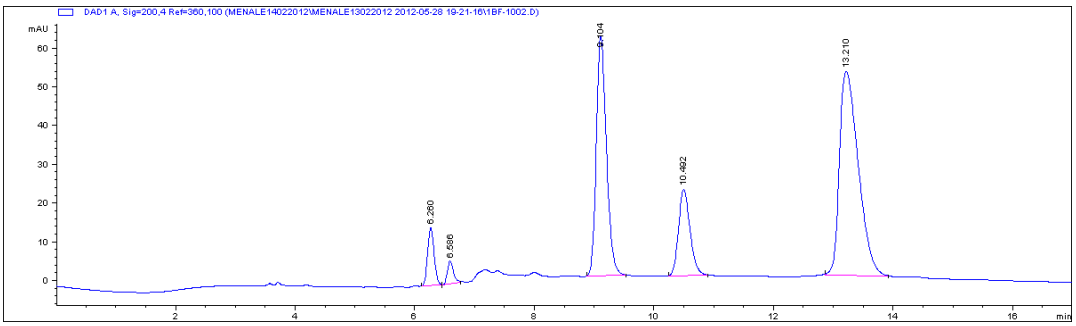
(a) Standard (left to right: oxalate, norphedrine, Cathinone)



(b) Untreated sample



(c) Eluted from MIP



(d) Spiked

Figure 3.4.10 Chromatogram of khat alkaloids (a) standard, (b) untreated khat extract (c) eluted from MIP, (d) Spiked khat extract

3.4.6.5 Evaluation of the MIP cartridge capacity

The capacity of the MISPE column was determined as described above. It has been found that co-extracted matrix components in the plant extract can influence the capacity of MISPE column (Dong *et al*., 2005). Thus, khat extract, instead of khat alkaloids standard solution were used for MISPE capacity tests. As it was determine by MISPE-HPLC, the amount of the alkaloids in 1 mL of green khat extract were 60 µg cathinone, 44 µg cathine and 4 µg (-) norephedine. Aliquots of 1.0 mL extract were applied to the cartridge until either of the alkaloids was detected in the eluent collected from the loaded extract. Each time, the collected fractions (loading, washing and elution) were evaporated and reconstituted with the mobile phase and determined by HPLC. It has been observed that only traces of the alkaloids were released from the MISPE column in the washing step. Thus, the total amount of the alkaloids (1548 µg) for green khat and (1042 µg) for red khat eluted with 5% acetic acid in methanol was taken as the capacity of the column (Table 3.4.1).

The capacity of the non-imprinted polymer SPE column (NISPE) for the alkaloids was also determined. The same procedure was followed as above. The result showed that MIP column has relatively higher loading capacity than NIP for both of the samples (Table 3.4.1).

Table 3.4.1 Binding capacity of MIP and NIP column for khat alkaloids from different Sike type khat varieties

	Green khat		Red khat	
	MIP	NIP	MIP	NIP
Total volume of extracts loaded on the column (mL)	16	11	13	10
Amount of total alkaloids loaded, in µg	1728	1188	1209	930
Amount of recovered alkaloids (µg)				
in loading step	113	104	63	76
in washing step	46	82	31	69
in elution step	1548	1042	1140	831

The results are the average of two measurements. The amount of total alkaloids in green khat and red khat varieties in 1.0 mL of the extract were 108 and 93 µg, respectively. The quantities of the alkaloids eluted from each step were determined by HPLC-DAD.

3.4.6.6 Evaluation of the analytical method

Once conditions for the cleanup of extracts were optimized, known concentrations of cathinone and norphedrine were spiked to the khat sample and extraction was conducted. Table 3.4.2 depicts the recoveries together with the precision results. As can be seen, good recovery and precision have been demonstrated that the MISPE process is a suitable method for the sample pre-treatment for the determination of khat alkaloids in khat extract. Due to lack of pure cathine standard, recovery was not performed for it and its concentration in the sample was determined using the calibration curve parameters of norephedrine since cathine and norephedrine are diasteriomers and expected to have comparable molar absorptivity.

Table 3.4.2 Average recoveries of norephedrine and cathinone from spiked khat (n = 4)

	Spiked alkaloids	
	(-) Norephedrine	Cathinone
Amount in the sample (µg/mL)	3.17	56.9
Amount of spiked (µg/mL)	100	50
Amount after spiking (µg/mL)	94.3	97.7
Recovery (%)	91.1	81.5
% RSD	2.27	2.93

3.4.6.7 MISPE and determination of khat alkaloids in selected samples

Two khat varieties were analyzed by HPLC after extraction with ultrasonication described above and MISPE clean up. Results are given in Table 3.4.3.

Table 3.4.3 MISPE extraction of khat alkaloids from Sike type khat varieties (mean ± SD µg/g, n = 4)

	MISPE		
Khat type	Norephedrine	Cathine	Cathinone
Red khat	251 ± 10	562 ± 24	2908 ± 133
Green khat	160 ± 5	1760 ± 81	2400 ± 65

3.4.6.8 Regeneration of the MIP

To evaluate the reusability of the cartridges, both the MIP and NIP cartridges were cleaned and regenerated by washing with 10 mL of methanol–acetic acid (9:1 v/v) followed by rinsing with 5 mL of methanol, and then dried under vacuum. Reusability studies were carried out for times. Results of the analysis are given in Figure 3.4.10.

From the result, no significant change in concentration of the analytes was observed and hence the cartridge can be used repeatedly at least four times.

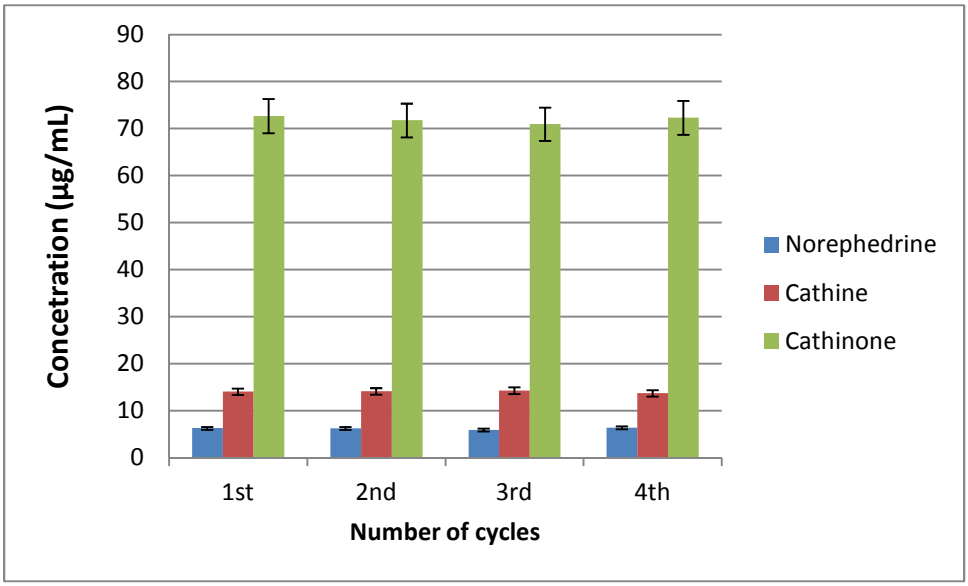


Figure 3.4.11 Regeneration of the SPE cartridge

3.4.6.9 Conclusion

In view of the need for a group selective and sensitive methods to determine psychoactive khat alkaloids in khat samples, in this work, a non-covalently MIP was synthesized by bulk polymerization using (-) norephedrine as a mimic template in a chloroform system, and applied as sorbent in SPE to obtain a selective extraction and clean up of cathinone, cathine and norephedrine with high recoveries. The extraction method is cost effective and does not consume large amounts of organic and toxic solvents which offer the advantages of selectivity towards the three structurally analogue psychoactive alkaloids, enabling an effective sample pretreatment of the plant extract and simplifies the subsequent chromatographic analysis. Reusability study of the cartridge revealed that the cartridge can be used repeatedly at least four times. The presented approach demonstrates the application of MISPE for the analysis of khat samples for the first time and can be applicable in pharmaceutical forensic and biomedical investigation.

3.5 DEVELOPMENT OF MODIFIED QUICK, EASY, CHEAP, EFFECTIVE, RUGGED, AND SAFE (QUECHERS) EXTRACTION METHOD FOR THE DETERMINATION OF KHAT ALKALOIDS

3.5.1 Background

In the analysis of bioactive natural products, one of the fundamental problems arising from the complexity of the matrices is analyte extraction prior to chromatographic determination. In the determination of psychoactive alkaloid compounds in khat plant material, sample preparation is a critical step and sometimes limits the development of analytical methodologies.

So far, for the extraction of khat alkaloids, maceration (solid-liquid extraction) and ultrasonic assisted extraction (UAE) have been frequently reported in the biomedical as well as forensic crime laboratories. Since khat extracts contain a wide range of co-extractives (simple phenolics, flavonoids, tannins and cathedulins), additional sample

clean-up is a must before chromatographic determination of the alkaloids. Thus, liquid–liquid extractions (LLE) and solid phase extraction using C_{18} sorbent were the only and repeatedly performed protocols to clean- up the interfering matrices (Geisshusler *et al.,* 1987; Mathys *et al*., 1993; Ripani *et al*., 1996; Krizevski *et al*., 2007 and 2008; Gambaro *et al*., 2012; Chappell *et al*., 2010; Laussmann *et al*., 2010; Dagne *et al*., 2010).

Maceration (solid-liquid extraction) is a time consuming and likely to cause cathinone degradation when prolonged extraction time is applied as the method requires 2 - 24 h for efficient extraction of khat alkaloids (Dagne *et al*., 2010; Gambaro *et al*., 2012; Geisshusler *et al.*, 1987; Ripani *et al*., 1996; El-Domiaty *et al*., 1994; Laussmann *et al*., 2010).

Ultrasonication is the most powerful and efficient extraction technique for a wide range of analytes from biological samples including khat alkaloids from khat plant. However, the methods still suffered from limitations as extraction and clean-up steps were carried out separately and conditioning, washing and elution steps were time consuming during SPE clean-up. Also LLE usually produces an emulsion that may decrease the extraction efficiency and lengthen the time the analyst needs to complete the procedure and hence leads to analyte loss.

The development of faster, more cost effective and environmentally friendly procedures as well as incorporating both extraction and clean up steps simultaneously is therefore a pressing demand.

Anastassiades *et al*.(2003) developed an approach that dubbed quick, easy, cheap, effective, rugged, and safe (QuEChERS) in contrast to the traditional methodology with multiple stages and the use of large amounts of sample. The authors questioned the conditions previously used for pesticide residues analysis, and through extensive experiments and novel use of $MgSO_4$ for salting out extraction/partitioning and dispersive solid-phase extraction (d-SPE) for cleanup; they devised a highly streamlined sample preparation method with excellent results for a wide range of pesticides in many types of samples.

The original procedure consisted of the sample being extraction by hand-shaking or vortex mixing with the 10 mL of acetonitrile (MeCN). Gram quantities of salts (4 g of $MgSO_4$ and 2 g of NaCl) are then added to the sample by mixing, to drive partitioning of the analytes between the aqueous residue and the solvent. After vortex mixing and centrifugation, clean-up and removal of residual water is performed using a d-SPE procedure (PSA adsorbent and anhydrous $MgSO_4$ are mixed with the sample extract), that requires less time than the traditional SPE and simultaneously removes residual water and many polar matrix components, such as organic acids, some polar pigments, and sugars (Anastassiades *et al*.,2003; www.quechers.com). Initially the methodology was developed for the analysis of veterinary drugs (anthelmintics and thyreostats) in animal tissues, but after realizing its great potential in the extraction of polar and particularly basic compounds, it was also tested on pesticide residue analysis in plant materials with great success.

Because of some limitations on the original method, modifications have been done in the subsequent years, depending on the properties of the analyte, the matrix composition, and the techniques and equipment available in the laboratory. The major changes include the addition of buffers like citrate and acetate salts (Lehotay, *et al*., 2005; Li *et al*., 2002; Lehotay, *et al.,* 2007) to avoid the degradation of certain pesticides, and the addition of water, to dry samples, to obtain the necessary moisture (Lehotay, *et al*., 2005; Sirhan *et al*., 2011; Khan *et al*., 2009; Pérez-Burgos *et al*., 2012). In the following clean-up step, dispersive SPE has been modified through the use of graphitized carbon black (GCB), C18 sorbent, or Florisil cartridges (Khan *et al*., 2009, Shi *et al.,* 2012). Other modifications proposed have been the use of dry ice to separate phases without the need for salting-out (Lee *et al*., 2011).

In general, QuEChERS based methods involve two steps: the first one is an extraction step based on partitioning via salting-out extraction involving the equilibrium between an aqueous and an organic layer, and the second one is a dispersive SPE step that involves further clean-up using combinations of $MgSO_4$ and different sorbents, such as C_{18}, primary–secondary amine (PSA) or graphitized carbon (GCB) to remove interfering substances.

It is very important to homogenize the sample prior to extraction so as to maximize the available surface area of the sample for better extraction efficiencies. During extraction, an exothermic reaction occurs between the magnesium sulphate and water, which can lead to low recoveries of the pesticides and hence the extraction tube is usually immersed in an ice bath (Khan *et al.,* 2009).

In the recent years, QuEChERS has shown its usefulness in the analysis of residues in foods, presenting some advantages, such as simplicity, minimum steps, and effectiveness for cleaning up complex samples.

The QuEChERS method makes it easier and less expensive to examine contaminants in food than other pretreatment methods and has been successfully used for the extraction and purification of a variety of chemicals, including pesticides, polycyclic aromatic hydrocarbons, antibiotics, mycotoxins, and veterinary drugs in a wide range of matrices (Tomasini *et al*., 2012; Shi *et al*., 2012; Paya *et al*., 2007; Pinto *et al*., 2010; Romero-González *et al*., 2011).

However, to the best of our knowledge, no studies were reported to apply this technique for natural products extraction possibly, because of the strong analyte-matrix interaction and the poor recoveries. However, one paper has been published very recently after we have modified and applied the method for alkaloids extraction (Delgado-Zamarreno *et al*., 2012). The authors applied the already established procedure for extraction of isoflavones (naturally occurring flavonoids) from plant material and have obtained appreciable result and recovery.

3.5.2 Objectives of the study

The aim of this study was to modify and develop the conventional QuEChERS extraction for the analysis of psychoactive phenylpropylamino alkaloids from khat (*Catha edulis* Forsk) chewing leaves. The purpose of modification was due to the fact that under acidic condition, the alkaloids are easily protonated and hardly partitioned into the acetonitrile phase. Under alkaline condition, the alkaloids exist in free amine base form and can be solubilized into the acetonitrile layer but phase separation cannot be achieved between

the aqueous layer containing the salt and the acetonitrile phase. Thus, an alternative organic solvent was thus devised. Furthemore, this study aimed to compare the extraction efficiency of QuEChERS extraction with that of matrix solid phase dispersion (MSPD) (optimized by our group) and with the already estabilished ultrasonic assisted extraction followed by solid phase extraction (UAE/SPE) reported by Mathys *et al.* (1993).

3.5.3 Experimental

3.5.3.1 Chemicals and reagents

All the reagents used were of analytical or HPLC grade reagents. Acetonitrile (Merck KGaA, Darmstadt, Germany), orthophosphoric acid, hydrochloric acid, diethyl ether, ethylacetate and NaOH (Merck Chemicals, Gauteng, South Africa), tripropylamine (Fluka, Switzerland), (–)-norephedrine [(1*R*) (2*S*)-norephedrine and (+) ephedrine hydrochloride were purchased from Sigma Aldrich. QuEChERS extraction tube and SampliQ QuEChERS AOAC Extraction kit, p/n 5982-5755 (Agilent Technologies Inc., Wilmington, DE, USA). (–)-Cathinone oxalate was isolated from the fresh leaves of the plant (Dagne *et al*., 2010). The water used was from MilliQ system (Millipore Milford, Mass, USA). The mobile phase was filtered through a Whatman membrane filter (47 mm diameter and 2 µm pore size) while all the plant extracts were filtered through Acrodisc syringe filter (PVDF membrane with 0.45 µm pore size).

3.5.3.2 Apparatus and equipments

The analysis was performed on an Agilent 1200 Series HPLC, Agilent Technologies Inc. (Santa Rosa, CA, USA) equipped with a binary pump and a DAD set at 200 nm. Separation of the compounds was achieved on an Agilent ZORBAX SB-Phenyl column (4.6 x 250 mm, 5 micron). The data was processed by the Agilent ChemStation for LC/MS 2D system software. A reverse-phase method developed by Mathys *et al.,* (1993) was employed to separate and quantitate the alkaloids. The mobile phase consisted of

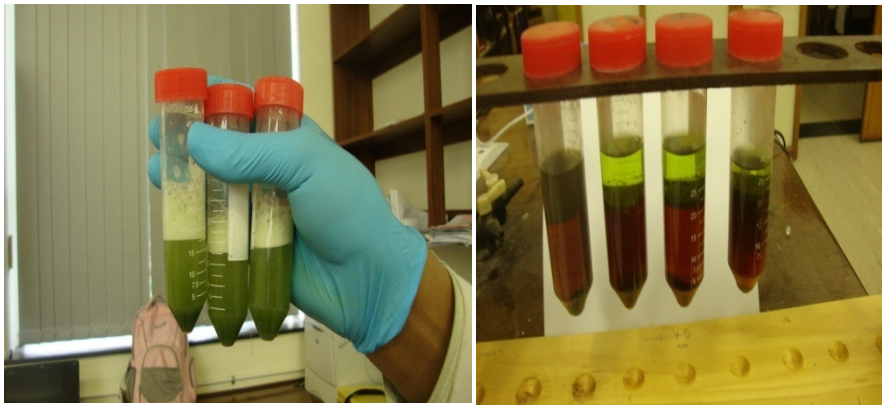
aqueous buffer of pH 2.65 (8.5 g/L orthophosphoric acid and 0.3 mL/L tripropylamine) and 5% acetonitrile in water at a flow of 1.5 mL/min in a gradient mode.

3.5.3.3 Plant materials collection

Khat samples were collected from Ethiopia and processed in the laboratory of the Department of Chemistry, Addis Ababa University, Ethiopia. Samples were brought from the sampling site in ice box and kept in the deep freeze. The chewable parts of the samples were taken and freeze dried within 5 h of arrival in to the laboratory, blended to fine particles in electrical blender and passed through 0.5mm sieve. The ground sample was packed in to polyethylene bag and taken to Chemistry Department of Rhodes University, South Africa for the analysis.

3.5.3.4 Procedure for modified QuEChERS based extraction

A 0.25 g sample was weighed into a 50 mL centrifuge tube containing 100 µL (500 µg) of ephedrine hydrochloride as an internal standard and 15 mL of 1% acetic acid (HAc) in water and kept for 15 min so as to allow the solvent to penetrate the cell wall of the plant material. A QuEChERS salt kit was added and vigorously shaken for 6 min. About 2 mL of 20% aqueous NaOH solution was added to make the solution alkaline (about pH 10, checked by universal paper indicator) followed by 10 mL of ethyl acetate and vigorously shaken for 2 min. The solution was kept for 2 min, or centrifuged for 30 s, to enhance phase separation. 5 mL of the greenish organic layer (Figure 3.5.1) was taken, using a pipette, placed in to 10 mL round bottomed flask and evaporated to dryness using Rota Vapor (BUCHI Rotavapor R-134, Switzerland). The alkaloids were re-constituted with 2.5 mL of the mobile phase (water containing 8.5 g/L phosphoric acid) while those fat soluble components were retained in the flask. The resulting solution was filtered through Acrodisc syringe filter (PVDF membrane with 0.45 µm pore size) and about 1 mL of the resulting filtered solution was placed in an auto sampler vial for HPLC-DAD analysis at 200 nm.



(a) (b)

Figure 3.5.1 Picture showing a) before addition of salt and organic solvent b) after addition of salt and organic solvent

3.5.3.5 Optimization of experimental parameters

To ensure method simplicity, speed, high recovery, and adequate selectivity, factors affecting these analytical requirements were optimized. Looking at the literatures for QuEChERS based extraction; usually 10 to 20 mL of water or 1% HAc and 10 mL of acetonitrile, acetone or ethyl acetate have been reported. Thus, having this in mind, various factors like extraction solvents (H_2O and 1% HAc), extraction solvent volume, type of organic solvents, pH of the media, soaking time and shaking time were optimized.

Selection of extraction solvent and solvent volume

A 0.25 g of freeze dried and powdered khat sample was placed in to QuEChERS extraction tube containing 100 µL of ephedrine hydrochloride (IS) and 10, 15, 20 and 25 mL of (water or 1% aqueous acetic acid). The mixture was then kept for 15 min so as to allow the solvent to penetrate the cell wall of the plant material. The QuEChERS salt (1.0 g of CH_3COONa and 4.0 g of $MgSO_4$) was added to the mixture and shaken vigorously for 4 min and 1 to 2 mL of 20% NaOH was added to adjust the pH to 10. Subsequently, 10 mL of ethyl acetate was added and shaken for 1 min. Finally 5 mL of the supernatant

was taken after centrifugation of the mixture for 1 min or kept as such for 2 min. The organic layer was removed and reconstituted with the mobile phase.

Optimization of soaking time

Before adding the QuEChERS salt, the sample was soaked in to the extraction solvent (1% HAc) for a specified time (0-15 min) so as to allow the solvent to swollen the cell wall of the plant material and facilitate the release of analytes from the matrix to the solution. Thus, four soaking conditions (0, 5, 10, and 15 min) were selected while the remaining procedures were same as above. Duplicate analysis was carried out.

Optimization of shaking/extraction time

Shaking the mixture after soaking the sample and adding the QuEChERS salt is a critical stage whereby the alkaloids are expected to be released from the matrix into the aqueous phase. The QuEChERS methodology allowed analyte extraction in about 1 min. Taking into account that the alkaloids were present in their natural form in the samples analyzed, we, tested whether the increase in the extraction time might increase the efficiency of extraction. Thus, portions of 0.25 g of powdered sample were taken and placed in a 50 mL centrifuged tube containing 15 mL of 1% HAc. After soaking the mixture for 15 min, QuEChERS salt was added and shaken vigorously for the set periods of time (2, 4, 6 or 10 min). Then the rest of the procedures were followed as above. Each analysis was done in duplicate.

Effect of pH and organic solvent

Once parameters like solvent volume (15 mL), soaking time (15 min) and shaking time (6 min) were optimized using ethyl acetate as organic solvent, the pH of the media and alternative organic solvents were evaluated for quantitative extraction of the alkaloids. The original QuEChERS method has previously been modified by the addition of acetate or citrate buffers to prevent the degradation of certain pesticides (Lehotay *et al*., 2005). In our case, however, acidic condition stabilizes the alkaloids in the aqueous phase due to protonation of the amine nitrogen. Thus 20% NaOH was added to make the solution alkaline and enhance solubility of the alkaloids in the organic phase. Three different pH conditions (8, 10 and 12) were investigated to select a pH-value that could be adequate for the quantitative extraction of the analytes from the aqueous phase in to the organic layer. Furthermore, diethyl ether was also compared with ethyl acetate under identical conditions so as to select optimum solvent for extraction.

Effect of salt addition on the extraction yield of the alkaloids

In the QuEChERS methodology, phase separation was induced by the addition of various salts–avoiding the use of potentially toxic and expensive co-solvents. The salt most commonly used is $MgSO_4$, which reduces the volume of the aqueous phase and facilitates the partitioning of polar analytes in to the organic phase (Lehotaya *et al.*, 2010). In order to evaluate the significance of the QuEChERS salt on the extraction efficiency and phase separation, a duplicate extraction was conducted following the same procedure as above without the addition of QuEChERS salt, i.e. 0.25 g of sample was soaked in to 15 mL of water for 10 min and then vigorously shaken for 6 min. After adjusting the pH to 10 an organic solvent was added and shaken for 2 min.

3.5.3.6 Ultrasonic assisted extraction followed by SPE (UAE/SPE)

For the UAE/SPE experiments, the procedure developed by Mathys *et al*. (1993) was used. Namely, 0.25 g of sample was mixed with 500 µg of ephedrine hydrochloride (IS)

and extracted 3 times with a total of 50 mL 0.1 M HCl using ultrasonic bath for 45 min. The combined filtrate was evaporated to dryness at 40 ^{o}C using a vacuum rotary evaporator. The residue was dissolved in the mobile phase and passed through a pre-conditioned SPE cartridge. Then the cartridge was eluted with the mobile phase.

3.5.3.7 Matrix solid phase dispersion (MSPD)

For the MSPD experiment, the procedure developed by our group and reported in this thesis under section 3.2 was used**.** A 0.25 g aliquot of the sample was placed in a mortar and mixed with 0.75 g of sorbent and 100 µL of (+)–ephedrine hydrochloride (IS). The mixture was then homogenized in an agate mortar using an agate pestle to obtain a homogenous mixture. The blend was then transferred into a 10 mL syringe with a paper frit at the bottom. The sample was covered with another paper frit and the flow of the solution through the syringe was driven by the pressure of a vacuum manifold pump. The alkaloids were eluted directly with aqueous solution of 0.1 M HCl and the residue, after evaporation to dryness was dissolved in the mobile phase. Eluents were filtered through a PVDF membrane and injected into the HPLC system.

3.5.3.8 Reproducibility and recovery

The reproducibility of the analytical methods and the repeatability of the extraction procedure were assessed by evaluating the peak area ratios of the three alkaloids present in the extracts. Two replicates were performed for each extraction assay and two replicate LC–DAD analyses were performed on each filtrate. The recovery of the QuEChERS extraction was assessed by measuring the recovery of a sample containing about 4 times of the original norephedrine concentration (125 µL of 1 mg/mL) and about half of the original cathinone oxalate concentration (300 µL of 1mg/mL) and 100 µL of 5 mg/mL ephedrine hydrochloride stock solution after passing all the processes mentioned above.

3.5.4 Result and discussion

3.5.4.1 Sample comminution

The mechanical force generated during shaking the mixture and the exothermic heat produced during the hydrolysis of the salt added is responsible for the extraction of the alkaloids from the matrix in to the aqueous phase. These mechanisms of extraction seemed to be lower compared with other extraction approaches. Thus, it is important to ensure that the sample is powdered to fine particles to maximize the surface area and ensure better extraction efficiency. It has been observed that sample particles size significantly affect efficiency of the process (results not shown).

3.5.4.2 Selection of extraction solvent

To quantitatively liberate the analytes from the matrix and subsequently enrich them in the organic phase, optimum solvent type and its volume was deemed important, since it was observed in previous experiments (matrix solid phase dispersion and maceration) that nature of the solvent could affect the extraction yield of the alkaloids from khat samples (section 3.2). Thus, H_2O and 1% HAc in H_2O were compared by varying the volume of the solvents. Results of the analysis are shown in Figure 3.5.2.
Looking at the figure, both water and 1% HAc have successfully extracted the three alkaloids from the plant material under identical conditions. However, 1% HAc was found to be more efficient as compared to pure water. This result corroborates earlier reports on other extraction protocols like matrix solid phase dispersion and ultrasonic assisted extraction (Section 3.2).

Study of the extraction solvent volume revealed that extraction with a volume of solvent higher than that proposed in the original QuEChERS methodology improved the extraction yields. It has noticed that an increase in solvent volume (up to 20 mL) caused slight increment in the extraction yield of the alkaloids. Further increase in solvent volume yielded lower concentration of the alkaloids when both of the solvents were considered. This might be due to lowering of the heat generated in the system as a result

of exothermic reaction between water and the added salt which is supposed to be sufficient to liberate the alkaloids from the cell wall of the plant material in addition to the mechanical force applied during shaking the mixtures. Therefore, 15 mL of 1% HAc was selected as optimum solvent and solvent volume for this study.

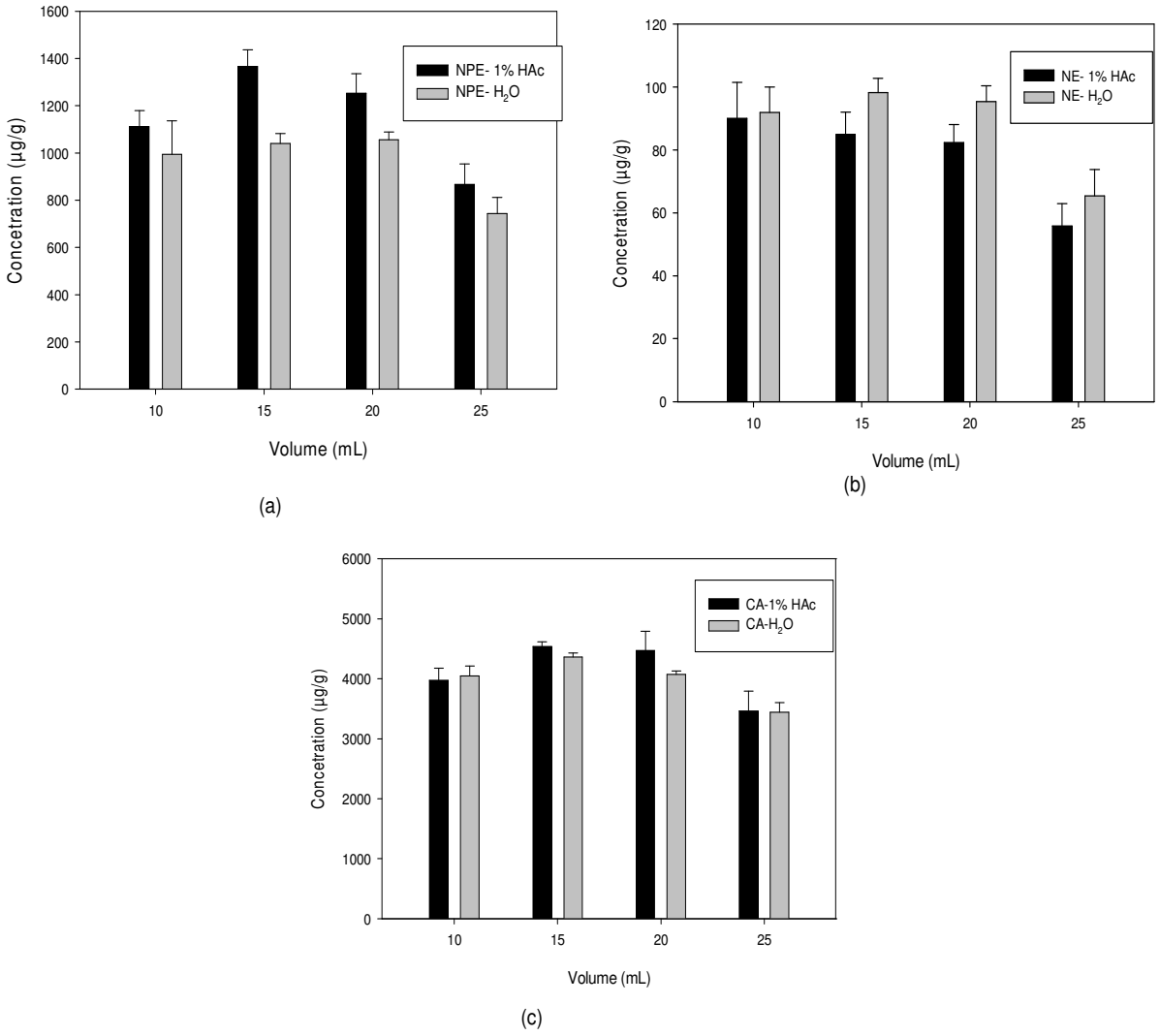


Figure 3.5.2 Optimization of volume of 1% HAc and H_2O for extraction of (a) norpseudoephedrine (NPE) or cathine, (b) norphedrine (NE) and (c) cathinone (CA)

3.5.4.3 Optimization of soaking time

Since the matrix analyte interaction is much stronger in case of natural products compared with other synthetic compounds like pesticides in foods, it is absolutely important to soak the sample in the extraction solvent so as to give more room to the solvent to penetrate the cell wall of the plant material and increase swelling so as to ensure better extraction efficiency of the alkloids. Results of the analysis are shown in Figure 3.5.3.

Looking at the graph, the extraction efficiency of the alkaloids was found to increase with soaking time. Even though, soaking of the sample for 20 min gave better result, the difference in yield with the 10 min soaking time was not significant. So, soaking of the sample between 10 to 20 min can be recommended as optimum time for soaking the samples. Thus 15 min was selected as optimum soaking time for the study.

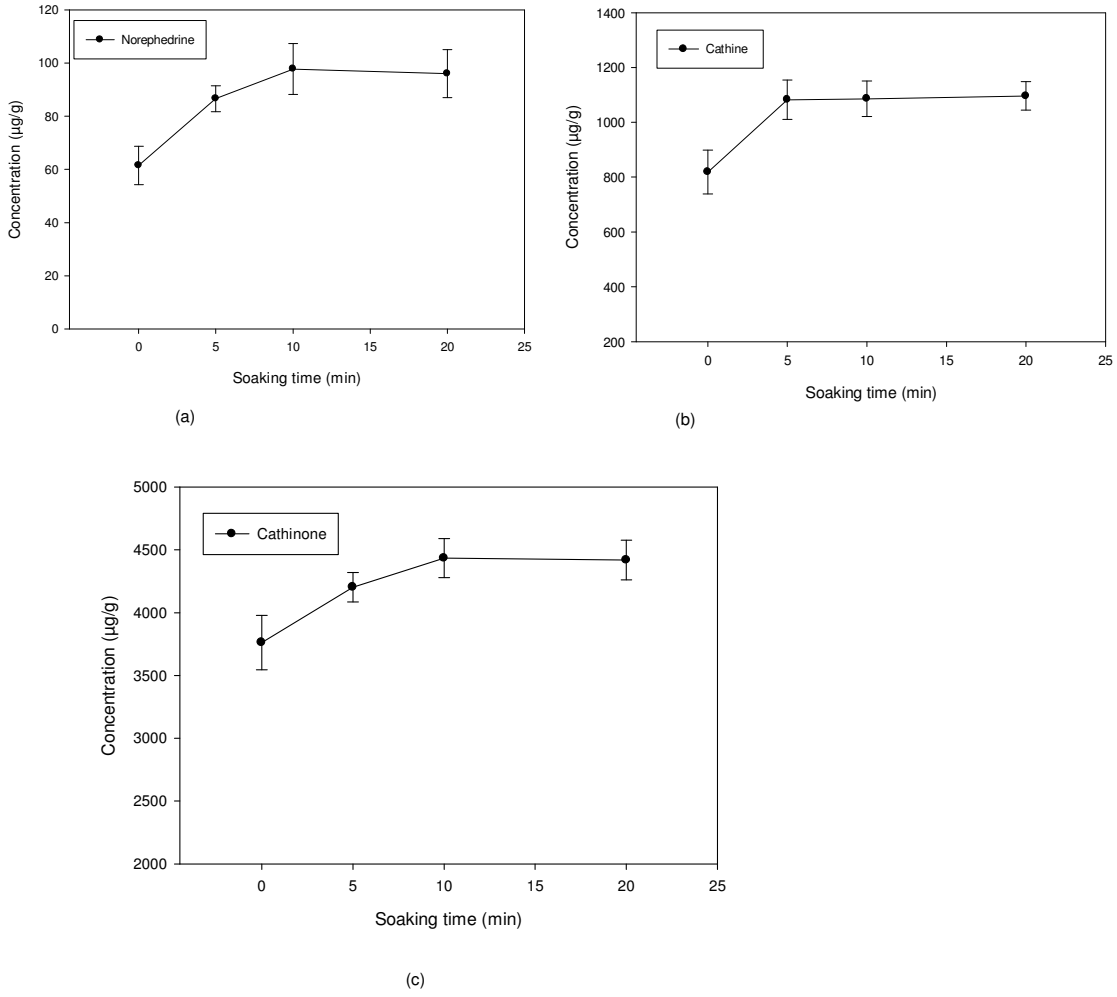


Figure 3.5.3 Effect of soaking time on the extraction efficiency of (a) norphedrine, (b) cathine and (c) cathinone

3.5.4.4 Optimization of shaking time

As it has been mentioned above, shaking the mixture for a set period of time is a critical step to enhance the extraction efficiency of the alkaloids. Shaking time was varied between 2-10 min (Figure 3.5.4). When the shaking/extraction time increased, a rise in the analytical signal was observed and hence 6 min was considered as efficient time for shaking the alkaloids.

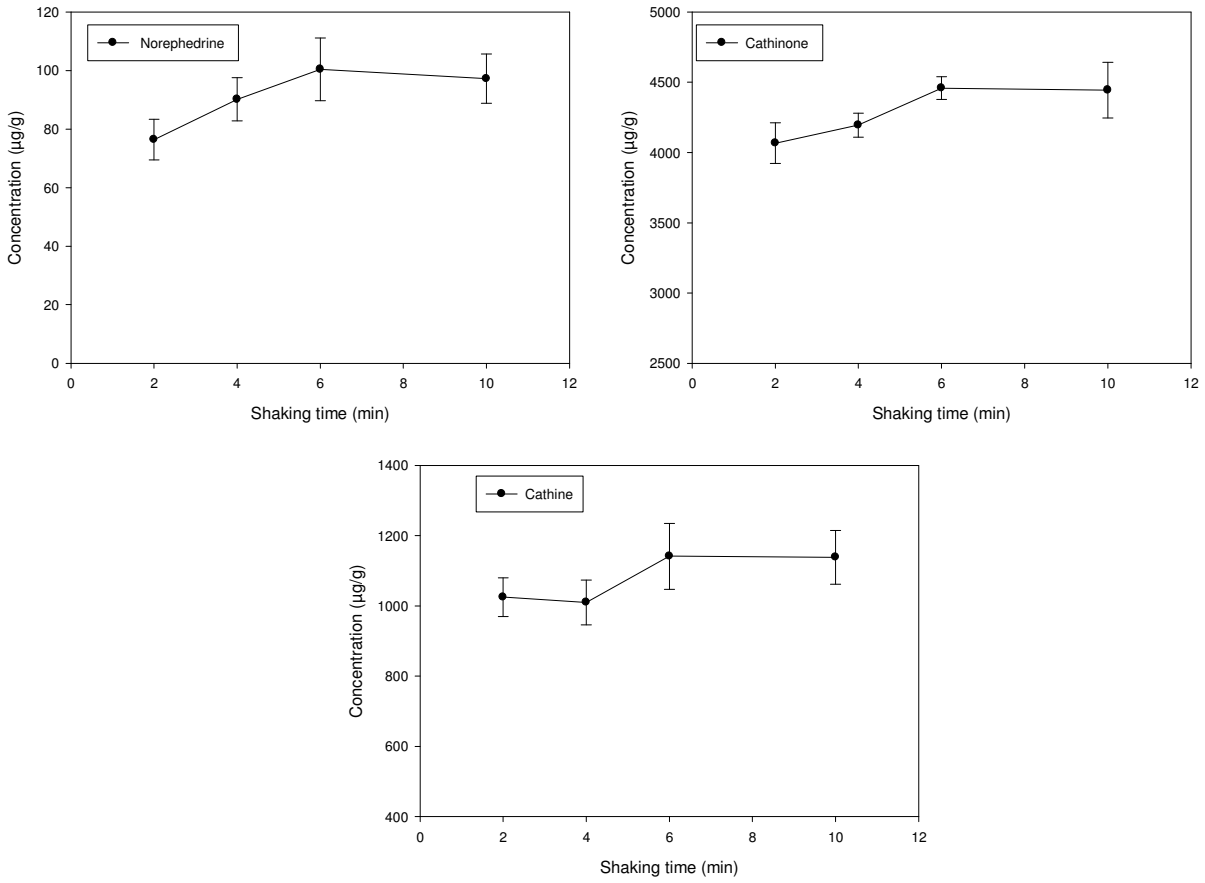


Figure 3.5.4 Effect of shaking time on the extraction efficiency of the alkaloids

3.5.4.5 Effect of pH and organic solvent selection

The pH of the extraction must be controlled. Unlike the conventional QuEChERS based extraction, partitioning of the alkaloids was carried out in an alkaline solution since alkaloids are easily partitioned in to the organic phase when the solution is basic. This is due to alkaline solution liberating free amine bases of the alkaloids. But, it has been noted that cathinone is sensitive to alkaline conditions (Szendrei 1980) and may undergo artifact formation. Thus, selection of optimum pH is mandatory so as to ensure quantitative recovery of the alkaloids in the organic phase without significant cathinone degradation. Figure 3.3.5 shows the effect of pH on the extraction yield of the alkaloids. As it can be noted from the figure, a significantly better analytical signal was found with

the solution at pH 10, rather than pH 8 and 12. Therefore, it was regarded as optimum pH for the study (Figure 3.5.5).

In this study, three organic solvents were compared, acetonitrile, ethyl acetate and diethyl ether. The QuEChERS method reported so far for pesticides extraction recommended acetonitrile as good solvent for partitioning of the analytes from acid solution upon the addition of salts. However, phase separation has been diminished as the pH of the media became alkaline. Thus acetonitrile was not useful for the present study. Diethyl ether was found to be more efficient in terms of phase separation, unlikely to cause emulsion formation and lesser tendency to extract co-extracts like lipids. However, it is more volatile and susceptible to affect the repeatability of the measurement and the reliability of the data. Finally, a less volatile solvent, ethyl acetate was found to be good solvent for this study. But, it co-extracts lipids and waxes. Fortunately, the co-extractives were remaining un-dissolved on the wall of the round bottomed flask when re-constituting the khat amines with the acidic mobile phase after removing the organic solvent.

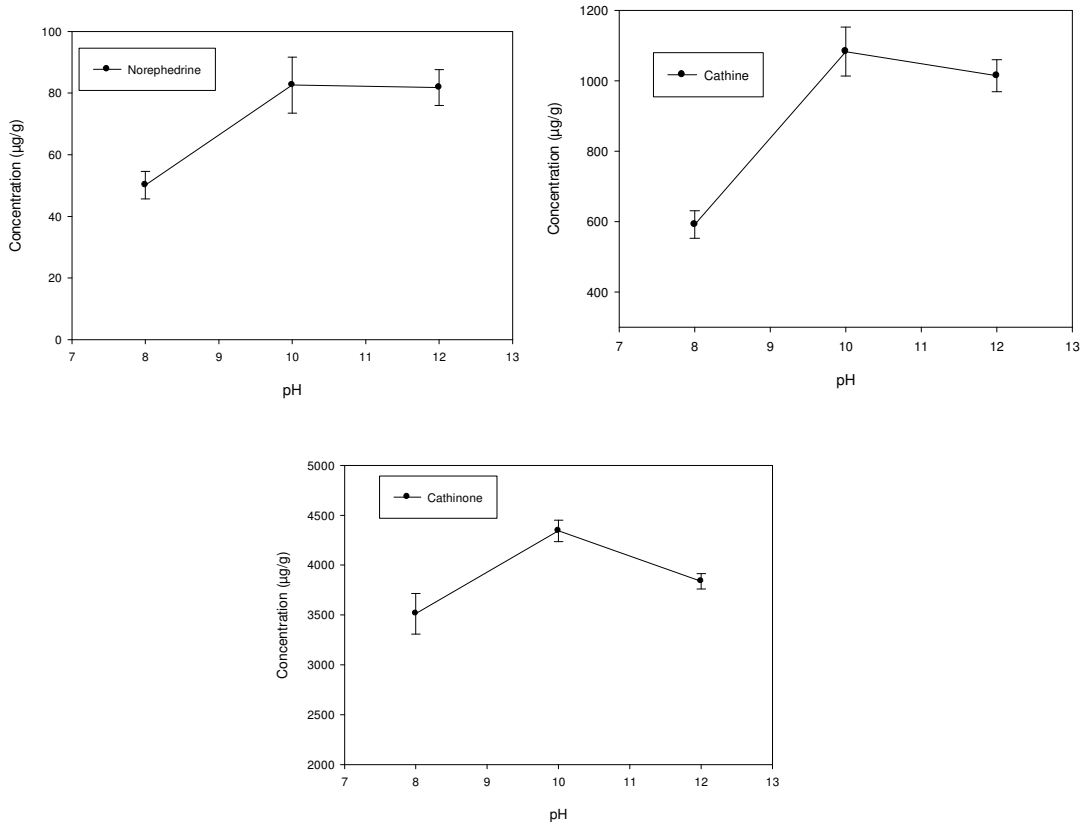


Figure 3.5.5 Effect of pH on the extraction efficiency of norephedrine, cathine and cathinone

3.5.4.6 Effect of addition of salt

In the QuEChERS methodology, phase separation was induced by the addition of various salts mostly $MgSO_4$, thus avoiding the use of potentially toxic and expensive co-solvents (Anastassiades *et al.*, 2003). The salting-out effect influences analyte partition, which is dependent upon the solvent used for extraction. Extraction was conducted with and without salt using ethyl acetate as organic solvent and it was noted that phase separation could not be achieved in the absence of salt. A very thick layer (emulsion) was formed on top of the aqueous phase and hence the contribution of the salt was significant to induce phase separation (Figure 3.5.6).

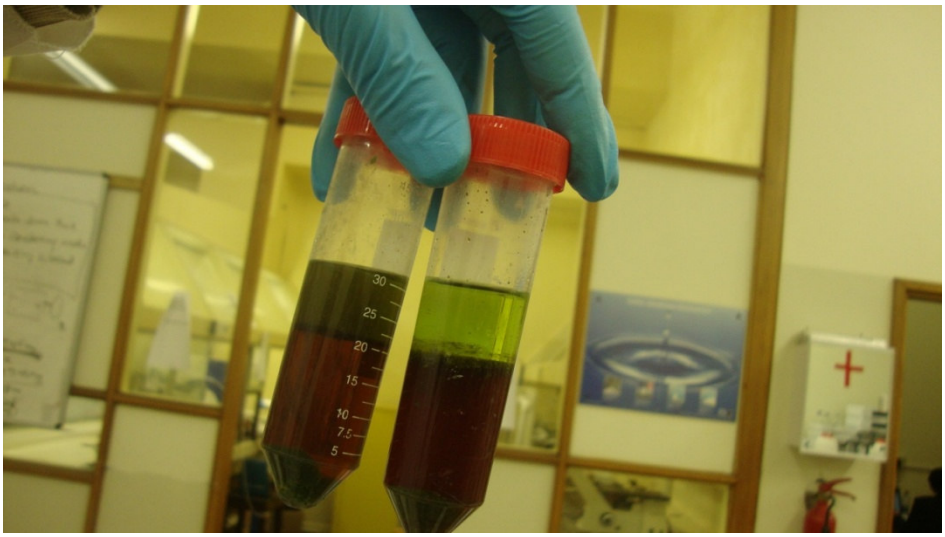


Figure 3.5.6 Effect of salt addition on phase separation (the left and right side tubes indicates mixtures without and with salt addition respectively)

It was observed that diethyl ether can form phase separation without salt addition but there is emulsion formation at the interface between the aqueous and organic phase. Furthermore, diethyl ether is a highly volatile solvent and it is difficult to handle during the extraction process.

Apart from phase separation, it was also anticipated that the heat librated due to hydrolysis of the salt could enhance the extraction yield and hence the analytical signal. For confirmation, diethyl ether was used as a partitioning solvent instead of ethyl acetate and extraction was carried out, with and without, salt. Unlike ethyl acetate, two distinct phases were obtained in both cases (with and without salt) when diethyl ether was used. However, a light emulsion was formed at the interface between the aqueous and the organic phase when no salt was added. Table 3.5.1 indicates the results obtained from the analysis. From the table, it can be inferred that the QuEChERS salt has a significant role in speeding up the rate of extraction and enhances the yield and hence the analytical signal under a given condition.

Table 3.5.1 Effect of QuEChERS salt on the extraction efficiency of khat alkaloids. Results are mean ± S (n = 4)

Without salt (µg/g)			In the presence of salt (µg/g)		
Norephedrine	Cathine	Cathinone	Norephedrine	Cathine	Cathinone
32 ± 4	764 ± 49	2138 ± 170	90 ± 10	1095 ± 59	4157 ± 208

3.5.4.7 Comparison of QuEChERS extraction with MSPD and ultrasonic assisted extraction followed by SPE

The QuEChERS extraction was compared with two extraction methods, ultrasonic assisted extraction followed by SPE and matrix solid phase dispersion (MSPD). Results are shown in Table 3.5.2. From the table, it was observed that comparable yield of the norephedrine but slightly higher value for cathinone could be obtained when QuEChERS based extraction was applied. But significantly lower concentration of cathine was noticed on the other hand than did the MSPD and UAE/SPE. QuEChERS based extraction is much easier, faster and more than four samples/aliquots can be handled at a time. Furthermore, a less noisy chromatogram could be obtained compared with MSPD and UAE/SPE. However, the problem with QuEChERS based extraction is that shaking the sample by hand is laborious and hence it might be improved if a vortex mixer was used as it has been applied successfully to the same extraction technique for pesticide analysis elsewhere (Tomasini *et al*., 2012).

It is important to note that, lower concentrations of the three alkaloids were noticed when analysis was carried out without internal standard. Thus, to minimize error generation in the multiple steps of the QuEChERS method, an internal standard should be added before extraction.

Table 3.5.2 Comparison of QuEChERS extraction with UAE/SPE and MSPD (results are mean ± S µg/g, n = 4)

Protocol	Norephedrine	Cathine	Cathinone
QuEChERS	96 ± 11	1127 ± 130	4096 ± 355
MSPD	101 ± 7	1538 ± 89	3525 ± 130
UAE	101 ± 8	1888 ± 109	3616 ± 122

3.5.4.8 Evaluation of the analytical method

Once the conditions for the extraction were optimized, known concentrations of the alkaloids were spiked to the khat sample and extraction was conducted. Figure 3.5.7 shows the chromatograms of the QuEChERS extracts of unspiked khat sample, the same khat spiked with khat amine (norephedrine, cathine and cathinone) and the standards. Table 3.5.3 reports the recoveries together with the precision. As can be seen, adopting the optimal conditions of the MSPD extraction to khat samples, all the three compounds were extracted very efficaciously and showed good recoveries (82-91%). The limit of detection (LOD) of the method was calculated using the calibration curve parameters. The LOD obtained for cathinone and norephedrine were 1.3 µg/mL and 1.7 µg/mL, respectively (Table 3.5.3). Due to the lack of pure cathine standard, recovery was not performed for it and its concentration in the sample was determined using the calibration curve parameters of norephedrine due to the fact that cathine and norephedrine are diateriomers and expected to have comparable molar absorptivity.

Looking at Figure 3.4.7, QuEChERS based extaction gave a clear chromatogram and all the interfering moieties remained in the bulk of the solution (polar components) and the coextractive less polar components were remain on the wall of the round bottom flask during evaporation of the organic solvent and reconstituting the alkaloids with the mobile phase.

Table 3.5.3 Reproducibility, average recovery (n = 4) and limit of detection of khat sample spiked with the two khat alkaloids

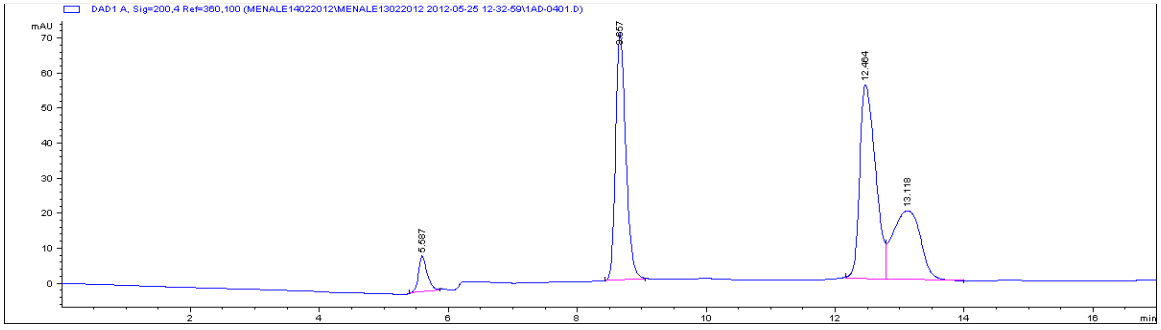
Compound	Before spiking (µg/g)	Amount added (µg/g)	Amount after spiked (µg/g)	% Recovery	Reproducibility, %RSD (n = 4)	LOD (µg/mL)
Cathinone	2620	1002	3442	82	14	1.3
Norephedrine	126	500	581	91	12	1.7

Drawbacks of QuEChERS based method

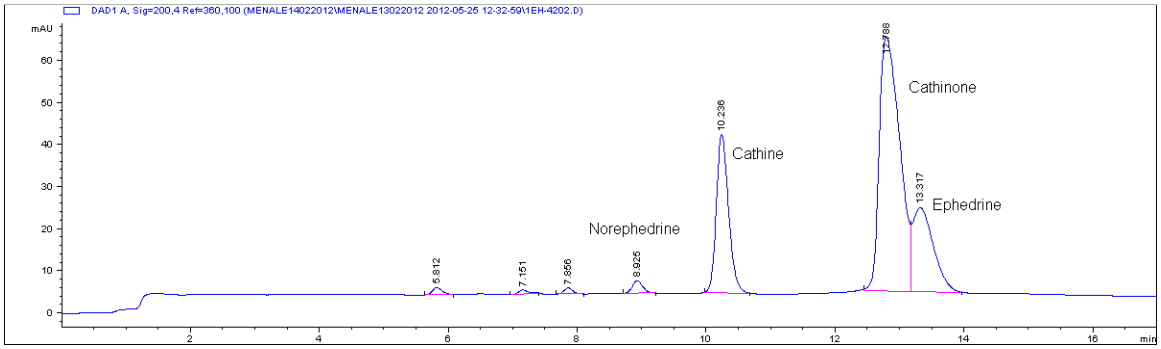
The reproducibility and repeatability of this method is affected by many factors. As it has been observed, use of internal standard is mandatory so as to control lose of analytes during the multiple steps of extraction and clean up protocols. Furthermore, powdered samples should be used so as to increase the surface area of the samples so as to enhance the efficiency of the method. In addition, it would useful if a vortex mixer is tried instead of manual or hand shaking the mixtures in the extraction tube.

3.5.4.9 Conclusion

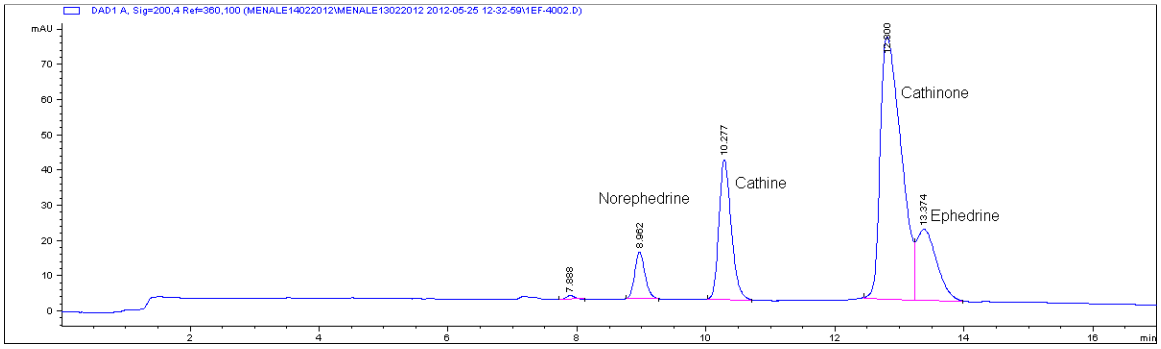
In this report, a modified QuEChERS based extraction and LC–DAD method was developed, optimized and evaluated for the extraction of naturally present alkaloids from khat (*Catha edulis* Forsk) leaves samples. The method follows two extraction steps where the anlytes are first extracted into the aqueous phase followed by partitioning in to the organic phase after pH adjustment. The results showed that the method was satisfactory in terms of selectivity and reproducibility with a less noisy chromatogram being obtained without any clean up step. It was simple, cost-effective, and can be used as a useful analytical extraction method to measure the khat alkaloids concentration in forensic and biomedical investigations.



(a) Standard (Left to right: oxalate, norephedrine, Cathinone, ephedrine)



(b) unspiked khat



(c) spiked khat

Figure 3.5.7 Chromatograms representing a) the standard, b) unspiked khat sample, c) spiked khat sample

4. SELECTED SECONDARY METABOLITES AND ANTIOXIDANT ACTIVITY OF KHAT PLANT EXTRACT

4.1 Background

Khat is one of the most widely consumed stimulant vegetable in Ethiopia among millions of individuals on a daily basis. However there is paucity of information about its major secondary metabolites (total phenolics, flavonoids and tannins) and the information about the antioxidant effects of khat is equivocal.

Because of excellent antimicrobial, antioxidant and anti-cancer activity of flavonoids and other polyphenols, research is undergoing to identify plants with such potential secondary metabolites content. It is been confirmed that deep colored fruits and vegetables have been reported to be good sources of phenolics, including flavonoids, anthocyanins and carotenoids and recognized as more healthy to humans (Qian *et al*., 2004; Sass-Kiss *et al*., 2005; Crozier *et al*., 2007). Thus we anticipated that khat could be one of a good source of such metabolites. This is due to the fact that colors of khat plant (leaves and stem) vary in color from purely red to green and, for some cultivars, mixed colored leaves have been observed (Section 2, Figure 2.1). Thus, there is a need to investigate the composition of phenolic compounds in khat cultivars since the concentrations of bioactive compounds in plant foods are known to vary with such factors as the variety or cultivar, stage of maturity, geographic or climatic effects, agricultural practices, soil composition etc (Qian *et al*., 2004; Sass-Kiss *et al*., 2005; Crozier *et al*., 2007).

In addition, the benefit and side effect of khat tannins are equivocal. Chronic chewers often complain of symptoms suggestive of stomatitis, oesophagitis, gastritis and constipation. Though no systematic work has been done, there seems to be agreement among researchers as well as khat users themselves concerning the gastrointestinal problems brought about by khat-chewing. These are due probably to the astringency of khat tannins ingested (direct effect) and the sympathomimetic action of khat amines (indirect effect) (Kennedy *et al*., 1983; Karunamoorthi *et al*., 2010; Craddock, 1993; Halbach, 1972; Hassan *et al*., 2002). Thus, it is important to quantitatively investigate tannin contents in different khat cultivars for further study on this aspect or to generate

supportive evidence for further clinical studies. One reported study indicated that khat chewing reduced the absorption of the antibiotics amoxicillin and ampicillin from the gastrointestinal tract (Attef *et al.* (1997). Though, no experimental justification has been given, the authors suspected that the effect might be due to the binding of tannins with the antibiotics. Thus, there is a quest to investigate the distribution of khat tannins in different khat cultivars for futures pharmacological studies.

Furthemore, beside their excellent antioxidative property, tannins affect protein and mineral utilization by forming insoluble complexes and prevent bioavailability and hence it is a recommended practice to take protein containing food some hours before or after ingesting tannin rich foods/beverages (http://www.herbs2000.com/h_menu/tannins.htm)

Vanillin-HCl and Protein precipitation methods using gelatin and Bovine serum albumin (BSA) are most frequently used to quantify tannins in different foods of plant origin. Vanillin-HCl is non-specific as it reacts with simple phenols; Gelatin method requires about three days to completely precipitate tannins and BSA is highly expensive (Herderich and Smith, 2005). Thus, development of an alternative but a plausible, cheap and fast method is a pressing demand.

4.2 Objectives of the study

The aim of this study was

(i) Optimization of appropriate sample preparation conditions for khat phenolic compounds analysis;

(ii) To develop colorimetric method for tannins determination in plant extract using ovalbumin as a precipitating agent; Folin denis reagent and Feric chloride solution as color forming moiety;

(iii) To quantify the distribution of total polyphenols, total flavonoids and total tannins in khat cultivars; and

(iv) To determine the antioxidant activity of khat cultivars extract.

4.3 Materials and method

4.3.1 Apparatus and equipments

A T60 UV-Vis Spectrophotometer equipped with 1-cm pathlength quartz cells was used for the absorbance measurements.

4.3.2 Chemicals and reagents

All of the reagents were of high purity. Gelatin, ferric chloride, anhydrous $AlCl_3$ and Na_2NO_2 (Fluka, Switzerland); sulfuric acid, NaCl, ovalbumin, sodium carbonate (x 10 H_2O) and ethanol (Research Lab Fine Chem, Mumbai, India); tannic acid, sodium acetate, NaOH and ascorbic acid (BDH, England); D-catechin, HCl, 1,1-diphenyl-2-picryldrazyl (DPPH), glacial acetic acid, methanol and acetone (Sigma Aldrich); sodium tungstate ($Na_2WO_4.2H_2O$) and phosphomolybdic acid (Scharlau Chememia S.A.) were used as received. Folin Denis reagent: to 75 mL of water, 10 g of sodium tungstate ($Na_2WO_4.2H_2O$), 2 g of phosphomolybdic acid and 5 mL of concentrated phosphoric acid were mixed and refluxed at 100 ^oC for 2 h and then diluted to 100 mL. The golden yellow solution was kept in a brown bottle in a refrigerator. Fresh solution of the reagent was prepared every week. Gelatin solution: 0.25 g of gelatin was soaked in a saturated sodium chloride solution for 1 h, then warmed until the gelatin dissolves and diluted to 100 mL with saturated sodium chloride. Acid sodium chloride solution: 25 mL of concentrated sulfuric acid was added to 375 mL of saturated sodium chloride solution, sodium dodecyl sulfate solution (SDS) (1% w/v): 1 g of SDS was dissolved in 100 mL of deionized water. SDS-triethanolamine (TEA) (1% SDS (w/v) and 7% (v/v) triethanolamine in distilled water) solution: 1 g SDS was dissolved in 7 mL of triethanolamine and 93 mL distilled water. Ferric chloride reagent (0.01 mol/L ferric chloride in 0.1 M HCl).

4.3.3 Sampling

Specific areas of sampling site with respective trade name of khat cultivars analyzed are already given under the section 3.2. Few of the samples were collected from khat markets of Addis Ababa. These include: Hirna, Liyu, Awadai and Gelemso khat cultivars. The rest of the samples were collected from the farm lands.

For those samples collected from the particular farms, three to five nearby farming areas were selected. From each farm, samples were collected and wrapped with banana leaf and brought to the laboratory. For samples collected from the market, six kiosks were randomly selected and a minimum quantity available in the kiosk was bought and brought to the laboratory.

4.3.4 Sample preparation

Upon arrival at the laboratory, different parts of the plant (chewable parts or young leaves, tips of tender stem near to the young shoot and older leaves) were isolated from each sample. Similar cultivars were mixed and a representative one bulk sample was taken for each cultivar. A total of 21 bulk samples were obtained for each plant part. Stems were dried in the oven at 50 ^{o}C. Parts of the young leaves and older leaves samples were freeze-dried in a lyophilizer while portion of it was oven dried under the same conditions as the stem parts for comparison purpose. After drying, the dried plant materials were ground to a fine powder (0.5 mm) with ceramic mortar and pestle. Ground powders were packed in polyethylene bags and stored at room temperature in dark until use.

4.3.5 Chemical analysis of samples

4.3.5.1 Total phenolic analysis

Optimization of extraction solvent

In order to select optimum procedure for extraction of phenolic compounds from different parts of the khat plant, various solvents were evaluated for their extraction efficiency. Pure solvents (water, acetone, methanol, and ethanol) and their binary mixtures with water were evaluated. Each analysis was made in triplicates.

Procedure for extraction and determination of total phenolic compounds

Dried (finely ground) plant material (100 mg) was placed in an Erlenmeyer flask of approximately 50 mL capacity. Ten mL of extracting solvent was added and the flask was kept on magnetic stirrer and stirred continuously for the prescribed of time. The contents of the flask were then transferred to centrifuge tubes and subjected to centrifugation for 10 min. The supernatant (10 mg/mL) was then collected and kept in the refrigerator until analysis. A portion of the centrifugate was taken and a tenfold dilution with the extracting solvent was made. The diluted samples were taken and total phenolic contents were determined by Folin Denis reagent as reported by Padda and Picha with slight modification (Padda and Picha, 2007). Namely, 100 µL of sample was taken and the volume was adjusted to 8 mL with de-ionized water. Then 1 mL of freshly prepared Folin Denis reagent was added followed by 1 mL of 20% Na_2CO_3. Few minutes later, absorbance was measured at 760 nm using UV-Vis spectroscopy against reagent blank. Finally 70% acetone was selected for optimal extraction of phenolic compounds.

Optimization of extraction time and effect of drying method

After optimum solvent was selected, extraction time was evaluated following the above procedure. Finally 2 h was selected as optimum extraction time. The effect of drying

(oven drying at 50 ^{o}C) and freeze drying method was compared for young leaves of Sebeta khat cultivar. It was found that Freeze drying gave relatively better result than oven drying.

Analysis of total phenolic compounds in different khat cultivars

Following the optimized procedure, 100 mg of powdered sample (young leaves and tender stems near to the young leaves) was taken and extracted with 10 mL of 70% (v/v) acetone for 2 h. After a set period of time, the extract was centrifuged and the supernatant was taken for analysis as above. Tannic acid was used as a standard solution for calibration curve and results were interpreted as tannic acid equivalent (TAE). The completeness of the extraction was checked by re-extracting the residue left in the centrifuge tube with 5 mL of the solvent. Recovery of total phenols in the second supernatant was < 5% of that in the first supernatant. Therefore, the second extraction step was omitted.

To compare leaf maturity with phenolic compounds distribution, two khat cultivars, Sebeta and Awadai type khat were analyzed following the same procedure as above.

Calibration curve construction for total phenolic analysis

Series of standard solutions of aqueous tannic acid was prepared from the stock solution of 0.1 mg/mL of tannic acid. After the volume was adjusted to 8 mL, 1 mL of each of Folin Denis reagent and carbonate solution was added and the absorbance was read at 760 nm.

4.3.5.2 Method development for the determination of total tannin

In this study, a method of total tannins determination in plant extract was developed using ovalbumin solution and Folin Denis reagent as a precipitating reagent and color forming moiety respectively. As reported by Hagerman *et al*. (1978), tannin protein precipitation is affect by various factors like ratio of tannin to protein, precipitation time and pH of buffer solution. Thus, these parameters were optimized using standard tannic

acid solution before being applied to real sample. Two routes of quantification strategies were followed.

In the first case, the in indirect approach was followed. Briefly, total phenolic compounds were determined as mentioned above (Folin Denis reagent method) and then, the same solution was treated with ovalbumin solution under optimized conditions. The tannin-protein precipitate was separated via centrifugation and the supernatant (the non-tannin solution was treated with Folin Denis reagent as stated above (Padda and Picha, 2007). The difference in absorbance value before and after tannin removal was regarded as absorbance value of total tannins. The second approach was a direct method whereby the above precipitate was dissolved in an appropriate solvent and treated with ferric chloride reagents. The resulting pink color was determined spectrophotometrically as reported by Hagerman *et al*., (1978). The resulting absorbance value is proportional to tannin concentration in the standard/plant extract.

Ovalbumin method optimization

In this report, ovalbumin was used to precipitate tannins. Both Folin Denis reagent and ferric chloride reagents were validated using standard tannic acid solution for determination of protein precipitable phenolics and non-protein precipitable phenolics. Thus, before applying the method, extraction time, ratio of tannin to protein and pH of the buffer solution were optimized.

Preparation of ovalbumin solution

Acetate buffer (0.1 M) at a particular pH was prepared and 1 g NaCl was added per 100 mL of buffer solution. Then 0.25 g of ovalbumin was taken and suspended in 50 mL of acetate buffer and kept for 2 h, without stirring and shaking, until it was dissolved completely (concentration = 5 mg/mL).

Optimization of tannin to protein ratio

5 mg/mL of tannic acid was prepared in de-ionized water. The following ratio of tannin to protein was taken to select optimum concentration of ovalbumin for efficient removal of protein precipitable tannins. 1:1, 1:2, 1:3, 1:4, 1:5, 1:6 and 1:10 concentration ratio of 2 mL of each of tannic acid and protein were taken by diluting the tannic acid solution with water to the desired concentration. The mixture was shaken for 5 min and kept for 40 min and centrifuged. 0.5 mL of the supernatant was analyzed for non-precipitated tannins following the above procedure (Folin Denis reagent). Finally, a 1:5 ratio of tannin to protein concentration was deemed adequate for complete precipitation of the tannins and selected as optimum ratio for the analysis.

Optimization of precipitation time

Between 20 min to 72 h have been reported for precipitation of tannins with different protein (Llaudy *et al*., 2004). Thus in this work, the time for complete precipitation of tannins was evaluated. An equal volume, but 1:5 ratio of tannin to protein was mixed and shaken for 5 min and kept for 10, 20, 30 and 40 min and then centrifuged for 10 min. Then the absorbance of the supernatant was measured as above. Results of the analysis (not shown) revealed that precipitation was completed within 10 min and no change in absorbance was observed for prolonged reaction time. Consequently, 15 min was selected as optimum time for all samples analyzed.

Optimization of pH for tannin precipitation

Acetate buffer (0.1mol/L) was prepared at different pH levels (2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6 and 6.5) as above. A 4 mg/mL portion of ovalbumin was prepared with each buffer solution. 0.8 mg/mL tannic acid was prepared in water. 2 mL of each of tannic acid and ovalbumin was mixed and kept for 15 min after shaking for 5 min. Thereafter, it was centrifuged for 10 min and 0.5 mL of the supernatant was diluted to 8 mL with water. 1 mL of each of Folin Denis reagent and carbonate solution was added and the absorbance

was read at 760 nm. Finally acetate buffer (0.1 M, pH, 4.5) was deemed optimum for the analysis.

4.3.5.3 Comparison of Folin Denis and ferric chloride methods of tannin analysis after ovalbumin precipitation

Following the optimized procedure, the efficiency of ferric chloride and Folin Denis reagents were compared. Namely; 0.8 mg/mL tannic acid (TA) in water and 4 mg/mL ovalbumin in acetate buffer (0.1 M, pH, 4.5) were prepared. 2 mL of each of the reagents were mixed for 5 min and kept for 15 min, centrifuged for 10 min, filtered, and kept for analysis. The precipitate was carefully washed twice with 2 mL of the buffer solution and centrifuged for 10 min each time. Finally, the precipitate was dissolved in 3 mL of sodium dodecyl sulfate solution (SDS) (1% w/v). A 0.5 mL portion of it was taken and diluted to 1 mL with SDS solution followed by 3 mL SDS-triethanolamine solution and 1 mL of Fe^{3+} reagent and kept for 30 min. Finally, the absorbance was measured at 510 nm. Similarly, 0.5 mL of the supernatant and 0.1 mL of the stock solution was taken and each of them were treated with 1 mL of freshly prepared Folin Denis reagent, and followed by 1 mL of 20% Na_2CO_3 solution. The blue solutions were analyzed at 760 nm. It should be noted that amount of precipitate should not be so large as to minimize volume error. Thus, diluted extracts are advisable to use.

Calibration curve construction for tannin determination using ferric reagent

Series of standard solutions of tannic acid in (1%) SDS solution was prepared from the stock solution of 0.2 mg/mL tannic acid. After the volume of the solution was adjusted to 1 mL using 1% SDS, 3 mL of SDS-TEA solution and 1 mL of ferric chloride solution were added and the absorbance of the resulting solution was measured at 510 nm after 30 min of reaction time.

4.3.5.4 Tannin determination using gelatin

Total tannins in four different khat cultivars were determined using the methodology described by Bajaj and Devsharma (1984) with slight modification. Following the above procedure, samples were extracted with 80% methanol to obtain 2 mg/mL. Then 1 mL extract was taken and mixed with 0.2 g kaolin in an Erlenmeyer flask. 2 mL gelatin and 2 mL acid sodium chloride solutions were added and the content was stirred continuously for 2 h and kept at room temperature for 3 days. Then, centrifuged at 3000 rpm for 10 min. 200 µL and 100 µL of the supernatant and the original solutions were individually mixed with 7.8 mL and 7.9 mL of water, respectively to have 8 mL of solution. Then absorbance of the original solution and the supernatant were measured at 760 nm after the addition of 1 mL of Folin Denis reagent and 1 mL of 20% aqueous sodium carbonate solution. Total tannins were determined by the difference between total phenolics and non-protein precipitable phenolics.

4.3.5.5 Total tannin content in different khat cultivars

After selecting optimum conditions, total tannin concentration of methanolic extract of each khat cultivar was determined using ovalbumin and Folin Denis reagent in triplicates. 2 mL of diluted khat extract (1 mg/mL) was mixed with 2 mL of 5 mg/mL ovalbumin solution in acetate buffer of pH 4.5. After 15 min, the mixture was centrifuged and the filtrate was taken. 0.3 mL of the supernatant was taken and mixed with 7.7 mL of water and 1 mL of freshly prepared Folin Denis reagent followed by 1 mL of 20% Na_2CO_3 solution. Similarly, 0.1 mL of the original solution (plant extract) was treated in the same way as the supernatant solution after adding 7.9 mL of water to adjust the volume of the solution. Results were interpreted as tannic acid equivalent (TAE).

Freeze drying and oven drying methods were compared for Bahir Dar type khat and the following results were obtained: freeze dried sample, (139 ± 4 mg TAE/g) of dried leaves; and oven dried sample (134 ± 7 mg TAE/g of dried leaves.

4.3.5.6 Total flavonoids analysis

Total flavonoid contents in different khat varieties were analyzed using colorimetric aluminum chloride method proposed by Zhishen *et al.* (1999). 0. 5 ml of the above 2 mg/mL khat extract was taken and added to 5.5 ml of water followed by 0.3 ml Na_2NO_2. 5 min later, 3 ml of 5.5% of anhydrous $AlCl_3$ was added. At 6 min, 2.3 ml of 1.0 M NaOH solution was added. After repeated shaking, absorbance of the resulting reddish solution was measured at 510 nm. The results of the analysis are expressed in mg catechin equivalent (CE)/g of dry matter. Following the same procedure, calibration curve was constructed using D-catechin as a standard (Table 4.3 and Figure 4.3)
Total flavonoids in young and matured leaves of Bahir Dar type khat was determined and found to be 70.1 ± 6.8 and 61.7 ± 4.0 mg CE/g of dry leaves, respectively.

4.3.5.7 Antioxidant activity of khat

Antioxidant activity was measured by a radical scavenging assay, using 1,1-diphenyl-2-picryldrazyl (DPPH) (Molyneux, 2004). The scavenging activity of khat cultivars was measured by monitoring the reduction of DPPH in the presence of khat leaves and stem extract. A 0.4 mmol/L DPPH solution was prepared by placing 7.84 mg of DPPH in a 50 mL volumetric flask and diluting to volume with methanol. 20 mg of ascorbic acid was dissolved in 100 mL of deionized water. The sample of freeze-dried phenolic extract was prepared (2 mg/mL) in 70% acetone as mentioned above.

An aliquot of 1 mL of ascorbic acid of different concentrations (2, 4, 6, 8, 16 and 20 µg/mL) was mixed with 4 mL of methanol and 0.8 mL of 0.4 µmol/L DPPH (dissolved in methanol). The mixture was vigorously shaken and left to stand at room temperature for 60 min in a dark room. Similarly, an aliquot of 1 mL of diluted khat extract at different concentrations (20, 40, 60 and 80 µg/mL) was mixed with 4 mL of methanol and 0.8 mL of 0.4 mmol/L DPPH. The control contained only 0.8 mL of 0.4 mmol/L DPPH solution and 5 mL of methanol instead of sample while pure methanol was used as the blank. Absorbance was read at 517 nm by using UV-Vis spectrophotometer. The scavenging effect was calculated using the following equation:

$$\text{Scavenging effect } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} x\, 100\% .$$

Calibration curve was constructed for the standard ascorbic acid as % scavenging activity vs mass of standard ascorbic acid and the resulting calibration curve is shown in Figure 4.6.

4.4 Results and discussion

4.4.1 Effect of variable parameters on extraction of total phenolic compounds

Pure water, pure methanol, pure ethanol, pure acetone and their binary mixture of various combinations with water are commonly used to extract phenolic compounds from samples (Dai and Mumper, 2010). The ability of different solvents to extract phenolic compounds was compared by performing Folin Denis assay. The results were expressed as tannic acid equivalents (mg TAE/g of dry leaves). Table 4.1 shows that all the solvents were capable of extracting phenolics but aqueous acetone (70%) was more effective solvent than the remaining solvent combinations for extracting phenolic compounds from khat leaves samples. Acetone (70%) gave the highest TPC (mg TAE/g of dry leaves) which was 206, followed by 80% acetone (196) and 80% methanol (191 mg/g) of dry leaves. Where as pure water and other combination of aqueous binary solvents showed lower extraction efficiencies. Except 70% and 80% acetone and 80% methanol, the different types of solvent have a significant effect ($p < 0.05$) on total phenolic content (TPC).

Acetone is a more effective solvent for the extraction of condensed tannins as have tannins have a relatively high molecular weight compounds (Alasalvar *et al*., 2009; Dai and Mumper, 2010). It is strongly believed that the higher the molecular weight of the solvent, the lower the polarity, which allows other substances of about the same molecular weight to be easily extracted. This can be correlated to “like dissolve like” or “polarity versus polarity” principle as both acetone and tannins are of high molecular

weight. Solubility of phenolics is affected by the polarity of solvents used which is why it is very difficult to develop an extraction procedure suitable for the extraction of all plant phenolics (Naczk and Shahidi, 2004; Dai and Mumper, 2010).

Mixtures of acetone and methanol with water have been found to be more efficient in extracting phenolic constituents when compared to mono-component solvent system. Addition of small quantity of water to organic solvent usually creates a more polar medium which facilitate the extraction of polyphenols as suggested by Spigno *et al*. (2007). By increasing the proportion of water to acetone, the polarity of the solvent also increases. When this is achieved, the solvent system is able to extract phenolic substances from both ends of the polarity (highest polarity substances and low polarity substances), as well as those of moderate polarity (Zhang *et al*., 2007). Generally, 70% and 80% acetone and 80% methanol exhibited comparable extraction efficiency. Therefore, in case where acetone is not preferable for a particular analysis like protein precipitation assay of tannins as acetone interfere with this assay (FAO/IAEA, 2000), 80% methanol will be the best choice of interest. Thus, acetone (70%) was chosen for the determination of total phenolic compounds and total flavonoids extraction while 80% methanol was used for extraction of protein perceptible tannins.

As can be seen from Table 4.2, the extent of extraction of TPC increased with time until 90 min and no further increment was observed for a prolonged extraction time. Thus 120 min was selected as an optimum extraction time for all the khat cultivars including leaves and stems.

Effect of drying on quantitative extraction of phenolic compounds was compared for young leaves of Sebeta type khat cultivar. Yield of the extract was high in the freeze dried leaves (172 ± 4 mg TAE/g of dry leaves) followed by oven dried samples at 40 ^{o}C (164 ± 4 mg TAE/g of dry leaves). The slight decrease in total phenolic content in oven-dried sample might be due to instability of some phenolic compounds at relatively higher temperature (Dai and Mumper, 2010).

Table 4.1 Effect of different solvents on extraction of total phenolic compounds

	(mg/g) TAE (X ± SD)				
Solvent	100%	90% (solvent)	80% (solvent)	70% (solvent)	50%
Water	122 ± 3	-	-	-	-
Ethanol	131 ± 7	139 ± 5	153 ± 6	136 ± 2	134 ± 3
Methanol	148 ± 6	153 ± 9	195 ± 5	161 ± 1	169 ± 1
Acetone	109 ± 4	138 ± 3	196 ± 2	206 ± 9	187 ± 4

Table 4.2. Optimization of extraction time for total phenolic compounds in khat

Time (min)	10	20	30	40	60	90	120	240	360
mg/g TAE	104± 5.9	135 ± 10	177 ± 10	200 ± 8	205 ± 10	211 ± 9	212 ± 9	211± 5	209 ± 8

4.4.2 Total phenolic compounds (TPC) content in different khat cultivars

In this work, the total phenolic content of 20 samples of khat cultivars grown in Ethiopia, belonging to 20 different brands, were analyzed. For each cultivar, both the young leaves and the tender stem tips near to the young shoot have been analyzed. Tannic acid was used as a standard solution and parameters of the calibration curve are shown in Table 4.3 and Figure 4.1.

Results of TPC in khat leaves and tender stem are presented in Table 4.4. TPC was expressed in terms of tannic acid (TA) equivalents per gram of the dry plant part used. As shown in Table 4.4, the total phenolic content in khat plants significantly vary among most of the cultivars both in young leaves and stems. The mean concentration of phenolics ranged from 129 to 274 mg TAE/g for young leaves and 89.3 to 175 mg TAE/g for tender stem tips.

Khat leaves from Guragie, Bahir Dar, Sike, Hirna, Suke, Gelemso, Awadai, Mokonisa, Chengie, and Belechie showed the higher levels of total phenolics (274, 263, 242, 239, 237, 220, 219, 213, 203, 201 mg TAE/g for dry leaves, respectively). While Berdaye and Gerbicho (145 and 129) mg TAE/g for dry leaves, respectively, showed lower levels of total phenolic compounds in the edible portion of young leaves. However, the remaining khat leaves cultivars contained between 150 and 200 mg TAE/g of dry leaves.

The tender stem tips of khat accumulated relatively higher concentration of phenolic compounds but for most of the cultivars it was 1.5 to 2 folds less than the corresponding phenolics in leaves. These results are in agreement with previous works reporting high leaf/stem polyphenol proportions in other plants (Lizcano *et al*., 2010). Our results reveal that khat leaves are rich in phenolics comparable to unprocessed fresh tea plants (Erturk *et al*., 2010).

The large difference of khat cultivars in terms of total phenolics in khat plant could be due to variation in seasonal, genetic, and agronomic factors. Furthermore, variations in age of harvested khat cultivars have also profound effect on phenolic compounds accumulation. Even chewers prefer khat leaves from old tree than those collected from recently cultivated ones. This might be related not only to the alkaloids content but also with phenolic compounds in the leaves.

Erturk *et al*., (2010) reported that harvesting season has a significance influence on tea leaves phenolic content. They noticed that those harvested in sunny season had accumulated two folds phenolic content than those harvested in rainy season. The same might be true of khat leaves since khat growing regions in Ethiopia have significant variation in climatic conditions throughout the year.

Fortunately, those khat cultivars widely and commonly consumed in Ethiopia and exported to different countries accumulated more phenolic compounds. Thus it is possible to say that phenolic concentration in the leaves could be one of the biomarkers for consumer preference.

Effect of leaf maturity was compared for phenolic distribution in two khat cultivars. Results are given in Table 4.5. Looking at the table; young leaves accumulated more

phenolics than the corresponding older leaves. This result also corroborates other reports (Chutichudet *et al*., 2010).

Compared with results reported by Dudai *et al*., (2008), about thousand times higher concentrations of TPC have been found in Ethiopian khat. This could be due to variation in environmental factors listed above and some possible problems mentioned under the introduction section 'review of literature'. However, the data reported by Vinokur *et al*. (2008) are comparable (TPC = 180 mg TAE/g of dry khat sample) with some of the present results.

Table 4.3 Data for construction of calibration curve for total phenolics analysis using tannic acid

V. of 0.1 mg/mL tannic acid (mL)	amount of TA in (µg)	V. of water in (mL)	V. of Denis reagent (mL)	V. of 20 % Na_2CO_3 (mL)	Absorbance
0.00	0.00	8	1	1	0.00
0.04	4	7.96	1	1	0.026
0.08	8	7.92	1	1	0.053
0.12	12	7.88	1	1	0.076
0.16	16	7.84	1	1	0.107
0.2	20	7.8	1	1	0.127
0.4	40	7.6	1	1	0.250
0.8	80	7.2	1	1	0.453

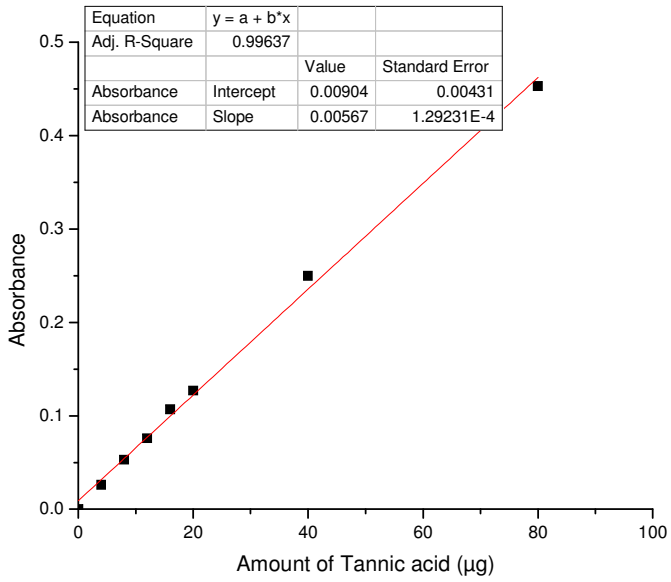


Figure 4.1 Calibration curve for tannic acid after reacting with Folin Denis reagent

Table 4.4 Average concentration (X ± SD, mg/g TAE), n = 3) of total phenolic compounds and total tannins in different khat cultivars in dry weight basis

Khat cultivars	Total phenolic compounds (mg/g)		Total tannins (mg/g)	
	Young leaf	Stem	Young leaf	Stem
Anferara	198 ± 10^{ch}	99.3 ± 4.3^{an}	109 ± 4	65.3 ± 5.4
Awadi	220 ± 9^{fg}	173 ± 4^{c}	118 ± 6	76 .4 ± 5.9
Bahir Dar	263 ± 5^{d}	175 ± 5^{c}	139 ± 4	87.4 ± 3.2
Basha	151 ± 6^{e}	121 ± 4de	69.1 ± 5.7	34.4 ± 6.7
Belechie	201 ± 4^{h}	119 ± 7^{d}	101 ± 2	74.4 ± 1.7
Berdaye	145 ± 6^{e}	102 ± 4^{abm}	68.4 ± 3.5	39.0 ± 1.2
Bole	171 ± 4^{b}	129 ± 3^{g}	88.0 ± 4.6	58.2 ± 4.2
Chengie	203 ± 10^{h}	133 ± 4^{fgl}	115 ± 7	98.4 ± 6.2
Damile	200 ± 5^{h}	142 ± 5^{h}	129 ± 3	87.1 ± 3.3
Debo	156 ± 5^{e}	104 ± 7^{abm}	70.2 ± 4.4	58.1 ± 4.0

Gelemso	221 ± 17^{f}	144 ± 3^{hkq}	113 ± 6	70.3 ± 5
Gerbicho	148 ± 7^{e}	92.4 ± 3.5^{jn}	88.3 ± 2.3	76.7 ± 3.5
Guragie	274 ± 10^{d}	139 ± 6^{hl}	143 ± 7	85.3 ± 5.1
Hirna	240 ± 4^{a}	129 ± 3^{eg}	128 ± 7	62.1 ± 1.5
Liyu	184 ± 4^{bcg}	109 ± 7^{bm}	108 ± 5	60.0 ± 4.4
Mokonisa	213 ± 9^{afh}	159 ± 9^{p}	119 ± 7	94.0 ± 4.4
Sebeta	172 ± 4^{b}	119 ± 6^{d}	91.0 ± 5.6	61.0 ± 3.3
Sike	242 ± 6^{a}	127 ± 4^{defg}	95.0 ± 3.5	69.3 ± 4.3
Suke	238 ± 7^{a}	151 ± 7^{kpq}	135 ± 3	78.4 ± 6.3
Yirba	173 ± 8^{b}	123 ± 5^{de}	92.1 ± 4.9	59.3 ± 5.3

Values in the same column with different letters are significantly different at $p<0.05$.

Table 4.5 Total phenolic compounds in young and matured leaves of selected khat cultivars, results are given as (X ± SD, mg/g TAE), n = 3)

Khat cultivar	(young leaf)	Matured leaves(mg/g) DW
Sebeta	172 ± 4	150 ± 3
Awadai	220 ± 9	191 ± 8

4.4.3 Total tannin analysis

In this report, ovalbumin was evaluated for successful precipitation of tannin from khat cultivars. Before applying for real sample analysis, various parameters were evaluated like ratio of tannin to protein (Figure 4.2), pH of the buffer solution (Figure 4.3) and reaction time (not shown) have been optimized. Once tannin is removed, the non-tannin supernatant was reacted with Folin Denis reagent in the presence of carbonate solution.

Looking at Figure 4.2, binding ability of ovalbumin increased with increasing pH of the buffer solution and reaches its maximum binding affinity at about pH value of 4.5, i.e. the absorbance of the supernatant at pH = 4.5 was minimal due to the fact that most of tannic acids have been precipitated from the solution. At this pH value, tannin to protein ratio of 1:5 for precipitation time of 15 min gave maximum precipitation efficiency and recorded as optimum ratio for real sample analysis (Figure 4.2).

After precipitation, analysis of total tannins was evaluated using Folin Denis reagent and ferric chloride reagent (Table 4.6). In both cases, comparable protein precipitable tannic acid was recovered. And hence we recommend that Folin Denis method is the method of choice as it will be applicable for lower concentration of tannins, minimize analysis time and analyte loss that may arise in case of the ferric chloride method which is accompanied by multiple washing steps after filtration.

Following the optimized procedure, total tannin content in the khat cultivars were assayed using ovalbumin precipitation and Folin Denis method. Results are presented in Table 4.4. For comparative purpose, gelatin method was used for selected khat cultivars. Results are presented in Table 4.7 on dry weight basis as tannic acid equivalent (TAE). Calibration curve parameters for tannins determination using ferric chloride reagent is given in Table 4.8 and Figure 4.4, while for the Folin Denis based method, the calibration curve result is given in Table 4.3 and Figure 4.1.

Our study has shown that young leaves of khat (Table 4.4) possess high tannins content ranging from 70.2–135 mg TAE/g of dry matter. The high content of tannins in khat leaves correlates with the bitterness of the leaves. For example, chewers claim that Suke

type khat is highly astringent and less likely be used by them frequently unless shortage of khat occurs in the market. Similarly, tips of tender stems accumulated lower concentration of tannin ranging from 49–98 mg TAE/g of dry matter which is evident that the stem is less bitter than the leaves.

Similar to total phenolic compounds significant difference ($p < 0.05$) has been noticed for tannin content in matured leaves and young leaves. Tannins accumulation in young leaves and matured leaves of the Sebeta type khat was found to be 91.0 ± 5.6 and 74.6 ± 3.5 mg TAE/g of dry matter, respectively.

Considering with gelatin method (Table 4.8), less tannins were found than with the ovalbumin method. This might be due to the increased precipitation time and/or increased equilibrium gelatin/tannin concentration ratio resulting in a soluble tannin-protein complex instead of insoluble ones (Hagerman *et al*., 1987). Thus, ovalbumin will be the better choice for the determination of khat tannins. Thus, our results showed that, appropriate pH, precipitation time and gelatin/tannin concentration ratio should be optimized for plant phenolic analysis if gelatin is used as a precipitating agent.

The occurrence of high tannin content in khat cultivars may lead us to support the earlier hypothesis, i.e., association of gastrointestinal problem and cellular toxicities mentioned so far with such high concentration of tannins in the plant. Habitual users try to attenuate the gastrointestinal problem by food adaptation, notably by eating a meal with high fat content prior to the khat session in order to facilitate intestinal transit (Hassan *et al*., 2007) and they prefer to spend the chewing session accompanied by excessive water and more preferably with milk. But still some chewers dislike incorporating milk in the chewing session while chewing because they believe that they feel less stimulation effect of khat if milk is simultaneously used.

Most khat chewers are suffer from mouth (lips) drying which could be due to the fact that proline rich proteins in the mouth might easily be removed via precipitation with the high tannin content in the leaves during chewing.

Results of the present study reveals that care should be taken because of high concentration of khat tannins which are responsible for precipitation of proteins, starch,

digestive enzymes and essential mineral nutrients from the diet (Chung *et al*., 1998b; Mitjavila *et al*., 1977) and prevent their bioavailability and digestibility. Thus chewers should take their meal a few hours before and after chewing.

On the other hand, many health benefits of polyphenols (tannin and flavonoids) have been reported. Review paper by Chung *et al*., (1998a) reported that *in-vivo* and *in-vitro* studies directly and indirectly support the preventive polyphenol effect against oral cancer and are responsible for its positive antioxidant, anti-inflammatory and antimicrobial effects. Concentration and types of tannins may dictate the importance and/or health impact of khat tannins. Therefore, further studies with respect to individual members' identification as well as toxicological effects should be required. Generally, our finding is an input for further study to clearly understand the effect of tannins on health.

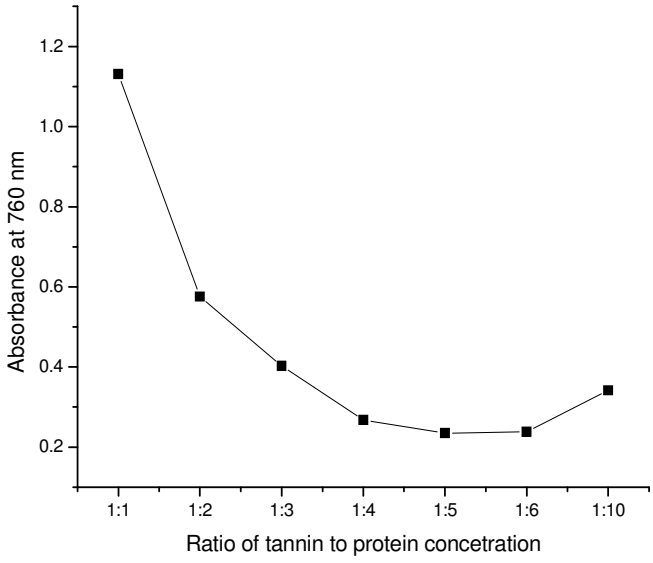


Figure 4.2 Absorbance of supernatant solution vs. ratio of tannin to protein

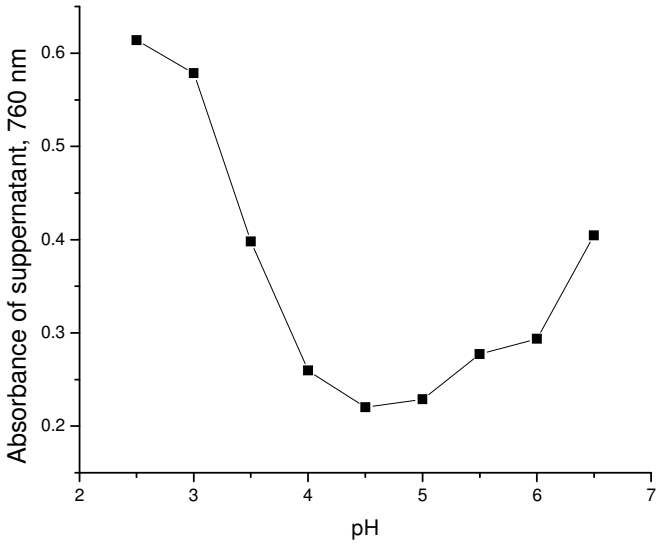


Figure 4.3 Absorbance vs. pH of buffer solution

Table 4.6 Comparison of Folin Denis reagent and ferric chloride reagents for tannic acid analysis for the stock solution of 20 mg TA in 25 mL of solution

Method	TA (mg/25 mL)	Unprecipitated TA in filtrate (after removal with protein) (mg/25 mL)	Tannic acid content mg/25 mL
Folin Denis reagent	[a]20.9 ± 0.2	4.31 ± 0.17	[c]16.8 ± 0.3
Ferric chloride reagent	[b]15.5 ± 0.2	-	15.5 ± 0.2

[a]In the stock solution; [b]in dissolved precipitate, [c]protein precipitate-able TA

Table 4.7 Data for construction of calibration curve for tannins analysis using Ferric reagent method

V of 0.2 mg/mL tannic acid in 1 % SDS (mL)	Conc. of tannic acid (µg)	1 % SDS, (mL)	SDS- TEA (mL)	Ferric chloride (mL)	Absorbance at 510 nm
0.00	0.00	1	3	1	0.00
0.2	40	0.8	3	1	0.097
0.4	80	0.6	3	1	0.199
0.6	120	0.4	3	1	0.305
0.8	160	0.2	3	1	0.412
1.0	200	0.00	3	1	0.510

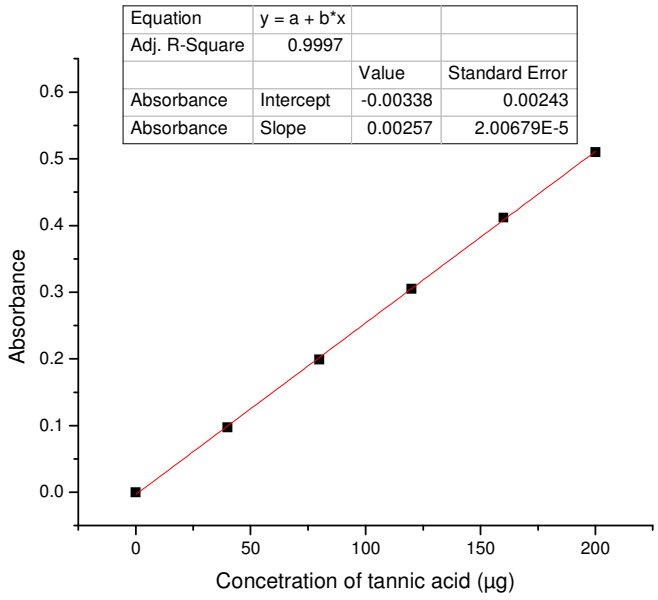


Figure 4.4 Calibration curve for tannic acid after reacting with ferric chloride reagent

Table 4.8 Total tannin concentration of selected khat cultivars, mg/g, dry weight basis (X ± SD) using gelatin method

Khat cultivar (leaves)	Gurage	Hirna	Awadai	Chengie	Gelemso
mg/g	119 ± 10	94.3 ± 8.6	92.8 ± 7.8	96.7 ± 8.7	102 ± 8

4.4.4 Total flavonoids content

In this work, total flavonoids content of 20 khat cultivars grown in different parts of Ethiopia have been investigated and results are given in Table 9 on dry weight basis as catechin equivalent (CE). The calibration curve parameters are also shown in Table 4.10 and Figure 4.5

Flavonoid constitutes one of the most important groups of phenolics in plants. In this respect a significant correlation ($r = 0.70$; $p < 0.05$) was observed between flavonoids and total phenolic content of khat leaves. Flavonoids contents of the different khat leaves cultivars evaluated varied ranging from 26 to 70 mg CE/g of dry matter (Table 4.9). In the tender stem tips, the concentration of flavonoids were between 26–56 mg CE/g of the dry stem. ANOVA results revealed that for some of the cultivars, there is a wide variation in flavonoid contents ($p < 0.05$) within the leaves and stems. In some of them, the difference is not so pronounced ($p \geq 0.05$). Presence of at least one similar letter within a column of Table 4.9 indicates absence of significant difference for pair wise ANOVA test and otherwise the data are significantly different. As of total phenolic compounds and tannins, flavonoids in leaves are significantly higher than the corresponding flavonoids in stem.

Apart from mentioning the antioxidant, anti-inflammatory and antimicrobial activity of khat, at the best of our knowledge, literature is scarce on the quantitative finding of flavonoids of khat cultivars. Thus, this report will be an input for further study on health benefit of khat flavonoids.

Table 4.9 Total flavonoid contents and antioxidant activity in different khat cultivars (X ± SD mg/g, n = 3, dry weight basis of triplicate analysis)

	Total flavonoids (mg/g CE)		Antioxidant activity (mg/g AAE dry wt)	
Sample type	Young leaf	Stem	Young leaf	Stem
Anferara	45.0 ± 3.2^{ac}	40.0 ± 2.4^{a}	208 ± 13^{d}	154 ± 10^{c}
Awadai	60.1 ± 2.3^{b}	25.6 ± 1.2^{bc}	263 ± 34^{bf}	211 ± 10^{k}
Bahir Dar	70.1 ± 6.8^{dg}	46.1 ± 2.0^{fn}	281 ± 21^{a}	191 ± 10^{ac}
Basha	42.9 ± 1.6^{ac}	38.8 ± 2.1^{a}	173 ± 9.1^{c}	147 ± 10^{d}
Belechie	51.2 ± 1.3^{e}	33.1 ± 2.1^{d}	242 ± 9^{fg}	119 ± 8^{h}
Berdaye	47.4 ± 0.4^{ae}	29.3 ± 1.0^{bcd}	172 ± 11^{c}	145 ± 8^{cdf}
Chengie	60.9 ± 4.3^{bf}	53.1 ± 3.2^{gh}	232 ± 6^{g}	148 ± 6.2^{cf}
Damile	52.2 ± 2.3^{e}	45.3 ± 2.3^{f}	228 ± 8^{g}	173 ± 15^{af}
Debo	43.0 ± 2.2^{ac}	32.3 ± 2.0^{d}	173 ± 14^{c}	145 ± 14^{cd}
Gelemso	40.7 ± 1.6^{c}	33.0 ± 2.3^{cd}	290 ± 16^{a}	130 ± 9^{dh}
Gerbicho	34.2 ± 1.3^{k}	29.0 ± 1.2^{bd}	182 ± 10^{c}	134 ± 7^{dfg}
Guragie	66.0 ± 5.1^{dh}	44.6 ± 3.1^{g}	270 ± 17^{a}	180 ± 12^{ab}
Hirna	55.4 ± 6.2^{d}	20.0 ± 0.5^{bd}	279 ± 17^{a}	143 ± 23^{c}
Liyu	55.0 ± 3.6^{be}	33.4 ± 3.1^{d}	281 ± 8^{ab}	118 ± 7^{gh}
Mokonisa	52.2 ± 4.2^{e}	49.4 ± 3.4^{fh}	252 ± 12^{fg}	179 ± 7^{ab}
Sebeta	50.7 ± 2.5^{e}	51 ± 3.3^{gh}	186 ± 10^{cd}	135 ± 2^{dfgh}
Sike	66.8 ± 2.3^{fgh}	25.2 ± 2.1^{c}	284 ± 17^{ab}	195 ± 10^{bk}
Suke	60.4 ± 5.1^{b}	40.4 ± 4.3^{ghmn}	263 ± 26^{b}	174 ± 12^{a}
Yirba	48.3 ± 6.3^{ae}	47.4 ± 6.7^{fm}	189 ± 12^{c}	133 ± 4^{fh}

CE = Catechin Equivalent; AAE, Ascorbic Acid Equivalent)

Table 4.10 Data for construction of calibration curve using D-catechin for flavonoids analysis

V of 0.6 mg/mL cathechin (mL)	Amount of cathechin (µg)	V of Water (mL)	V of Na_2NO_2 (mL)	V of $AlCl_3$ (mL)	V of 4 % NaOH (mL)	Absorbance
0.00	0.00	6	0.3	3	2.3	0.00
0.04	24	5.98	0.3	3	2.3	0.07
0.08	48	5.92	0.3	3	2.3	0.135
0.12	72	4.88	0.3	3	2.3	0.209
0.16	96	5.84	0.3	3	2.3	0.28
0.2	120	5.8	0.3	3	2.3	0.34

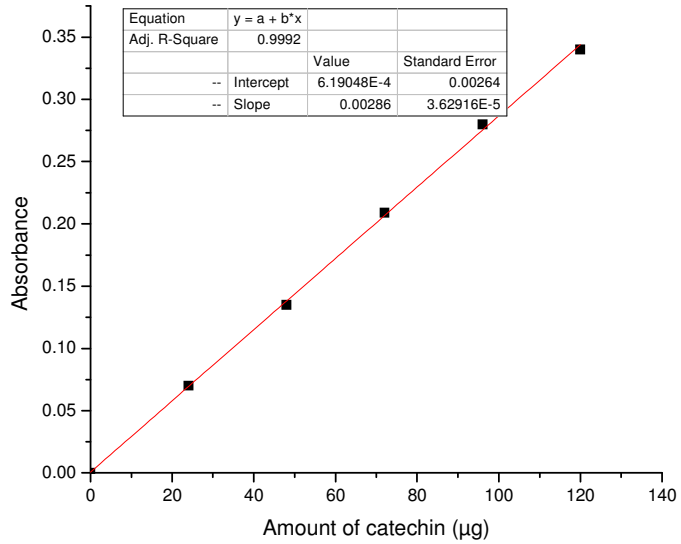


Figure 4.5 Calibration curve for D-catechin solution for flavonoids analysis

4.4.5 Antioxidant activity analysis

Most reports on khat were on its health impact. However, its health benefit to the chewers has had little attention. As other phenolic rich foods, *Catha edulis* cultivars were found to possess a substantial antioxidative activity.

Antioxidant activity of fresh leaves and tips of tender stem of 20 khat cultivars is given in Table 4.9, and calibration curve parameters of the standard ascorbic acid is shown in Figure 4.6. From the calibration curve (Figure 4.6), a good correlation (R^2) between the concentration of ascorbic acid and its percent scavenging activity of the DPPH has been noticed. In a comparison between 20 *Catha edulis* varieties grown in most parts of Ethiopia, all the samples had a substantial antioxidative activity, ranging 173–290 mg AAE/g of dry matter in the young leaves and 118–211 mg AAE/g of dry matter in stems (Table 4. 9). Looking at the table, there is significant variation ($p < 0.05$) among some of the cultivars investigated while no significant difference was observed ($p \geq 0.05$) for the remaining cultivars for both the leaves and stems. The same letter(s) within a particular column indicates those varieties which have no significant variation in antioxidant activity among the cultivars while lack of at least one similar letter within a given column indicates there is significant variation in antioxidant activity.

The results for antioxidant activity clearly outline that khat shoots could be one of the richest sources among plants in terms of antioxidant activity. The significant difference of khat shoots for antioxidant activity across varieties is supposed to the effect of change of ecological parameters like climatic conditions, seasonal variation, age of harvested cultivars, as well as physiochemical nature of the soil are responsible factor beside cultivar variation. Traditionally khat infusion which was known as Abyssinia tea was widely used for treating certain diseases (Paris *et al.,* 1958; Getahun *et al.,* 1973). To date also, khat leaf infusions particularly the matured leaves infusion are used by some chewers. The infusion is locally known as “Hawuza”. This might be due to the realization of its health-promoting antioxidant potential of the plant by the chewers.

The effect of leaf maturity on the antioxidative activity was also determined. Dried young leaves and matured leaves of Bahir Dar khat were found to contain 281 and 209 mg AAE/g of dry matter, respectively. Young leaves had higher activity than mature leaves which is also correlated with tannins, flavonoids and total phenolic compounds. The high antioxidant activity of young leaves is in agreement with previous reports (Dudai *et al*., 2008; Vinokur *et al*., 2008). However, for some of the cultivars significantly higher antioxidant activity has been obtained in this study.

In general, high antioxidant activity in a khat extract and increased case study report on cancer consequence among khat chewers is an indication of health impact of khat chewing.

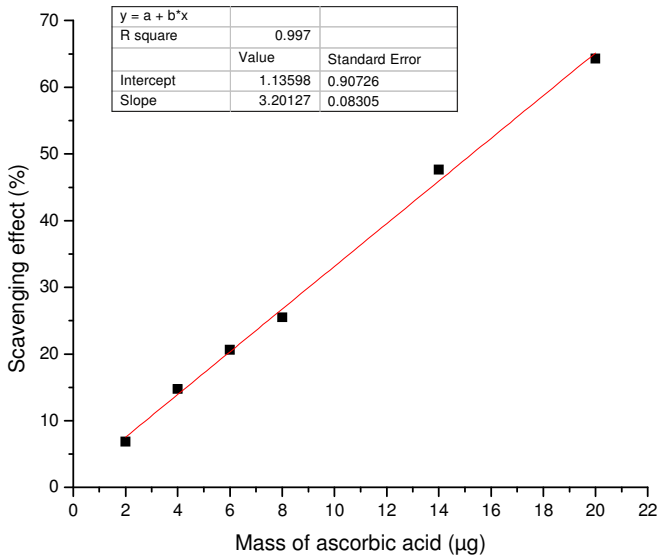


Figure 4.6 Calibration curve for antioxidant activity assay using ascorbic acid standard solution

4.4.6 Correlation between the antioxidant capacity and secondary metabolites content

Correlation between total phenolic, tannins and flavonoid contents in young leaves and tips of tender stems and radical scavenging activity of 20 khat extracts were determined. The correlation curves are depicted in Figures 4.7-4.12 with correlation coefficient (r^2) of 0.62–0.80. In general, extracts with a high radical scavenging activity showed a high phenolic, flavonoid and tannin contentl, but good correlations could not be found among them (Figures 4.7-4.12). A direct correlation between radical scavenging activity and phenolic, tannin and flavonoids content of the samples was not demonstrate by linear regression analysis. This lack of relationship is in agreement with other literatures (Dudai

et al., 2008; 23, Lizcano *et al*., 2010). It is known that only phenolic compounds with a certain structure and particular hydroxyl position in the molecule can act as proton donating and show radical scavenging activity (Rice-Evans *et al*., 1996).

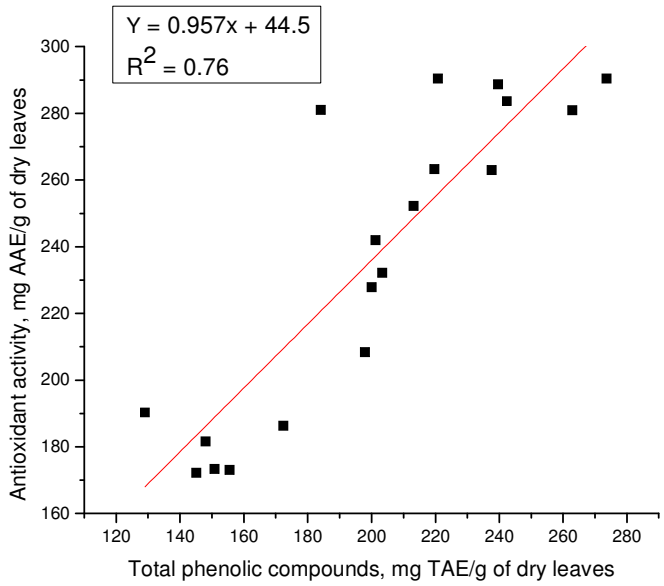


Figure 4.7 The correlation between antioxidative activity and total phenolic content of young leaves of 20 khat cultivars

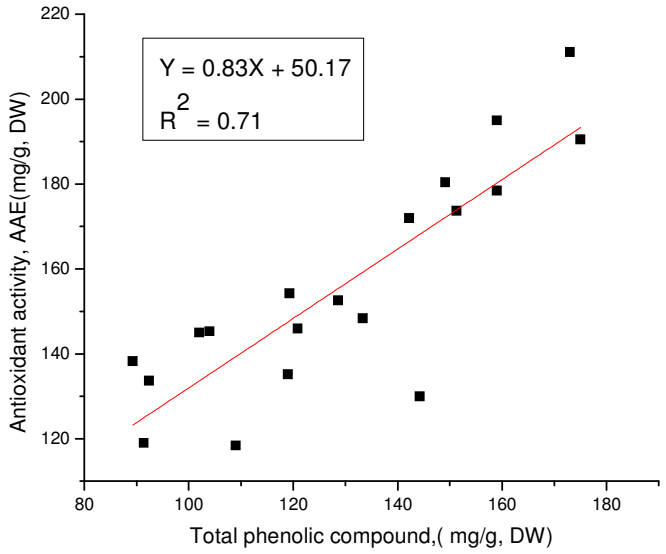


Figure 4.8 The correlation between antioxidative activity and total phenolic content of tips of tender stems of 20 khat cultivars

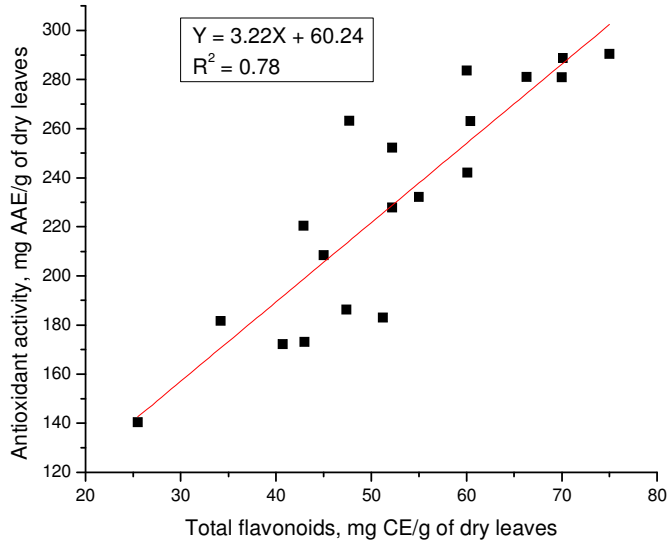


Figure 4.9 The correlation between ant oxidative activity and total flavonoids content of young leaves of 20 khat cultivars

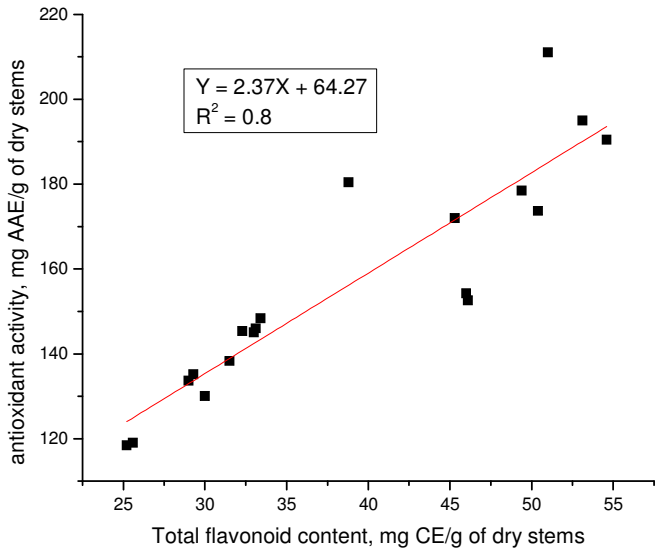


Figure 4.10 The correlation between antioxidative activity and total flavonoids content of tips of tender stems of 20 khat cultivars

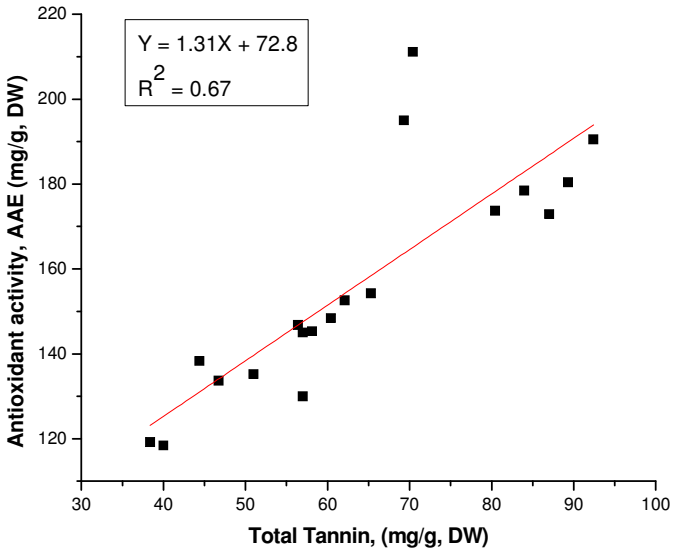


Figure 4.11 The correlation between antioxidative activity and total tannins content of tips of tender stems of 20 khat cultivars

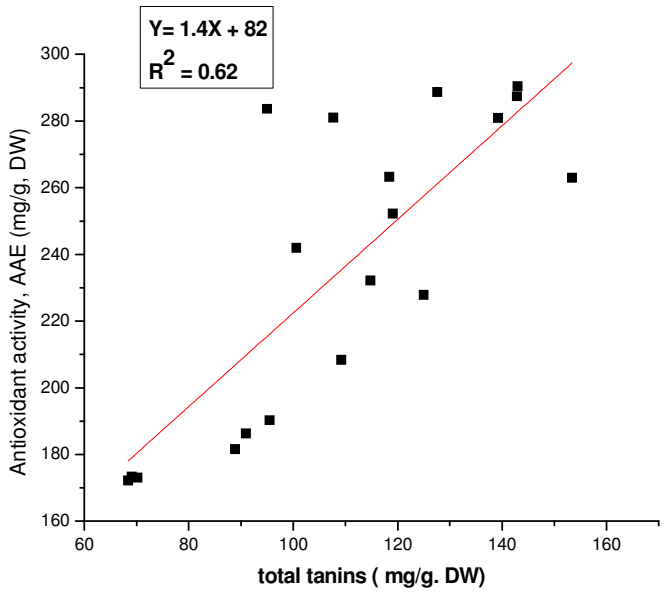


Figure 4.12 The correlation between antioxidative activity and total tannins content of young leaves of 20 khat cultivars

4.5 Conclusion

This study documents thet total phenolic compounds, flavonoids, tannins I khat as well as the antioxidative properties of the different cultivars grown in the country. The method of extraction procedure and quantification technique for future *in-vivo* and *in-vitro* studies was also studied.

The extraction of khat phenolic compounds depended largely on the drying technique, type of solvent used and extraction time. Among the methods tested to extract the total phenolic compounds of khat cultivars, the aqueous acetone and aqueous methanol extraction is recommended for being the most suitable method, for this type of samples regarding the distribution of its phenolic compounds. In addition, we proposed a plausible tannin assay method using ovalbumin as a precipitating agent and Folin Denis reagent for color forming moiety, for quantification. The results indicate that, the ovalbumin method gave significantly better efficiency than gelatin method. Furthermore, Folin Denis reagent and ferric chloride based assays yield comparable protein perceptible tannin concentration.

Ethiopian khat accumulated substantially high concentration of phenolic compounds, tannins and flavonoids having wide variation across cultivars. Our results confirmed that the frequent complaint about gastric problem among chronic khat chewers might be due to the high concentration of khat tannins. The khat extracts analysed in this study had high antioxidant activity in terms of free radical scavenging, thus indicating possible benefit to human health.

5. DISTRIBUTION OF FLUORIDE IN KHAT LEAVES

5.1 Background

Currently chronic khat consumption among all age levels is becoming more prevalent in east African countries, particularly in Ethiopia. Information about khat chewing and dental fluorosis is equivocal. Some reports indicate that long-term khat chewing causes intrinsic and extrinsic stain on the surface of the teeth (Hill *et al.,* 1987; Hassan *et al*., 2002; Hailu *et al*., 2006). This could be partly due to khat fluoride. Others believe that long term chewing of khat cannot cause dental fluorosis.

Furthermore, there is paucity of information about the distribution of fluoride in khat leaves grown in different geographical localities. Hill *et al.* (1987) conducted case studies on chronic Yemeni khat chewers and they found positive correlation of khat chewing and tooth staining. They reported as khat leaves accumulated 360 ppm of fluoride, which would be one cause of staining. However, they did not mention method of sampling, extraction and analysis. Whereas contradicting data have been reported from the same country by Hattab *et al.* (2000) where almost negligible concentration of fluoride was found.

Apart from this controversy, we anticipated that Ethiopian khat could conceivably accumulate fluoride in the different parts of the leaves of khat plants since some khat producing areas of Ethiopia are in the rift valley region where fluoride is predominantly exists in the soil and water (Malde *et al*., 1997; Kloos *et al.,* 1999). Furthermore, agricultural practices, environmental conditions and soil parameters usually vary from country to country and significantly affect the accumulation of fluoride in plants.

Therefore, khat cultivars grown in the rift valley and non-rift valley regions should be systematically assessed for their accumulation of fluoride in different position of the leaves so as to resolve the controversies and to evaluate the contribution of khat for total fluoride intake to the chewers per day. Furthermore, systematic extraction procedure should be optimized for quantitative estimation of the analyte in different leave positions.

5.2 Objectives of the study

The aim of this study was to measure the fluoride concentration of the major khat varieties/cultivars grown in the country and commonly chewed by most individuals in the region as well as being exported to neighboring countries. This study, therefore, becomes important in determining the exposure of human body to fluoride through khat. This also stresses the need of inclusion of fluoride level to determine the total dietary intake of fluoride for chewers. The result of the present study will also fill the gap and/or controversy that existed in studies on dental effects of khat due to fluoride concentration in the plant.

5.3 Materials and method

5.3.1 Apparatus and equipments

A pH/ISE meter (Orion model, EA 940 Expandable Ion Analyzer, USA) equipped with combination fluoride selective electrode (Orion Model 96-09, USA) was employed for the determination of fluoride in the samples and standard solutions. The pH was measured with pH/ION meter (WTW Inolab pH/ION Level 2, Germany) using a pH glass electrode.

5.3.2 Chemicals and reagents

Reagents used were of analytical grade reagents. Anhydrous sodium fluoride (99.0% NaF, BDH Chemicals, England) was used to prepare the fluoride stock and calibration standard solutions. Glacial acetic acid (Techno Pharmchem, Delhi, India), sodium chloride (Oxford Laboratory, Mumbai, India), sodium citrate (BDH Chemicals, England), EDTA (Scharlau Chemie S.A., Barcelona, Spain) and sodium hydroxide (Scharlau Chemie S.A., Sentmenat, Spain) were used to prepare the total ionic strength adjustment buffer (TISAB). TISAB II was prepared by mixing 57 mL of glacial acetic acid, 58 g of sodium chloride, 7 g of sodium citrate and 2 g of EDTA in volumetric flask and made to

500 mL with distilled-deionized water. The pH was adjusted to 5.3 with 6 M sodium hydroxide, and then made up to 1000 mL in a volumetric flask with distilled-deionized water. Distilled-deionized water was used throughout the experiment for sample preparation, dilution and rinsing apparatus prior to analysis.

5.3.3 Prevention of contamination

To minimize the risk of contamination, all glassware used for the analytical methods were washed with tap water followed by chromic acid and distilled-deionized water. Sterile disposable powder-free plastic gloves were worn when handling the khat sample during the sampling and analyses stages. The extracted solutions in plastic volumetric flasks were kept in a refrigerator until analysis.

5.3.4 Collection and preparation of khat samples

As stated above, there are various types of khat plant that grow in Ethiopia and brought to different cities of the country including the capital city Addis Ababa, and sold in kiosks in various locations in the city. The geographic locations of the studied samples are given in section 3.2. For this study, samples were selected on the basis of their popularity to consumers in the country and export to the neighboring countries. As a result, Bahir Dar, Wondo-Basha, Wondo-Kuto, Belechie, Awadai, Liyu, Gelemso and Guragie type khat samples were collected from different kiosks in Addis Ababa. Sike, Yirba and Chengie types were collected from different kiosks in Awassa (the capital city of southern nation nationality peoples Region of Ethiopia (SNNPR).

For Awadai, Liyu, Sike, Yirba and Chengie types of khat, eight kiosks were randomly selected while 14 kiosks were selected for collecting the remaining khat types. Around 50 g of the edible portion of the leaves were collected from each kiosk. After mixing the leaves of similar varieties of khat samples from the different sampling sites, 11 bulk samples were obtained and a portion of each sample was taken for extraction of the fresh leaves following the optimized procedure for the analysis of water extractable fluoride. The remaining portion of each sample was oven dried at 70 ^{0}C for 72 h (until constant

weight) and ground in a blender to homogenize and reduce the size of particles. Finally, 11 bulk samples were prepared for total fluoride analysis. Three 1 g aliquots from each sample were taken for ashing and final analysis.

It is reported that the lower part of the leaf next to the young shoot contains lower cathinone and more cathine content. It is also harder and difficult to chewing. As a result, chewers do not prefer it for consumption. But in some areas, they dry and use it after moisten with tea or cola drinks in the periods where shortage of khat is observed, particularly in the summer season. It is also taken as a tea infusion (Halbach, 1972). As a result, determination of fluoride content in such part of the leaf is also of interest because fluoride accumulation varies with positions of the leaves (Fung *et al.* 1999). As a result, the lower parts of the leaf next to the young shoot was collected for the Sike type khat and dried in the oven following the same procedure as above.

5.3.5 Optimization of sample preparation procedures

To select an optimum procedure (ashing temperature, extraction time and reagent volume) for extracting fluoride from the fresh and the dried leaves, previously reported methods were referenced and evaluated for the present study (Hattab *et al*., 2000; Malde *et al*., 1997; Malde *et al*., 2001; Yadav *et al*., 2007). Slight modification was made on the reported procedures for the present study.

5.3.5.1 Preparation of the samples for total fluoride content analysis

By slightly modifying the reported methods (Malde *et al*., 1997; Malde *et al*., 2001), the following method was employed for total fluoride content analysis; 1.0 g of khat leaf powder was transferred to a nickel crucible. 6 mL of 8 M NaOH was added and the crucible was placed in an oven (150 °C) for 1.5 h until NaOH solidified. The crucible was placed in a muffle furnace and set at 200 °C for 1.5 h after which the temperature was increased to 525 °C and kept there for 2.5 h in order to fuse the sample in the crucible. The crucible was allowed to cool and 10 mL distilled water was added. Then, 37% HCl solution (about 4 mL) was added slowly to adjust the pH to 8-9. The sample

solution was transferred to a plastic beaker. The crucible was rinsed successively with 5 mL water until the final volume reaches 50 mL. All the washings were mixed and filtered with Whatman filter paper (110 mm, diameter) and transferred to a 50 mL plastic volumetric flask. The filtrate was used for the fluoride content determination. A filtrate was prepared, in parallel, from a nickel crucible containing no sample to serve as a control. A portion of it was taken, mixed with an equal amount of TISAB II and the fluoride concentration was determined by F^--selective electrode following double standard addition method. All the determinations were done in triplicate.

5.3.5.2 Preparation of the samples for water extractable fluoride

The extraction time for water extractable fluoride was optimized by varying it from 0.5 to 10 min on two different khat leaves samples. Just like chewing, known amount of edible portion of the leaves (4 g) were taken and repeatedly crushed using pestle in a plastic beaker by adding a few drops of water until it became fine particles just like bolus is formed in the mouth. Then 24 mL (total) of distilled-deionized water was added and vigorously stirred with magnetic stirrer for the set period of time. A portion of it was taken and analyzed for fluoride concentration following the above procedure. All the determinations were made in triplicates. It was found that 5 min was deemed adequate to liberate the maximum of fluoride that is expected to be released in the mouth during chewing process (Table 1). Using the optimized procedure, water extract able fluoride was determined in different khat cultivars and results are shown in Table 5.3.

Table 5.1 Evaluation of extraction time on the extraction of fluoride from khat leaves (4 g of khat leaves sample in 24 mL of water)

Sample variety	Extraction time (min)	F^- (µg/g)	Sample variety	Extraction time min)	F^- (µg/g)
Wondo-Kuto	0.5	-	Yirba	0.5	0.19
	1	0.19		1	0.26
	2	0.21		2	0.31
	3	0.24		3	0.32
	4	0.25		4	0.32
	5	0.25		5	0.32
	10	0.25		10	0.32

5.3.5.3 Recovery test

The efficiency of the method for total fluoride and water soluble fluoride analysis was evaluated using recovery experiment, i.e. by adding known concentration of sodium fluoride solution to 4 g of the fresh leaves sample and to 1 g of dry khat leaves sample. The procedure was as follow: Each of a 4 g of fresh khat leaves and 1 g of dried khat leaves samples were spiked with a sodium fluoride solution of which the fluoride content was equivalent to 50%, 100% or 200% of the fluoride content of the original (un-spiked) khat leaves samples. After extraction, following the procedure, determination was made in triplicates. The results of the measurements are presented in Tables 5.2.

5.3.6 Conversion factor determination

To report the results obtained on dry weight basis in terms of fresh weight basis, a conversion factor was calculated by taking the weight of a known quantity of fresh edible part of the plant before and after drying (at 70 ^{0}C) to a constant weight. This procedure was repeated for all the 11 types of khat samples collected from the 11 different areas.

Thus, 3.73 (the mean value) was taken as conversion factors because 3.7 g of wet sample (on the average) yielded 1 g of dry sample.

5.4 Results and discussion

5.4.1 Evaluation of analytical method

In the absence of standard reference materials for khat, the validity of the applied procedure for khat was checked by carrying out spiking experiment and the results are presented in Table 5.2. As shown in Table 5.2, the percentage recoveries were between 93% and 103% from spiked Belechie and Wondo-Basha khat leaves samples, which are within an acceptable range. For all the measurements, CV (coefficient of variation) was less than 10% which confirms the reproducibility of the measurement.

Table 5.2 Recovery test results

Sample	[a]Concentration in sample (µg/g)	Amount added (µg/g)	[b]Concentration in spiked sample (µg/g)	[c]% Recovery
[d]Wondo-Basha	0.24	0.12	0.37±0.01	103±3
	0.24	0.24	0.47±0.02	98±4
	0.24	0.48	0.74±0.03	103±4
[e]Belechie	4.2	2.0	6.1 ±0.2	98±3
	4.2	4.0	8.0±0.2	97±3
	4.2	8.0	12±0.3	98±2

[a] Average value of triplicate analyses (µg/g).
[b] Values are mean ± SD of triplicate analyses.
[c] Values are mean ± SD of triplicate analyses.
[d]Fresh sample. [e]Dry leaf sample.

5.4.2 Water extractable and total fluoride distribution in khat cultivars

Three aliquots of khat leaves samples were taken from each bulk sample and the data on the different aliquots was combined (n = 9) to give one mean value. Total fluoride content in the dried leaves samples and water extractable fluoride in the fresh leaves of 11 varieties of chewing khat grown in different parts of the country were determined and the results are shown in Table 5.3. Table 5.3 also shows percentage of total fluoride that is extracted with water from the fresh leaves. Thus, even though it is not possible to tell how much of the RSD (relative standard deviation) in the results (Table 5.3) is from sample inhomogeniety and how much result from analytical error, the overall error (resulting from sample inhomogeniety and from analytical error) is within an acceptable range (RSD $\leq$ 10%).

Since the fresh part of the plant is consumed, the result in terms of the dry weight basis is converted to fresh weight basis so as to correlate intake of total fluoride with the amount of khat chewed using a conversion factor 3.7. Table 5.4 presents the mean concentration of total fluoride in 100 g of fresh khat leaves in terms of wet weight basis in the present study.

Table 5.3 Mean concentration (X ± SD, n = 9, μg/g) and relative standard deviation (% RSD) of water extractable and total fluoride in khat samples

Sample	Total F^- content (dry weight basis)	% RSD	Water extractable F^- (fresh weight basis)	% RSD	[a]% F^- extracted
Bahir Dar	3.4±0.2	5.9	0.19±0.01	3.3	21
Wondo-Kuto	3.9±0.3	7.7	0.25±0.02	8.0	24
Wondo-Basha	3.8±0.3	7.9	0.24±0.02	8.3	23
Guragie	4.0±0.3	8.0	0.23±0.01	4.3	21
Belechie	4.2±0.3	7.2	0.26±0.02	7.7	24
Liyu	4.7±0.4	8.5	0.25±0.02	8.0	20
Awadi	4.8±0.3	6.3	0.26±0.02	7.7	20
Gelemso	4.6±0.4	8.7	0.28±0.02	7.1	22
Yirba	5.8±0.3	5.2	0.32±0.01	3.1	21
Sike	6.5±0.2	3.1	0.31±0.02	6.5	18
Chengie	7.1±0.7	9.8	0.43±0.04	9.3	23

[a]Calculation was made after converting the dry weight basis of total fluoride in to fresh weight basis using a conversion factor of 3.7.

Table 5.4 Mean concentration (µg/100 g and µg/500 g fresh weight) of total fluoride and water soluble fluoride in khat samples

Sample	[a]Total fluoride content (wet weight basis)		Water soluble fluoride (wet weight basis)	
	µg/100 g	µg/500 g	µg/100 g	µg/500 g
Bahir Dar	91	455	19	95
Wondo-Kuto	104	520	25	125
Wondo-Basha	103	515	24	120
Guragie	106	530	23	115
Belechie	108	540	26	130
Liyu	126	630	25	125
Awadi	129	645	26	130
Gelemso	124	620	28	140
Yirba	155	775	32	160
Sike	175	875	31	155
Chengie	190	950	43	215

[a] Calculation was made after converting the dry weight basis of total fluoride in to fresh weight basis using a conversion factor of 3.7.

As can be seen from Table 5.3, water extractable fluoride concentration varies from 0.19–0.43 µg/g fresh weight and was highest in Chengie type khat variety, while, the lowest concentration was found in Bahir Dar khat. The table also shows that total fluoride concentration in different khat varieties ranged between 3.4–7.1 µg/g dry weight. Chengie type khat contained the highest total fluoride of the analyzed khat varieties. The variations in the fluoride content in the different varieties of khat analyzed was not very large, except for the last three, which could have been caused by wide fluctuations of fluoride concentration in the water and soils in which the plant was grown (Fung *et al*.,

1999; Miller *et al*., 1999). Whether variation is significant or not, it is discussed at the end of this section

Plants do not require F^-, and tissue concentrations from uncontaminated soils rarely exceed 30 mg kg^{-1} dry mass (Kabata-Pendis, 2001). Plant F^- uptake is dependent on solution F^- activity, pH (physical property of the soil), application of fertilizers and pesticides, substrate composition, and plant species (Häni, 1978; Elrashidi *et al.,* 1985, Singh *et al*., 1995; Fung *et al*., 1999; McLaughlin *et al*., 2001).

Generally, the natural sources are the major cause of fluoride pollution. An increase in population and industrialization also results in pollution of water, air and soil, which in turn, causes in unexpected concentration of fluoride in plants. Particularly agricultural activities such as use of fertilizers, pesticides, and irrigation with contaminated sewage are the major sources of contaminations (Miller *et al.*, 1999; McLaughlin *et al*., 2001).

Looking at Table 5.3, fluoride concentration varies with growth area. Chengie contains highest fluoride concentration followed by Sike Yirba, Awadai, Liyu, Gelemso, Belechie, Guragie, Wondo-Kuto, Wondo-Basha and Bahir Dar type khat. This variation is attributed to the many factors mentioned above. Fluoride concentration in Chengie, Yirba and Sike varieties of khat which are cultivated around Awassa region in Sidamo district was higher than the rest of khat varieties analyzed. This could be attributed to higher fluoride concentration in the water for irrigation and soil. Research indicates that this district is found in rift valley region of the country and up to 8.0 mg/kg of fluoride has been reported in the different parts of the water body within the district (Malde *et al.,* 1997). The same trend has been observed in vegetables, serials and other plants analyzed from the same district (Malde *et al*., 1997).

Comparison of the F^- content in khat with that of other plants such as vegetables, cereals and tea revealed that F^- in khat is close to that of vegetables and cereals (Bamidele Sanni, 1982, Malde *et al*., 1997), but much less than that found in tea, which ranges from 117–682 µg/g (Pires, *et al.*, 1996; Yadav *et al*., 2007). Even compared to chewing tobacco, khat leaves contains lower concentration of fluoride (Yadav *et al*., 2007).

Use of fertilizer and pesticides are a common practice among khat cultivars to increase their yield (Lemessa, 2001). This in turn contributes significant fluoride to the soil and hence to plants (Hillman *et al*., 1979; McLaughlin *et al*. 2001).

The result indicates that khat leaves from the same province seemed to have similar total F^- contents. Wondo-Kuto and Wondo-Basha are from the same province (Wondo province) and have similar distribution of fluoride. The same is true for Awadai, Gelemso and Liyu types, which are cultivated from Harari province.

For water extractable fluoride, higher F^- contents were released from Chengie and Sike type khat than those from the rest, due to the original higher total F contents in the khat leaves of these samples. However, lower percentage of fluoride were released from Sike and Liyu types khat (17–19%) than the others (22–24%), due to the fact that the Sike and Liyu type khat leaves had broader, harder with lower surface area than those with relatively soft, smaller in size and hence larger surface area. These small sized leaves were easier for crushing during extraction and therefore more efficient in releasing F^-. Research indicates that more fluoride is extracted in the mouth while chewing and in acidic media (Hattab *et al.*, 2000; Yadav *et al.,* 2007).

One paper reported on fluoride contents of Yemeni khat (Hattab *et al.*, 2000). The results are shown in Table 5.5. Looking at the report by Hattab *et al.*, (2000), less than 0.18 µg/g of water extract fluoride was reported while 0.19–0.43 µg/g was obtained in the present study. Similarly, there is significant variation in total fluoride content between the reported result (2.1 µg/g) and present investigation (3.4–7.1 µg/g). Only one varietiy of Ethiopian khat (Bahir Dar khat) has comparable results with the Yemeni khat. In general, Ethiopian khat contains higher fluoride than Yemeni khat. This might be due to any of the aforementioned factors. Furthermore, Ethiopian farmers use chemicals and traditional techniques such as tobacco extract, ash, garbage from houses clean, etc. to increase soil fertility and control pestes (Lemessa, 2001). This might contribute for higher fluoride content in the investigated samples.

Fluoride distributions in different parts of the leaves (young shoot and matured leaves) were investigated in Sike type khat and the result is shown in Table 5.6. From the table,

fluoride contents accumulated in leaves were proportionally related to the age of leaves (young leaves, 6.5 ± 0.2 µg/g; matured leaves 12 ± 0.7µg/g).

In most cases, the average quantity of khat chewed by Ethiopians ranges from 100 g to 500 g daily. Thus, the consumption of khat contributes 91 to 190 µg/100 g total fluoride and 19 to 43 µg/100 g of water extractable fluoride on wet weight basis per day (Table 5.4). These values increase with increasing weight of khat chewed and reach up to 950 µg/500 g. Thus, chewing khat contributes up to 27% of the recommended daily dose of fluoride depending on the varieties taken. Matured leaves contribute double of the stated concentration in young shoot. In general, the fluoride concentration in khat chewing was found to be low and implies no problem related to dental fluorosis. However, khat chewing is accompanied by smoking, use of sugar, excessive hot drinks such as tea and excessive amount of water. Some chewers' drinks more than four cups of tea during chewing session. Reports indicate that drinking about four cups of tea contribute up to 50 % of the recommended daily dose of fluoride (Amanlou *et al*., 2008).

Furthermore, khat chewers drink much more water during chewing because of excessive sweating as a result of excitement. It is also known that drinking fluoridated water contribute more fluoride to the daily intake. Chewers who are living in the rift valley district of the country are highly affected by fluoride problem since up to 8.0 mg/L of fluoride has been reported in different bodies of water for consumption (Malde *et al*., 1997). In addition to other possible sources of fluoride, such as fluoride-containing toothpaste, various beverages, and fish increase the daily fluoride intake to a level higher than the recommended value. Thus, we conclude that, it is not just khat leaves but a cumulative effect that may cause dental health problem among khat leaf chewers. Even if the concentration is low, it contributes a significant amount of fluoride to the daily intake of an individual. Therefore, we recommend that, khat chewers should try to minimize excessive use of the above mentioned additives during khat leaves chewing and should avoid matured leaves.

Table 5.5 Comparison of fluoride with the available data in literature

Khat origin	Water extractable fluoride in the fresh (µg/g)	Total fluoride in dry sample (µg/g)	References
Ethiopian	0.19-0.43	3.4-7.1	Present study
Yemeni	0.18	2.1	Hattab *et al.*,(2000)

Table 5.6 Fluoride distribution in different parts of the leaves (matured and young shoots) of Sike type khat cultivar

Leaf position	Total fluoride (µg/g) dry weight
Young leaves	6.5±0.2
Matured leaves	12±0.7

5.4.3 Statistical analysis

Statistical analysis of data was made to verify whether there was a significant difference in total fluoride concentration between a pair wise comparative investigation of the eleven varieties of khat analyzed. For the present study, the significance of variation within sample and between samples has been studied using one-way ANOVA and calculations were made using SPSS software.

No significant difference ($p > 0.05$) at 95% confidence interval was observed in fluoride concentration for the following pair wise analysis: Bahir Dar, Wondo-Kuto, Wondo-Basha, Guragie, and Belechie. The same was true for Gelemso, Awadai, Liyu; Yirba, Sike, Chengie; Kuto, Gelemso, Liyu; Guragie, Gelemso, Liyu; and Belechie, Gelemso and Liyu types of khat varieties. Whereas any other pair wise combinations other than the

mentioned groups have showed significant difference ($p \leq 0.05$) at 95% confidence interval in fluoride concentration

Absence of significant difference in fluoride concentration in different varieties of khat may indicate the presence of similarities in certain factors or variables such as climatic conditions, soil type, water for irrigation, age of the harvested khat, etc. Similarly, presence of significant difference in fluoride concentration indicates that either of the studied area contains higher concentration of fluoride in the soil or variation in agricultural activities, for example application of fertilizers, insecticides, irrigation, etc. In addition to these, difference in the age of the harvested khat may result in significance difference in level of fluoride.

5.5 Conclusion

The fluoride concentrations (total and water soluble fluoride) in selected eleven commercially available Ethiopian khat (*Catha edulis*) (Bahir Dar, Wondo-Kuto, Wondo-Basha, Awadai, Liyu, Gelemso, Belechie, Guragie, Yirba, Sike and Chengie types of khat varieties) were determined by a fluoride selective electrode. The total fluoride contents were determined to be 3.4–7.1 μg/g dry weight in khat leaves and 0.19–43 μg/g fresh weight of water extractable fluoride which was prepared by extracting fresh khat leaves in water for 5 min. Khat (Chengie, Yirba and Sike type khat) from Awassa District contained more fluoride than khat from other parts of the country. Matured leaves contain higher fluoride concentration which is nearly twice that of young shoot. Percentage of fluoride extracted from khat leaves depends on the size and age of harvested khat leaves and can be up to 24% of total fluoride contents of khat leaves. Though fluoride content in the khat leaves is low, should not be overlooked during the estimation of total dietary intake of fluoride for those who chew this stimulant. We also recommend that chewers should avoid in using the matured leaves of the plant in any case since it can contribute more fluoride than the young leaves. The ANOVA results suggest that there were significant variation in the level of fluoride concentration among some of khat varieties, which could be attributed to different factors such as, age of the harvested khat,

geographical and climatic variation, difference in physicochemical nature of the soil and different agricultural practices among khat cultivars. Whereas absence of significant variation has been observed in some khat varieties analyzed, which might be due to similarity in basic factors mentioned above.

6. LEVELS OF MAJOR, MINOR AND TOXIC METALS IN SOIL AND KHAT CULTIVARS IN ETHIOPIA

6.1 Background

Humans require a suite of mineral elements in varying amounts for proper growth, health maintenance, and general well-being. (National Research Council (US) and Food and Nutrition Board, 19890) and Plants can make a significant contribution to daily mineral needs at all stages of the life cycle (Dwyer, 1994).

The availability of trace elements in the soil to plant, or the capability of the soil to deliver trace elements to agricultural crops is essential for obtaining high quality agricultural products. The availability of the essential micronutrients depends on genetic differences between plant species and on soil properties and availability of the required nutrient in it. Some plants are also accumulators for selected essential and non-essential minerals in their different parts (Porębska *et al.*, 1999; Loftleidir *et al.*, 2005). For instance, tea plant is best known for accumulating aluminum and fluoride compared to other mineral nutrients (Wong *et al*., 2003). Due to the large variation in soil types between regions and between farms within the same region, the plant uptake of micronutrients will vary. Thus, studying the mineral composition of the particular plant species and the soil is important to provide useful information for future studies on agronomy and physiology, fertilizer applications, nutritional, medical and toxicological effects in relation to the plant under investigation.

Khat is the second largest cash crop in the country and consumed by millions of people on a daily basis in the country as well as in many parts of the world. There are worries in the literature concerning mineral accumulation patterns of the different cultivars of this stimulant and their toxicological implication to individuals who are chewing the leaves regularly. Furthermore, the mineral content of the soils where these varieties are grown and the correlation patterns of metals in the soil and in the plant have not yet been investigated.

6.2 Objectives of the study

The aim of this study was to analyze ten elements (Ca, Mg, Fe, Mn, Cu, Zn, Co, Ni, Cd, and Pb) content in edible portion of khat cultivars growing in different parts of Ethiopia and in soil where these varieties are cultivated.

6.3 Materials and methods

6.3.1 Study site description

Khat leaf and soil samples were collected from different regions of the country, namely: Oromiya region, Southern Nation nationality Peoples' Region (SNNPR) and Amhara region. These regions are best known for khat cultivation for local consumption and selling in the capital Addis Ababa, Ethiopia. Some of the khat varieties are also exported to the neighboring countries. Specific area of sampling site with respective trade name of khat variety analyzed is given in section 3.2.

6.3.2 Sample collection and preparation

For the present study two different type of sampling strategy was followed. For six samples (Bahir Dar, Wondo, Awadai, Liyu, Gelemso and Guragie type khat samples), sampling was done from different kiosks in Addis Ababa between January to February, 2009. For Awadai and Liyu types of khat, 6 kiosks were randomly selected while 14 kiosks were selected for collecting the remaining khat types. Around 50 g of the edible portion of the leaf and stem (the softest part of the steam which is found next to the young twig near the top of the plant) of the plant was collected from each kiosk for Awadai and Liyu types khat while around 20 g for the remaining varieties. These were oven dried at 70 ^{0}C for 72 h (until constant weight) and ground in a blender to homogenize and reduce the size of particles. Finally, six bulk samples (100 g each) were prepared for analysis. Three 0.5 g aliquots from each sample were taken for digestion and final analysis. It should be noted that one bulk sample of each of khat varieties were

prepared and analyzed because the geographical variation within the provinces (of smaller areas with similar latitude and longitude and comparable climatic condition) was assumed to be insignificant.

For the remaining 13 varieties/cultivars, samples were collected from khat farms. For each sample three nearby farming areas were selected. A total of 13 bulk khat leaf samples (edible portions of leaves and tender stem) were collected randomly from the selected farming areas between the months of January, 2010 to March, 2010. These were brought to the Department of Chemistry, Addis Ababa University, Ethiopia for further treatments and analysis. Pretreatment was done as above.

Soils samples were collected at the sites where the khat leaf samples were plucked, a total of 13 (about 4 kg each) corresponding soil samples (0–20 cm depth) were collected, air-dried and ground to pass through a 2 mm sieve and stored in clean polyethylene bags prior to analysis.

6.3.3 Equipment and apparatus

A stainless steel knife was used to mark and dig the area for the collection of the soil samples. Both the plant and soil samples were ground using a ceramic mortar and pestle. All of the khat leaves and soil samples were weighed on a digital analytical balance. Round bottom flasks (100 mL) fitted with reflux condenser and Kjeldahl digestion block were used for the total digestion of all the samples. The concentrations of Ca, Mg, Fe, Mn, Cu, Zn, Co, Ni, Cd, and Pb in both the khat leaves and soil samples were determined by flame atomic absorption spectrophotometer (Buck Scientific Model 210 VGP, East Norwalk, USA) using an air-acetylene flame. A potentiometric digital pH meter (WTW Inolab pH/ION Level 2, Germany) was used to determine the pH of soil samples after stirring by a magnetic stirrer.

6.3.4 Chemicals and reagents

Concentrated HNO_3 (69–72%, Spectrosol, BDH, UK) and concentrated $HClO_4$ (70%, Fine Chem Industries Mumbai, India) were used for the digestion of the khat leaf samples. Concentrated HCl (36–38%, Hopkin & Williams, UK) and HNO_3 (69–72%, Spectrosol, BDH, UK) were used for the digestion of soil samples. Lanthanum nitrate hydrate (98%, Aldrich, USA) was used to avoid refractory interference (for releasing calcium and magnesium from their phosphates). Distilled de-ionized water was used throughout the analysis.

6.3.5 Prevention of contamination

To minimize the risk of contamination, all glassware used for the analytical methods was washed with distilled-deionized water followed by acid-wash, and sterile disposable powder-free plastic gloves were worn when handling the khat sample during the sampling and analyses stages. The digested solutions were kept in the refrigerator until analysis.

6.3.6 Chemical analysis of samples

6.3.6.1 Analysis of khat leaves samples

Optimization of digestion procedure

To select an optimum procedure for digestion, parameters like digestion time, reagent volume, volume ratio of reagents, and digestion temperature were optimized by varying one parameter at a time and keeping the others constant. Parameters giving clear solution at lower temperature, requiring minimum reagent volume and digestion time was selected as an optimum procedure for digestion of khat sample.

Finally, for a complete digestion of 0.5 g of the dry sample, 2 mL HNO_3 (69-72%) and 2 mL $HClO_4$ (70%) for a total of 2 h and 30 min at variable interval was selected as optimized procedure for the digestion.

Digestion of khat samples

Applying the optimized procedure, 0.5 g of dried and homogenized khat sample was transferred into a 100 mL round bottomed flask. To this was added 4 mL of a mixture of HNO_3 (69-72%) and $HClO_4$ (70%) with a volume ratio of 1:1 and the mixture was digested on a micro-Kjeldahl digestion apparatus by setting the temperature first to dial 4 (120 ^{0}C) for 30 min and then increased to dial 9 (approximately 270 ^{0}C) for the remaining 2 h. The digested solution was allowed to cool and 20 mL of distilled-deionized water was added to dissolve the precipitate formed on cooling and to minimize dissolution of filter paper by the digested residue while filtering with Whatman, (110 mm; diameter), filter paper. The round bottom flask was rinsed subsequently with 5 mL distilled-deionized water until the total volume reached around 45 mL. To this final solution, 1% lanthanum nitrate solution was added and the solution was filled to the mark (50 mL) with distilled-deionized water. Triplicate digestions were carried out for each bulk sample.

6.3.6.2 Analysis of soil samples

Soil pH was measured in a suspension of a 1:1 soil: water mixture. About 10 g of air-dried soil (< 2 mm) was weighed and transferred into a 100 mL beaker and 10 mL of water was added. The sample was stirred by a magnetic stirrer and the pH was measured after allowing the suspension to stand for 1 h at room temperature (Tan, 1996). Each determination was made in triplicates. Results of the analysis are given in Table 6.3.

1.0 g of soil was accurately weighed and transferred in to a 100 mL round bottom flask and moistened with 1 mL of distilled de-ionized water. 10 mL of 1:3 mixture of conc. HNO_3 and HCl and 1 mL of H_2O_2 was added to the flask and kept for hours until it got stabilized. It was then, digested on a Kjeldahl digestion block under reflux condenser for

2 h at 140 °C. The digest was left to stand for 30 min to cool to room temperature. About 50 mL distilled deionized water was added to the flask, filtered through Whatman No. 41 filter paper into a 100 mL volumetric flask, and made up to the mark with rinsing the digestion flask. The solutions were used for the analysis of the total soil metal concentrations for Ca, Mg, Fe, Mn, Cu, Zn, Co, Ni, Cd, and Pb by flame atomic absorption spectrophotometer (AAS) (Nieuwenhuize *et al*., 1991; Kabata-Pendias and Pendias, 2001; Kisser, 2005; Pansu, *et al*., 2006; Guven *et al*., 2011).

6.3.6.3 Method detection limit

To determine method detection limit, triplicate analyses for six blank samples were performed following the same digestion procedure as the samples.

6.3.6.4 Recovery test

To check the efficiency of the procedure, appropriate amount of stock solution of Ca, Mn, Zn, Co, Cu, Cr and Ni were added together into 0.5 g of two different khat samples and appropriate amount of the remaining metals in to another digestion flask containing 0.5 g of the same varieties of khat samples. A recovery test was also performed for the soil samples using the same procedure. Each recovery test for both samples was performed in triplicate.

6.3.6.5 Conversion factor determination

To report the result obtained (in dry weight basis) in terms of fresh weight basis, a conversion factor was calculated for all the khat varieties after drying a known fresh edible part of the plant. The results are present in Table 6.3. For all khat varieties, a conversion factor lies between 3.67−3.90, i.e. 3.67−3.90 g of fresh (wet) sample yielded 1 g of dry weight depending on the khat variety.

6.4 Results and discussion

6.4.1 Optimization of digestion procedure

To select an optimum procedure for digestion, parameters like digestion time, reagent volume, volume ratio of reagents, and digestion temperature were optimized by varying one parameter at a time and keeping the others constant. Parameters giving clear solution at lower temperature, requiring minimum reagent volume and digestion time was selected as an optimum procedure for digestion of khat sample. Finally, for a complete digestion of 0.5 g of the dry sample, 2 mL HNO_3 (69-72%) and 2 mL $HClO_4$ (70%) for a total of 2 h and 30 min at variable interval was selected as optimized procedure for the digestion.

6.4.2 Method detection limit

To determine method detection limit, replicate analyses for 6 blank samples were performed and the pooled standard deviation of the 6 reagent blank was calculated. The detection limits were obtained by multiplying the pooled standard deviation of the reagent blank by three. The method detection limits of each metal are given in Table 6.1. The method detection limits are generally comparable with that of the instrument for both khat leaves and soil samples.

Table 6.1 Method detection limits for khat leaf and soil sample (n = 6)

Metal	Ca	Mg	Cu	Zn	Mn	Ni	Fe	Co	Cr	Cd	Pb
MDL (µg/g)[a] for khat leaf	3.1	2.6	2.1	0.85	1.1	4.2	4.6	1.0	5.1	0.79	7.5
MDL (µg/g)[a] for soil	3.4	2.3	2.1	0.78	1.1	4.1	4.2	1.2	5.1	0.71	8.8

[a]Values are mean of 3 x standard deviation of seven blank determinations each measured three times.

MDL: Method detection limit

6.4.3 Evaluation of analytical method

The validity of the digestion procedures for khat and soil samples was checked by carrying out with a lower level of traceability, such as spiked samples. The results are presented in Tables 6.2a, 6.2b and 6.2c. The percentage recoveries of khat sample are between 90% and 105%, which are within the acceptable range. whereas the values of percentage recoveries for the studied macro- and micronutrient and the toxic metals in the soil samples were within the range of 90–106% which are still within the acceptable range.

Table 6.2a. Recovery test results for khat leaf sample 1

Metal	Conc. in sample (µg/g)a	Amount added (µg/g)	Conc. in spiked sample (µg/g)a	% Recoveryc
Ca	6120	2000	7999 ± 33	94 ± 2
Mg	2710	1000	3634 ± 29	92 ± 3
Fe	121	40	158 ± 2	93 ± 5
Zn	29.1	15	42.7 ± 1.3	91 ± 8
Mn	14.3	8	21.5 ± 0.8	90 ± 9
Cu	13.8	8	22.2 ± 0.6	105 ± 8
Ni	9.3	5	13.9 ± 0.2	91 ± 3
Co	2.1	2	3.93 ± 0.08	91 ± 4

[a]Average value of 9 measurements (µg/g). [b]Values are mean ± SD of triplicate readings of triplicate analyses. [c]Values are mean ± SD of triplicate readings of triplicate.

Table 6.2b. Recovery test results for khat leaf sample 2

Metal	[a] Conc. in sample (μg/g)	Amount added (μg/g)	[b] Conc. in spiked sample (μg/g)	[c] % Recovery
Ca	3311	660	4409 ± 22	90.8 ± 5.8
Mg	2571	660	3185 ± 25	93 ± 5.7
Fe	259	78	329 ± 6	90 ± 8.5
Zn	29.1	12	40.4 ± 0.2	95.0 ± 7.7
Mn	27.2	12	38.6 ± 0.3	95.2 ± 3.8
Cu	20.3	10	29.3 ± 0.2	90.3 ± 1.4
Cr	5.22	0.8	6.05 ± 0.05	104 ± 6.1
Co	1.49	0.8	2.31 ± 0.01	102.1 ± 3.0

[a] average value of 9 measurements (μg/g).

[b] values are mean ± SD of triplicate readings of triplicate analyses.

[c] values are mean ± SD of triplicate readings of triplicate.

Table 6.2c. Recovery test results for soil samples

Metal	Conc. in sample (μg/g)[a]	Amount added (μg/g)	Conc. in spiked sample (μg/g)[a]	% Recovery[c]
Ca	1040	500	1504 ± 9	93 ± 2
Mg	2030	1000	2995 ± 39	97 ± 4
Fe	20600	2300	22796 ± 95	96 ± 4
Zn	79.2	40	121 ± 3	105 ± 7
Mn	1920	600	2548 ± 34	105 ± 6
Cu	22.3	10	32 ± 0.9	97 ± 9
Ni	34.7	15	50.5 ± 1.3	106 ± 9
Co	12.7	5	17.4 ± 0.3	94 ± 7
Cd	1.2	1	2.33 ± 0.06	91 ± 6

[a]Average value of 9 measurements (μg/g). [b]Values are mean ± SD of triplicate readings of triplicate analyses. [c]Values are mean ± SD of triplicate readings of triplicate.

6.4.4 Level of metals in soil sample

Concentration of selected macro, micro and toxic elements in the selected khat farms are given in Table 6.4. The values of pH of the soils for the selected fields of the thirteen khat growing farms are presented in Table 6.3. The soil pH of the farms was within the range of 5.28–7.34, which categorizes the soils under weakly acidic to weakly basic. Farmers are using using of fertilizers to boost their production (Lemessa, 2001). Thus, one of the reasons for the slight acidity of soils of some of the farms might be due application of NPKS fertilizers.

Reports indicate that increasing rates of nitrogenous fertilizers generally increase soil acidity (Ishibashi *et al*., 2004). Soil pH is one of the most influential parameters controlling the conversion of metals from immobile solid-phase forms to more mobile and/or bioavailable solution-phase forms. As reported by many authors, the solubility of heavy metals is generally greater as pH decreases within the pH range of normal

agricultural soils (approximately pH 5.0 to 7.0) (Kabata-Pendias, 2004; Wang *et al*., 2006). The high pH values of soils could have accounted for a low transfer of metals from soil to plant.

The macro-, micro- and toxic metal contents analysed in the khat farm soils varied significantly from site to site. Among the macro-elements, the Mg content of the soils was highest within a range of 1.13–2.20 mg/g, followed by Ca (0.78–1.82 mg/g). Some Ethiopian khat producing soils are classed under the category of vertisols, kandic paleustalfs, hyperdystric acrisol, oxisols, ferralsols, and plinthic alisols. Some of them are with clay like texture and dark reddish brown colour and others are with reddish colour (Solomon *et al*., 2001; Beyene, 1988; Atengo *et al*., 2006), which is indicative of the presence of excess amounts of hematite (Fe_2O_3) (Tan, 1996). Soils with low pH contain high amounts of Fe and Al oxides (Hu *et al*., 2002). Thus, Fe is the predominant metal within the concentration range of 9.88–26.6 mg/g in these soils whereas Mn content is in the range of 0.70–1.77 mg/g. The concentration of Zn in the soil samples ranged from 49.9–131.3 mg/kg. Similarly, Co (12.7–38.1 mg/kg), Ni (8.27–41.7 mg/kg), and Cu (9.13–29.8 mg/kg) have been obtained in the analysed soil samples. On the other hand, Cd was detected in most of the analyzed soils of farm lands, except in Mokonisa, Debo and Damile farms. In the remaining soils, the level of the toxic heavy metal Cd, ranged from 0.73 mg/kg for Sebeta farms and up to 1.20 mg/kg for soils from Chengie farms. The level of Pb, the other tested toxic metal, in the soils of all studied farms, was found to be below the detection limit of the method used in this study.

Comparing the metal concentration in soil with guidelines for soils showed that all the metal concentrations were below the guidelines for toxic soils (Begerow *et al*., 2004) ; Kabata-Pendias and Pendias, 2001; Itanna, 2002; Environmental Agency, 2009 (a and b)). Examining the table, most soils taken from the farms follow a similar trend in metal accumulation. For the majority of the analyzed soils, the mean concentrations of the ten metals for this study were found to follow decreasing order: Fe > Mg > Ca > Mn > Zn > Co > Ni >Cu > Cd > Pb.

Comparing with some other parts of the world, major khat producing areas of Ethiopia have accumulated lower concentrations of heavy metals which implies that the

investigated farm soils are relatively free from heavy metals, contamination (Awode *et al*., 2008; Khairiah, *et al*., 2009)

Statistical analysis (both one-way ANOVA and Pearson correlation matrices) have been conducted to see the correlation of metal accumulation in soils of the different sites and correlation between metals respectively. Results of the analysis are given in the next section.

Table 6.3. Average value of soil pH and moisture content of khat varieties.

Soil sampling sites	Soil pH (1:1 H_2O)	Khat varieties	% Moisture content of khat samples	Conversion factor
Anferara	5.28	Anferara	73.1	3.72
Aleta-Wondo	7.34	Gerbicho	73.9	3.83
Sebeta	6.60	Sebeta	73.1	3.72
Yirba	6.56	Yirba	73.2	3.87
Chengie	7.18	Chengie	73.1	3.72
Wondo-Basha	6.74	Basha	74.4	3.9
Mokonisa	6.12	Mokonisa	73.0	3.71
Debo	6.24	Debo	73.6	3.79
Damile	5.99	Damile	73.5	3.77
Belechie	6.57	Belechie	73.4	3.76
Bole	6.59	Bole	74.3	3.89
Sike	6.51	Sike	72.8	3.67
-	-	Bahir Dar	74	3.85
-	-	Awaday	72.2	3.6
-	-	Gelemso	72.2	3.6
-	-	Wondo	73	3.7
-	-	Guragie	72.7	3.66
-	-	Liyu	72.1	3.58

-Not studied

Table 6.4 Average metal concentrations in the soils of selected khat growing farms

Sampling site	Ca (mg/g)	Mg (mg/g)	Mn (mg/g)	Fe (mg/g)
	Metal concentration of soils (dry weight basis)			
Anferara	1.82± 0.15	1.98 ± 0.01	0.70 ± 0.08	17.1±0.27
Aleta-Wondo	1.70± 0.05	2.0 ± 0.09	1.68 ± 0.02	22.9±1.24
Sebeta	1.45± 0.04	1.41 ± 0.11	1.62 ± 0.03	27.1±0.75
Yirba	1.33± 0.10	2.20 ± 0.06	1.57 ± 0.05	26.6±0.31
Chengie	1.04± 0.07	2.03 ± 0.08	1.92 ± 0.04	20.6±1.32
Wondo-Basha	1.09 ± 0.04	1.42 ± 0.10	1.44 ± 0.05	14.2±0.11
Mokonisa	0.91± 0.01	1.52 ± 0.11	1.77 ± 0.06	16.7±0.10
Debo	0.86 ± 0.04	1.13 ± 0.02	1.03 ± 0.01	10.9±0.08
Damile	0.78 ± 0.05	1.85 ± 0.07	1.25 ± 0.06	12.9±0.38
Wondo-Belechie	0.88 ± 0.03	1.61 ± 0.09	1.40 ± 0.01	12.0±0.26
Bole	0.99 ± 0.02	1.46 ± 0.03	0.96 ± 0.07	12.8±0.32
Sike	0.90 ± 0.01	1.55 ± 0.06	1.42 ± 0.03	14.1±0.61

	Zn	Co	Cu	Ni	Cd	Pb
	µg/g					
Anferara	68.6 ± 3.85	37.5 ± 1.15	23.5 ± 0.39	25.0 ± 1.75	0.73± 0.07	ND[a]
Aleta-Wondo	85.9 ± 2.13	38.1 ± 0.10	24.0 ± 1.16	32.1 ± 2.21	0.96 ± 0.07	ND[a]
Sebeta	114.2 ± 3.06	22.7 ± 0. 89	22.7 ± 1.24	28.7 ± 1.66	0.76 ± 0.07	ND[a]
Yirba	131.3 ± 4.31	30.0 ± 1.67	25.9 ± 0.30	41.7 ± 0.93	1.23 ± 0.20	ND[a]
Chengie	79.2 ± 1.27	12.7 ± 2.10	22.3 ± 1.89	34.7 ± 1.47	1.20 ± 0.08	ND[a]
Wondo-Basha	63.9 ± 1.43	34.8 ± 1.21	15.2 ± 1.54	23.8 ± 1.53	0.94 ± 0.08	ND[a]
Mokonisa	67.1 ± 1.23	34.3 ± 0.6	17.3 ± 0.98	30.6 ± 2.86	[a]ND	ND[a]
Debo	56.9 ± 1.97	14.7 ± 1.35	11.1 ± 0.61	8.28 ± 0.45	[a]ND	ND[a]
Damile	52.3 ± 1.40	28.5 ± 0.3	19.1 ± 1.78	23.5 ± 0.22	[a]ND	ND[a]
Belechie	49.9 ± 1.1	23.3 ± 0.81	9.13 ± 0.87	19.4 ± 1.17	0.79±0.07	ND[a]
Bole	79.2 ± 3.2	15.2 ± 0.45	20.8 ± 1.45	15.3 ± 0.97	0.86 ± 0.04	ND[a]
Sike	91.1 ± 4.65	31.2 ± 1.38	14.3 ± 0.31	28.5 ± 1.71	0.78 ± 0.06	ND[a]

[a]Not detected (bellow the method detection limit)

6.4.5 Level of metals in khat cultivars

Three aliquots of khat samples were taken from each bulk sample and the data on the different aliquots was combined (n = 9) to give one mean value of the particular metal for one particular bulk khat sample. Table 6.5 shows the mean concentration values of each metal in each sample with its respective standard deviation.

As can be seen from Table 6.5, Wide variations were observed in the accumulation of metals by the different variety of khat samples which represented different contributing (both natural and anthropogenic) factors.

The results of total contents of the studied nutrient and toxic metals in the nineteen cultivars of khat (*C. edulis* Forsk) show the ability of these clones to accumulate high amounts of both macro- and micronutrient elements. The most abundant metal among the macroelements analysed was Ca followed by Mg whereas Fe content of the khat leaves was the predominant among the tested micronutrient heavy metals followed by Zn, Mn, Cu, Ni and Co. On the other hand, the content of the toxic non-essential heavy metals, Cd and Pb in the analysed clones were found to be below the method detection limit.

It can be deduced from the levels of all the metals in the studied khat varieties of all the sampling sites, that the concentrations of the macro- and the micronutrient metals followed similar trend for most of the samples. In general, ranges of concentrations of the studied macronutrient and micronutrient metals could be arranged according to their levels in the khat plant varieties of all the sampling sites in the following order in dry weight basis: Ca (3850−8750 mg/kg) > Mg (1670−3070 mg/kg) > Fe (121−336 mg/kg) > Zn (18.7−40.6 mg/kg) > Mn (11.4−31.0 mg/kg) > Cu (4.81−20.4 mg/kg) > Ni (4.43−10.2 mg/kg) > Cr(2.43-7.36) > Co (ND−3.67 mg/kg).

The higher levels of Ca and Mg in the studied khat plants is obvious since these nutrients are highly mobile in the plant tissue and are translocated from old leaves to young leaves

(Marschner, 2002). Furthermore, broad range of Ca-bearing minerals in soil and water and usually abundant in ground water and surface water which can easily be absorbed by the plant.

The concentration of Fe, Zn and Mn were higher than the entire trace metals in the samples. Since the soil types of khat growing areas of Ethiopia are moderately acidic to slightly basic with the pH ranges from 5.28 to 7.34 (Table 6.3), the plant is expected to have a better accumulation of micronutrients like iron and zinc (Wang *et al*., 2006; Kabata-Pendias *et al*., 2001; Kabata-Pendias, 2004). Iron was the most abundant trace metal in all the khat varieties analysed ranging from 171 mg/kg in Belechie type khat up to 336 mg/kg in Sebeta type khat. This might be due to higher concentration of iron in the soil (Table 6.4). Furthermore, khat contains high amount of polyphenols (see section 4) and hence it might facilitate Fe absorption through complexation. Higher concentration of manganese is expected because it is mostly accumulated in leaves of plants and also absorption of soluble Mn increases with decreasing soil pH (Ajasa *et al*., 2004; Kabata-Pendias, 2004).

On the other hand, Cu content ranged between 4.81 mg/kg in Mokonisa type khat and 20.4 mg/kg in Bahr Dar type khat, whereas the concentration range of Zn was found to be 18.7 mg/kg in Sike type of khat up to 40.8 mg/kg in Damile type of khat cultivar from Damile farm lands.

Our results revealed that khat clones accumulate appreciable amount of Ni, to the lesser extent Co and Cr, ranging from 4.43-14.2 mg/kg, ND-3.64 mg/kg and 2.43-12.7 mg/kg respectively in dry weight basis.

As reported by Dushenkov *et al*. 1995 and Grusak and Penna, 1999, Co and Cr are required by humans, but not by plants. Fortunately for humans, however, plants can acquire this element through non-specific influx processes using existing transporters localized to their roots. In fact, a wide range of plant's non-essential elements (both benign and detrimental) has been measured in plant tissues, with concentrations sometimes reaching dramatic levels if soil availability is high.

Only one paper has been reported dealing with Cu, Zn, Cd and Pb on Yemeni khat (Matloob, 2003). The concentration of Cu and Zn reported are comparable with the present study. Fortunately, the concentrations of toxic heavy metals, Pb and Cd in the studied khat leaves cultivars were too low to be detected by the analytical technique used in this study. However, there is a report of the availability of these metals at lower levels in Yemeni khat leaves with concentration range of Cd (0.007–0.018 mg/kg) and Pb (0.066–0.7 mg/kg) in *fresh weight* basis. In fact the concentration of the toxic heavy metals are expected to be very low in Ethiopian khat since these metals are related to environmental pollution caused by different industrial activities.

Comparing level of metals among the analyzed khat varieties, it is impossible to have a common trend for the distribution of all the metals in all the khat varieties, i.e. some khat varieties accumulate relatively higher concentration of some of the metals while lower concentration for remaining metals relative to the other khat varieties analyzed. This might be due various contributing factors like nature of chemical and physical property of the soil, climatic condition of the region, and application of fertilizers and pesticides. Furthermore, increase in population and industrialization results pollution to water, air and soil which in turn causes in unexpected concentration of trace metals in the plant. Particularly agricultural activities such as use of fertilizers, pesticides, and irrigation with contaminated sewage are the major source of contaminations.

Pair wise statistical analysis of data was made to verify whether there was a significant difference in metal contents between the khat varieties analyzed. For the present study, the significance of variation within sample and between samples has been studied using one-way ANOVA and calculations were made using SPSS software.

6.4.6 Analysis of variance

For most of the studied khat cultivars, significant difference ($p < 0.05$) at 95% confidence level was observed for the majority of the metals under investigation. While insignificant variation ($p > 0.05$) at 95% confidence interval was seen among some khat varieties for some of the metals when pair wise comparison was made. For example no significant difference was observed between Debo, Mokonisa and Damile type khat varieties for most of the analyzed metals. Similarly no significant difference at 95% confidence level was observed for Basha and Belechie types of khat varieties and Sike, Chengie, Yirba, and Bole type varieties for some of the mineral nutrients. Absence of significant difference might be due to the fact that the farm lands are found in the same province. For example, Basha and Belechie khat varieties are grown in Wondo-Genet province. Similarly, Debo, Mokonisa and Damile type khat varieties are grown in Sidama province while Chengie, Sike, Yirba and Bole type khat varities are grown in Hawassa province. Thus, the farms within the same province might share comparable climatic conditions and soil properties. Existence of significant difference in metal contents among khat cultivars might be due to variations in the aforementioned factors. Like that of the khat cultivars, more or less the same variations of metals content in the soils analysed have been observed.

Table 6.5 Average metal concentrations (X ± SD, n = 9) of analyzed khat cultivars from each selected farms

Khat variety	Metal concentration of khat varieties (dry weight basis)				
	Ca (mg/g)	Mg (mg/g)	Mn (µg/g)	Fe (µg/g)	Zn (µg/g)
Anferara	8.57 ± 0.19	2.80 ± 0.10	16.8 ± 0.82	200±14.4	37.2 ± 2.97
Gerbicho	7.98 ± 0.12	3.07 ± 0.06	18.2 ± 0.72	325±13.5	40.6 ± 1.71
Sebeta	8.45 ± 0.05	2.82 ± 0.04	22.7 ± 0.90	336±27.0	28.6 ± 1.62
Yirba	6.63 ± 0.48	2.04 ± 0.01	19.7 ± 0.50	144±8.17	38.6 ± 2.39
Chengie	6.12 ± 0.08	2.71 ± 0.20	14.3 ± 0.49	121±10.6	29.1 ± 1.99
Basha	5.03 ± 0.16	1.97 ± 0.01	23.9 ± 0.82	213±28.6	26.0 ± 2.0
Mokonisa	3.83 ± 0.10	2.70 ± 0.11	17.3 ± 0.81	220±5.58	22.1 ± 1.32
Debo	5.98 ± 0.33	1.67 ± 0.07	13.4 ± 0.32	203±11.7	19.8 ± 1.29
Damile	6.24 ± 0.32	2.60 ± 0.04	21.7 ± 0.69	236±34.9	40.8 ± 1.20
Belechie	5.12 ± 0.17	2.11 ± 0.10	24.8 ± 1.27	171±9.13	33.2 ± 2.11
Bole	6.34 ± 0.50	2.19 ± 0.08	17.2 ± 0.69	120±6.69	31.1 ± 1.45
Sike	6.23 ± 0.10	2.51 ± 0.11	11.4 ± 0.51	209±13.5	18.7 ± 1.78
Bahir Dar	3.81 ± 0.10	2.57 ± 0.03	27.2 ± 0.3	259 ± 4	29.1 ± 0.9
Wondo	5.88 ± 0.10	2.40 ± 0.05	31.8 ± 0.4	249 ± 4	19 ± 1.0
Awadai	4.18 ± 0.09	1.76 ± 0.02	31.1 ± 0.5	216 ± 4	29.2 ± 0.7
Liyu	5.51 ± 0.03	2.98 ± 0.04	28.8 ± 0.4	298 ± 4	34.5 ± 0.6
Geelemso	7.57 ± 0.04	2.35 ± 0.03	25.6 ± 0.4	198 ± 3	20.0 ± 0.7
Guragie	7.97 ± 0.03	2.60 ± 0.03	30.0 ± 0.4	304 ± 5	25.3 ± 0.6

	Co	Cu	Ni	Cd	Pb	Cr
	----------µg/g----------					
Anferara	1.80 ± 0.17	13.9 ± 1.12	11.9 ± 0.48	ND[a]	ND[a]	[b]NS
Gerbicho	2.79 ± 0.38	5.89 ± 0.41	7.7 ± 0.75	ND[a]	ND[a]	[b]NS
Sebeta	ND[a]	16.2 ± 1.62	14.2 ± 0.28	ND[a]	ND[a]	[b]NS
Yirba	1.9 ± 0.15	9.7 ± 0.94	7.1 ± 0.23	ND[a]	ND[a]	[b]NS
Chengie	2.11 ± 0.20	13.8 ± 0.43	9.3 ± 0.34	ND[a]	ND[a]	[b]NS

Basha	1.26 ± 0.07	6.09 ± 0.64	4.16 ± 0.33	ND[a]	ND[a]	[b]NS
Mokonisa	2.45 ± 0.23	4.81 ± 0.48	8.59 ± 0.80	ND[a]	ND[a]	[b]NS
Debo	2.5 ± 0.17	6.01 ± 0.16	6.81 ± 0.67	ND[a]	ND[a]	[b]NS
Damile	2.7 ± 0.25	14.8 ± 0.21	4.71 ± 0.38	ND[a]	ND[a]	[b]NS
Belechie	2.47 ± 0.18	7.02 ± 0.64	4.43 ± 0.43	ND[a]	ND[a]	[b]NS
Bole	1.72 ± 0.13	5.32 ± 0.08	10.8 ± 0.91	ND[a]	ND[a]	[b]NS
Sike	1.2 ± 0.01	8.12 ± 0.31	8.22 ± 0.74	ND[a]	ND[a]	[b]NS
Bahir Dar	1.49 ± 0.12	20.3 ± 1.3	[b]NS	ND[a]	ND[a]	5.22 ± 0.50
Wondo	1.72 ± 0.14	13.4 ± 0.4	[b]NS	ND[a]	ND[a]	9.09 ± 0.97
Awadai	1.78 ± 0.10	13.3 ± 0.4	[b]NS	ND[a]	ND[a]	12.7 ± 0.8
Liyu	2.57 ± 0.10	12.1 ± 0.6	[b]NS	ND[a]	ND[a]	6.10 ± 0.61
Geelemso	2.80 ± 0.10	7.3 ± 0.5	[b]NS	ND[a]	ND[a]	2.43 ± 0.23
Guragie	2.95 ± 0.12	6.8 ± 0.5	[b]NS	ND[a]	ND[a]	7.36 ± 0.72

[a]Below method detection limit.

[b]Not studied

6.4.7 Comparison of studied metals in plant and soil

Correlation test was carried out between soil and plant for each analyzed metals to evaluate the metals distribution in the soil and their availability and accumulation in the plant. The Pearson product moment correlation coefficient was calculated. Based on the results of r-value, the studied soils were found to correlate positively with the levels found in khat clones. The macronutrients and Cu from the trace elements were found to correlate more strongly than the rest of the nutrients analyzed.

Correlation test (Table 6.6) was also carried out between analyzed metals in soils, and then between tested metals and pH in khat cultivars (Table 6.7). This test may answer some of the question on how the metals interact among themselves in the plant and soil.

Examining Table 6.6, all the tested metals correlated positively with each other except Ca and Mn which has negative correlation. Some of the metals have strong correlations while others are weak. Positive correlation in the case of tested metals normally indicates their mutual existence and enrichment in soil, whereas negative correlation indicates their competition to occupy the same sites in soil exchange base or lattice. This means that some of the metals may enrich together, whilst for Ca and Mn, the Ca content may increase if the Mn content decrease or vice versa.

From Table, 6.7, it can be seen that Ni was negatively correlated with Fe and Mn. The same was true for Co between all the tested metals. Whilst the remaining tested metals correlated positively with each other. The pH of soil has a significant negative relationship with Fe, Mn, Zn, Cu, and Ni while weak positive relationship with Ca, Mg, and Co has been observed. Negative correlation implies that decrease in soil pH go together with increase in the solubility of Zn, Fe, Mn and Cu in the soil and increase in their bioavailability to the plant.

Table 6.6 Correlation between major, minor and trace metals in the soil

	Ca	Mg	Mn	Fe	Zn	Co	Cu	Ni
Ca	1							
Mg	0.434	1						
Mn	-0.034	0.322	1					
Fe	0.617	0.556	0.550	1				
Zn	0.439	0.284	0.294	0.729	1			
Co	0.460	0.349	0.074	0.165	0.036	1		
Cu	0.539	0.676	0.158	0.753	0.531	0.157	1	
Ni	0.379	0.675	0.689	0.734	0.558	0.501	0.580	1

Table 6.7 Correlation between pH, major, minor and trace metals in khat samples

	Ca	Mg	Mn	Fe	Zn	Co	Cu	Ni
Ca	1							
Mg	0.390	1						
Mn	-0.245	0.043	1					
Fe	0.218	0.426	0.429	1				
Zn	0.427	0.335	0.418	0.137	1			
Co	-0.268	-0.271	-0.214	-0.177	0.197	1		
Cu	0.127	0.426	0.443	0.296	0.294	-0.400	1	
Ni	0.55	0.50	-0.370	-0.02	0.205	-0.38	0.300	1
pH	0.134	0.020	-0.213	-0.063	-0.022	0.031	-0.424	-0.094

6.4.8 Contribution of khat to the individual's daily allowance of metals and toxicological implication

Since fresh khat is chewed for its stimulating property, the result in terms of dry weight basis is converted to fresh (wet) weight basis using a conversion factor of each cultivar given in Table 6.3. Furthermore, in most cases, the average quantity of khat chewed by Ethiopians ranges from 100 g to 500 g daily. Table 6.8 presents the range of metal concentrations of the tested metals in fresh weight basis in the analyzed khat cultivars. Looking at the table, chewing khat contributes 103–203 mg/100 g and 44.1–80.2 mg/100 g fresh weight basis per day of Ca and Mg, respectively. Similarly chewing khat contributes 0.51–1.06 mg/100 g and 0.12–0.44 mg/100 g of fresh weight basis per day of Zn and Cu, respectively. As per the 1989 recommended daily allowance (RDA) levels for Cu and Zn are respectively of 2–3 mg/day and 12–15 mg/day (National Research Council, 1989). These values do not pose a health risk if up to 100 g of the stated fresh khat are taken. Rather Zn from other source is required to satisfy the required amount. However, further increase in khat consumption may satisfy the maximum recommended

RDA value of Cu and additional intake of Cu from other source might pose health impact.

In Addition, chewing 100 g of fresh khat contribute the following concentration range of Fe, Mn, Ni, Cr and Co: 3.7–9.03 mg/100 g, 0.3–0.60 mg/100 g, 0.11–0.32 mg/100 g, 0.06-0.35 and 0.03–0.10 mg/100 g of fresh weight basis per day, respectively. Still these results are below the RDA recommended limit and require additional sources of these metals to satisfy the individual need (National Research Council, 1989). Furthermore, chewing the aforementioned khat varieties contribute insignificant amount of the toxic heavy metals Cd and Pb.

Generally, based on the current status, chewing Ethiopian khat in addition to its stimulating property, it contribute appreciable amount of macro and trace metals for the daily requirements of the individuals and are free from toxic heavy metals. Particularly, khat could be good source of Fe and Zn for individuals who are chewing this plant regularly.

Table 6.8 Concentration range of tested metals in the khat cultivars in fresh weight basis

Elements (mg/100 g) (wet weight basis)					
Ca	Mg	Fe	Zn	Mn	Cu
103–203	44.1–80.2	3.72–9.03	0.51–1.06	0.03–0.60	0.12–0.44
Ni	Co	Cr	Cd	Pb	
0.11–0.32	0.03–0.1	0.06-0.35	NDa	NDa	

[a]Not detected

It is the first time that the correlation of metals accumulation in the khat varieties and soil samples has been investigated. Thus, the present study fulfills the gap that existed between khat varieties/ cultivars/ clones and their accumulation patterns of the selected metals from the soil. In addition, it is the first time to analyze soil samples from major khat cultivation farms. Hence, results of our investigation is a newly added data concerning major, minor and toxic metals in khat clones, their toxicological implication and soil samples of major Ethiopian khat grown farms.

6.5 Conclusion

This study determined levels of macro- and micronutrient and the toxic heavy metals (Cd and Pb) in edible portion of khat leaves of nineteen khat cultivars grown in different areas of Ethiopia and their respective soil samples using FAAS.

The optimized and adopted wet digestion method for khat sample analysis was found to be efficient for the majority of the minerals and it was evaluated through the recovery experiment and a good percentage recovery was obtained (100 ± 10) for the majority of the minerals determined.

The results showed the ability of these plants to accumulate relatively higher amounts of Ca, Mg and Fe among the determined macro- and micronutrient metals, respectively. Heavy metals, Cu, Cr and Co was found to be at comparatively lower levels in most of the analyzed khat cultivars. The studied metal content of all cultivars followed similar trend across the varieties and could be arranged in descending order: Ca > Mg > Fe > Zn > Mn > Ni > Cu >Cr> Co.

The level of toxic heavy metals Cd and Pb in all clones was too low to be detected by the method used in this study. The results of the analysis showed that all the khat varieties analyzed contains appropriate concentration of essential major and minor metals and they could be source of mineral nutrients for those who are chewing khat regularly.

A study revealed that accumulation of most of the metals in khat plants correlate negatively with the pH of the soil whereas there were positive correlations among studied

metals in the khat cultivars. The ANOVA results suggested that there were significant variation in the level of some elements between the khat varieties which could be attributed to different factors such as age of the harvested khat, geographical and climatic variation, difference in physicochemical nature of the soil and different agricultural practices among khat cultivars. Insignificant variations were observed among some khat cultivars for some of the metals investigated.

The soils of the study farms were found to contain high levels of Fe followed by Mg, Ca, Mn, Zn, Co, Ni and Cu. In all of the soils, the level of Pb was below the method detection limit. However, unlike the khat leaves, the soils of all the farms were found to contain Cd, at relatively low levels except, the farms of Mokonisa, Damile and Debo. In general, the levels of most of the metals in the studied soils were found to correlate positively with the levels found in the khat leaves.

7. REFERENCES

Abascal, K., Ganora, L., Yarnell, E., 2005. The effect of freeze-drying and its implications for botanical medicine: a review. Phototherapy Research 19, 655-660.

Abdulsalam, A.F., Jian-kai, Z., Xue-feng, Y., 2004. Solid phase microextraction of flavor analysis in Harari Khat (Catha edulis) stimulant. Journal of Zhejiang University Science 5, 428–431.

Abdulwaheb, M., Muche A., 2007. Khat (*Catha edulis* Firsk) – an update review. Pharmacology Online 2, 12–25.

Agency for Toxic Substances & Disease Registry. 2003. Toxicological profile for fluoride, hydrogen fluoride and fluorine. Accessed at: http://www.atsdr.cdc.gov/toxprofiles/tp11.html. Accessed: 20 October 2012.

Ahmed, M.B., El-Qirbi, A.B., 1993. Biochemical effects of *Catha edulis*, cathine and cathinone on adrenocortical functions. Journal of Ethnopharmacology 39, 213–216.

Ajasa, A.O., Bello, M.O., Ibrahim, A.O., Ogunwandea, I.A., Olawore, N.O., 2004. Heavy trace metals and macronutrients status in herbal plants of Nigeria. Food Chemistry 85, 67–71.

Akinrinde, E.A. 2004. Soils: nature, fertility conservation and management. AMS Publishing Vienna, Austria, p 122.

Al-Ahdal, M.N., Mcgarry, T.J., Hannan, M.A., 1988. Cytotoxicity of khat (*Catha edulis*) extract on cultured mammalian cells: effects on macromolecule biosynthesis. Mutation Research 204, 317–322.

Alasalvar, C., Karamac, M., Amarowicz, R., Shahidi, F., 2006. Antioxidant and antiradical activities in extracts of hazelnut kernel (*Corylus avellana* L.) and hazelnut green leafy cover. Journal of Agriculture and Food Chemistry 54, 4826–4832.

Alem, A., Kebede, D., Kullgren, G., 1999. The prevalence and socio-demographic correlates of khat chewing in Butajira, Ethiopia. Acta Psychiatrica Scandinavica Supplement 397, 84–91.

Al-Halbori, M., 2005. The potential adverse effects of habitual use of Catha edulis (khat). Expert Opinon on Drug Safty 4, 1145–1154.

Al-Hebshi, N.N., Nielsen, O., Skaug, N., 2005. In vitro effects of crude khat extracts on the growth, colonization, and glucosyltransferases of *Streptococcus mutans*. Acta Odontologica Scandinavica 63, 136–142.

Al-Hebshi, N.N., Skaug, N., 2005. Khat (*Catha edulis*)—an updated review. Addiction Biology 10, 299–307.

Alia, M., Horcajo, C., Bravo, L., Goya, L., 2003. Effect of grape antioxidant dietary fiber on the total antioxidant capacity and the activity of liver antioxidant enzymes in rats. Nutrition Research 23, 1251–1267.

Al-Meshal, I.A., Tariq, M., Parmar, N.S., Ageel, A.M., 1986. Anti-inflammatory activity of the flavonoid fraction of khat (*Catha edulis* Forsk). Agents Actions 17, 379-380.

Al-Motarreb, A., Al-Habori, M., Broadley, K.J., 2010. Khat chewing, cardiovascular diseases and other internal medical problems: The current situation and directions for future research. Journal of Ethnopharmacology 132, 540–548.

Al-Motarreb, A., Baker, K., Broadley, K.J., 2002. Khat: Pharmacological and medical aspects and its social use in Yemen. Phytotherapy Research 16, 403–413.

Al-Obaid, A.M., Al-Tamrah, S.A., Aly, F.A., Alwarthan, A.A., 1998. Determination of (S) (−)-cathinone by spectrophotometric detection. Journal of Pharmaceutical and Biomedical Analysis 17, 321–326.

Al-Qirim, T.M., Shahwan, M., Zaidi., K.R., Uddin, Q., Banu, N., 2002. Effect of khat, its constituents and restraint stress on free radicalmetabolism of rats Journal of Ethnopharmacology 83, 245–250.

Al-Thobhani, M.A.H., Sathyanarayana, B.N., Simo, L., Sondur, S.R., 2008**.** First comparative genotypic study on khat Middle Eastern and Russian Journal of Plant Science and Biotechnology, 2, 1–8.

Al-Zubairi, A., Ismail, P., Pei, C.P., Wahab, S.I.A., Rahmat, A., 2007. Biochemical effect of sub-chronic administration of *Catha edulis* (Khat) crude extract in rat. Research Journal of Pharmacology 1, 84–90.

Amanlou, M., Nabati, F., Azizian, H., Farsam, H., 2008. Assessment of fluoride content and daily intake from different brands of tea bags in Iran. Research in Pharmaceutical Sciences 3, 55–59.

Amic, D., Davidovic-Amic, D., Beslo, D., Trinajstic, N., 2003. Structure-radical scavenging ctivity relationship of flavonoids. Croatia or Croatica ?? Chemica Acta 76, 55–61.

Anastassiades, M., Lehotay, S., 2003. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. The Journal of AOAC International 86, 412–431.

Anke, M.K., 2004. Transfer of macro, trace and ultratrace elements in the food chain. In: elements and their compounds in the environment: occurrence, analysis and biological relevance, Marian, E., Anke, A., Ihnat, M., Stoeppler, M. (Eds). Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, pp 1499–1522.

Antoine, L.M., Simon, C., Pizzi, A., 2004. UV Spectrophotometric method for polyphenolic tannin analysis. Journal of Applied Polymer Science 91, 2729–2732.

Aruoma, O.I., 2002. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. Mutation Research 523, 9–20.

Ashri, N., Gazi, M., 1990. More unusual pigmentations of the Gingival. Oral Surgery Oral Medicine and Oral Pathology 70, 445–449.

Asquith, T.N., Butler, L.G., 1986. Interactions of condensed tannins with selected proteins. Phytochemistry 25, 1591–1593.

Atengo, A.E., Zauyah, S., Hanafi, M.M., Rosenani, A.B., 2006. Genesis and classification of sesquioxidic soils from volcanic rocks in sub-humid tropical highlands of Ethiopia. Geoderma 136, 682–695.

Attef, O.A., Ali, A.A., Ali, H.M., 1997. Effect of khat chewing on bioavailability of ampicillin and amoxicillin. Journal Antimicrobial Chemotherapy 39, 523–525.

Awode, U.A., Uzairu, A., Balarabe, M.L., Okunola, O.J., Adewusi, S.G., 2008. Levels of some trace metals in the fadama soils and pepper (*Capsicum annuum*) along the bank of river Challawa, Nigeria. Asian Journal of Scientific Research 1, 458–463.

Ayoob, S., Gupta, A.K. 2006. Fluoride in drinking water: A review on the status and stress effects. Critical Reviews in Environmental Science and Technology 36, 433–487.

Badarinath, A.V., RAo, K.M., Chetty, C.M.S., Ramkanth, S., Rajan, T.V.S., Gnanaprakash, K., 2010. A review on in-vitro antioxidant methods: comparisons, correlations and considerations. International Journal of PharmTech Research 2, 1276–1285.

Bajaj, L.L., Devsharma, A.K., 1977. A colorometric method for the determination of tannins in tea. Mikrochimica acta [Wein] 2, 149–253.

Balint, G., Ghebrekidan, H., Balint, E., 1991. Catha edulis, an international sociomedical problem with considerable pharmacological implications. East African Medical Journal 68, 555–561.

Bamidele Sanni, S., 1982. The fluoride contents of common Nigerian vegetables. Journal of the Science of Food and Agriculture 33, 686–687.

Banjaw, M., Schmidt, W., 2005. Behavioural sensitisation following repeated intermittent oral administration of Catha edulis in rats. Behavioural Brain Research 156, 181–189.

Barghouthi, Z., Amereih, S., 2012. Spectrophotometric determination of fluoride in drinking water using aluminum complexes of triphenylmethane dyes. Water SA 38, 543–548.

Barker, S.A., 2000. Applications of matrix solid-phase dispersion in food analysis. Journal of Chromatography A 880, 63–68.

Barker, S.A., Long A.R., Short, C.R., 1989. Isolation of drug residues from tissues by solid-phase dispersion. Journal of Chromatography A 475, 353–361.

Barker, S.A., 2007. Matrix solid phase dispersion (MSPD. Journal of Biochemistry and Biophysics Methods 70, 151–162.

Begerow, J., Gerd, C., Ewers, U., Finck, M., 2004. Standards and regulations regarding metals and their compounds in environmental materials, drinkingwater, food, feeding-stuff, consumer products, and other materials. In: Elements and their compounds in the environment: occurrence, analysis and biological relevance, Marian, E., Anke, A., Ihnat, M., Stoeppler, M. (Eds). Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Belew, M., Kebede, D., Kassaye, M., Enquoselassie, F., 2000. The magnitude of khat use and its association with health, nutrition and socio-economic status. Ethiopian Medical Journal 38, 11–26.

Beyene, D., 1988. Soil fertility research on some ethiopian vertisols. In: management of vertisols in sub-Saharan Africa, Jutzi, S.C., Haque, I., McIntire, J., Stares, J.E.S. (Eds.). International Livestock Centre for Africa, Addis Ababa, Ethiopia, pp 223–231.

Bichel, J., Batch, A., 1967. Investigations on the toxicity of small chronic doses of tannic acid with special reference to possible carcinogenicity. Acta Pharmacologica et Toxicologica 26, 41–45.

Brenneisen, R., Geisshiisler, S., 1985. Phenylpentenylamines from *Catha edulis*. Journal of Natural Products 50, 1188–1195.

Campbell, A.D., 1987. Determination of fluoride in various matrices. Pure and Applied Chemistry 59, 695–702.

Capriotti, A.L., Cavaliere, C., Giansanti, P., Gubbiotti, R., Samperi, R., Aldo-Lagana, A., 2010. Recent developments in matrix solid-phase dispersion extraction. Journal of Chromatography A 1217, 2521–2532.

Chandravanshi, B.S., 2011. Monitoring the country's health. In: Coles, P. (Ed). Chemistry and life. The UNESCO Courier, January-March, p 21. I am very much surprised to see this Ref. ??

Chappell, J.S., Lee, M.M., 2010. Cathinone preservation in khat evidence via drying. Forensic Science International 195, 108–120.

Chu, X-G., Hu, X-Z., Yao, H-Y., 2005. Determination of 266 pesticide residues in apple juice by matrix solid-phase dispersion and gas chromatography–mass selective detection. Journal of Chromatography A 1063, 201–210.

Chung, K.T., Wei, C.I., Johnson, M.G., 1998b. Are tannins a double-edged sword in biology and health? Trends in Food Science and Toxicology 9, 168–175.

Chung, K.T., Wong, T.Y., Wei, C.I., Huang, Y.W., Lin, Y., 1998a. Tannins and human health: A review. Critical Reviews in Food Science and Nutrition 38, 421–464.

Colzato, L.S., Ruiz, M.J., Van den Wildenberg, W.P.M., Hommel, B., 2011. Khat use is associated with impaired working memory and cognitive flexibility. PLoS One 6, e20602.

Connett, P., Mullenix, P.J., Schatz, A., Foulkes, R., Hirzy, J.W., Burgstahler, A., Hill, D.R., Susheela, A.K., 2010. Fluoride's Neurological Effects: studies show there may be grave implications for Alzheimers, Dementia, Attention Deficit Disorder, reduced IQ in children. http://www.fluoridation.com/brain.htm. Accessed on February 19, 2010.

Cormack, P.A.G., Elorza, A.Z., 2004. Molecularly imprinted polymers: synthesis and characterization. Journal of Chromatography B 804, 173–180.

Cox, G., Rampes, H., 2003. Adverse effect of khat: a review. Advances in psychiatric treatment 9, 456–463.

Craddock, V.M., 1993. Cancer of the oesophagus: approaches to the etiology. UK, Cambridge, Cambridge University Press.

Crozier, A., Jaganath, I.B., Clifford, M.N., 2006. Phenols, polyphenols and tannins. In plant secondary metabolites occurrence, structure and role in the human diet; CroZier, A., Clifford, M.N., Ashihara, H., (Eds)., Blackwell, Victoria, Australia, p 386.

Dagne E., Adugna, Y., Kebede, E., Atilaw, Y., 2010. Determination of levels of cathine in khat (*Catha edulis*) leaves and its detection in urine of khat chewers: a preliminary report Ethiopian e-Journal for Research and Innovative Foresight 2, 7–22.

Dai, J., Mumper, R.J., 2010. Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. Molecules 15, 7313–7352.

Dawson, B.A., Black, D.B., Lavoie, A., LeBelle, M.J., 1994. Nuclear magnetic resonance identification of the phenylalkylamine alkaloids of khat using a chiral solvating agent. Journal of Forensic Science 39, 1026-1031.

Delgado-Zamarreno, M.M., Perez-Martın, L., Bustamante-Rangel, M., Carabias-Martınez, R., 2012. A modified QuEChERS method as sample treatment before the determination of isoflavones in foods by ultra-performance liquid chromatography–triple quadrupole mass spectrometry, Talanta in press.

DePaola P.F., Kashket, S., 1980. Prevention of dental caries. In: Fluorides, effects on vegetation, animals and humans. Salt Lake City: Paragon Press 199–211.

Dessie, G., Kinlund, P., 2008. 'Khat expansion and forest decline in Wondo Genet, Ethiopia'. Geografiska Annaler: Series B, Human Geography 90, 187–203.

Dhaifalah, I., Santavy, J. 2004. Khat habit and its health effect. a natural amphetamine. Biomedical Paper 148, 11–15.

Dietz, C., Sanz, J., Sanz, E., Mu˜noz-Olivas, R., C´amara, C., 2007. Current perspectives in analyte extraction strategiesfor tin and arsenic speciation. Journal of Chromatography A 1153, 114–129.

Dong, X., Wang, W., Ma, S., Sun, H., Li, Y., Guo, J., 2005. Molecularly imprinted solid-phase extraction of (−)-ephedrine from Chinese Ephedra. Journal of Chromatography A 1070, 125–130.

Dudai, N., Fischer, R., Segev, D., Chaimovitish, D., Rosenzweigh, N., Shimoni, M., 2008. Antioxidant activity of khat (*Catha edulis* Forsk). Acta Horticulturae 778, 85–92.

Dushenkov, V., Kumar, P.B., Motto, A.N., Raskin, H., 1995. The use of plants to remove heavy metals from aqueous streams. Environmental Science and Technology 29, 1239–1245.

Dwyer, J.T., 1994. Vegetarian eating patterns: science, values, and food choices where do we go from here? American Journal of Clinical Nutrition 59, 1255S–1265S.

Editorial, 2010. Introduction to the special issue: The changing use and misuse of khat (*Catha edulis*)—Tradition, trade and tragedy. Journal of Ethnopharmacology 132, 537–539.

Edmunds, W.M., Smedley, P.L., 1996. Groundwater geochemistry and health: an overview. Environmental Geochemistry and Health. Geological Society Special Publication 113, 91–105.

El-Domiaty, M.M., Elhag, H.M., El-Feraly, F.S., Al-Meshal, I.A., El-Olemy, M.M., 1994. Studies on (-)-cathinone formation in micro propagated plants and tissue cultures of *Catha edulis* (Khat). International Journal of Pharmacology 32, 135–141.

Elmi, A.S., Ahmed, Y.H., Samatar, M.S., 1987. Experience in the control of khat-chewing in Somalia. Bulletin on Nacrotics 39, 51–57.

Elrashidi, M.A., Lindsay, W.L., 1985. Solubility relationships of fluorine minerals in soils. Soil Science Society of American Journal 49, 1133−1136.

Erturk, Y., Ercisli, S., Sengul, M., Eser, Z., Haznedar, A., Turan, M., 2010. Seasonal variation of total phenolic, antioxidant activity and minerals in fresh tea shoots (*Camellia Sinensis*). Pakistan Journal of Pharmaceutical Science 23, 69–74.

FAO/IAEA., 2000. Quantification of Tannins in Tree Foliage- A laboratory manual, FAO/IAEA Working Document; IAEA, Australia, Vienna.

Fernandes, J.O., Soares, C., 2007. Application of matrix solid-phase dispersion in the determination of acryl amide in potato chips. Journal of Chromatography A. 1175, 1–6.

Feyissa, A.M, Kelly, J.P., 2008. A review of the neuropharmacological properties of khat. Progress in Neuro-Psychopharmacology & Biological Psychiatry 32, 1147–1166.

Fitzgerald, J., 2009. Khat: a literature review**.** Louise Lawrence Pty Ltd. http://www.ceh.org.au/downloads/Khat_report_FINAL.pdf, accessed on 10/11/ **2012.**

Franzle, S., 2010. Chemical elements in plants: Parameters controlling essentiality. Springer, New York. p 200.

Fung, K.F., Zhang, Z.Q., Wong, J.W.C., Wong. M.H., 1999. Fluoride contents in tea and soil from tea plantations and the release of fluoride into tea liquor during infusion. Environmental Pollution 104, 197−205.

Gambaro, V., Arnoldi, S., Colombo, M.L., Dell'Acqua, L., Guerrini, K., Roda, G., 2012. Determination of the active principles of *Catha Edulis*: Quali–quantitative analysis of cathinone, cathine, and phenylpropanolamine. Forensic Science International 217, 87–92.

García-López, M., Canosa, P., Rodríguez, I., 2008. Trends and recent applications of matrix solid-phase dispersion. Analytical Bioanalytical Chemistry 391. 963–974.

Geisshusler, S., Brenneisen, R., 1987. The content of psychoactive phenylpropyl and phenylpentenyl khatamines in *Catha edulis* Forsk of different origin. Journal of Ethnopharmacology 19, 269–277.

Getahun, A., Krikorian, A.D., 1973. Chat: coffee's rival from Harar, Ethiopia. Botany, cultivation and use. Economic Botany 27, 353–377.

Getahun, W., Gedif, T., Tesfaye, F., 2010. Regular khat (*Catha edulis*) chewing is associatedwith elevated diastolic blood pressure among adults in Butajira, Ethiopia: A comparative study. BMC Public Health 10, 390–397.

Griffiths, P., Gossop, M., Wickenden, S., Dunworth, J., Harris, K., Lloyd, C., 1997. A transcultural pattern of drug use: qat (khat) in the UK. British Journal of Psychiatry 170, 281–284.

Grotewold, E., 2006. The Science of Flavonoids. Springer, New York, USA.

Grusak, M.A., Penna, D.D., 1999. The nutrient composition of plants to enhance human nutrition and health. Annual Review of Plant Physiology and Plant Molecular Biology 50, 133–161.

Guo, L., Guan, M., Zhao, C., Zhang, H., 2008. Molecularly imprinted matrix solid-phase dispersion for extraction of chloramphenicol in fish tissues coupled with high-performance liquid chromatography determination. Analytical Bioanalytical Chemistry 392, 1431–1438.

Guven, D.E., Akinci, G. 2011. Comparison of acid digestion techniques to determine heavy metals in sediment and soil samples. Gazi University Journal of Science 24, 29-34

Hagerman, A.E., Robbins, C.T., 1987. Implications of soluble tannin-protein complexes for tannin analysis and plant defense mechanisms. Journal of Chemical Ecology 13, 1243-1259.

Hagerman, A.E., Butler, L.G., 1978. Protein precipitation method for the quantitative determination of tannins. Journal of Agricultural and Food Chemistry 26, 809–812.

Hailu, KLawoyin, D.O., Woods, D., Bailey, J.R., 2006. Khat chewing and dental staining. http://priory.com/den/khateeth.htm, Accessed on 01/ 28/ 2010.

Halbach, H., 1972. Medical aspect of chewing khat. Bulletin of the World Health Organization 47, 21–29.

Haldinmann, M., Zimmerli, B., 1993. Evaluation of ashing procedures for the gas chromatographic determination of fluoride in biological material. Analytica Chemica Acta 282, 589–601.

Halliwell, B., 1996. Antioxidants in human health and disease. Annual Reviews in Nutrition 16, 33–49.

Halliwell, B., Murcia, M. A., Chirico, S., Aruoma, O. I., 1995. Free radicals and antioxidants in food and in vivo: what they do and how they work. Critical Reviews in Food Science and Nutrition 35, 7–20.

Häni, H., 1978. Interactions by fluoride with a mineral soil containing illite and alterations of maize plants grown in this soil. Fluoride 11, 18−24.

Harborne, J.B., Williams, C.A., 2000. Advances in flavonoid research since 1992. Phytochemistry 55, 481–504.

Hart, A.D., Oboh, C.A., Barimalaa, I.S., Sokari, T.G., 2005. Concentrations of trace metals (Pb, Fe, Cu, and Zn) in crops harvested in some oil processing locations in river state, Nigeria. African Journal of Nutritional Science 5, 1–21.

Harun, N., Anderson, R.A., Cormack, P.A.G., 2010. Molecularly imprinted solid-phase extraction (MISPE) and liquid chromatography - tandem mass spectrometry (LC-MS/MS) analysis for ketamine and norketamine determination in hair samples. Analytical Bioanalytical Chemistry 396, 2449–2459.

Hassan, N.A.G.M., Gunaid, A.A., El Khally, F.M.Y., Murray-Lyon, I.M., 2002. The subjective effects of chewing qat leaves in human volunteers. Annals of Saudi Medicine, 22, 34–37.

Hassan, N.A.G.M., Gunaid, A.A., Murray-Lyon, I.M., 2007. Khat (*Catha edulis*): health aspects of khat chewing. Eastern Medeteranian Health Journal, 13, 706–718.

Hattab, F.N., Angmar-Mansson, B. 2000. Fluoride content in khat (*Catha edulis*) chewing leaves. Archives of Oral Biology 45, 253–255.

He, L., Su, Y., Shen, X., Zheng, Y., Guo, H., Zeng, Z., 2009. Solid-phase extraction of melamine from aqueous samples using water-compatible molecularly imprinted polymers. Journal of Separation Science 32, 3310–3318.

Heinecke, J.W., 1997. Mechanisms of oxidative damage of low density lipoprotein in human atherosclerosis. Current Opinions in Lipidology 8, 268–274.

Hem, J.D., 1989. Study and Interpretation of the chemical characteristics of natural water: Water Supply Paper 2254. 3rd ed. US Geological Survey, Washington, D.C.

Herderich, M.J., Smith, P.A., 2005. Analysis of grape and wine tannins:Methods, applications and challenges. Australian Journal of Grape and Wine Research 11, 205–214.

Hill, C.M., Gibson, A., 1987. The oral and dental effects of Q'at chewing. Oral Surgery, Oral Medicine and Oral Pathology 63, 433−436.

Hillman, D., Bolenbaugh, D. Convey, E.M., 1979. Fluorosis from phosphate mineral supplements in Michigan dairy cattle. Fluoride 12, 100−102.

Hoffman, R., Al'Absi, M., 2010. Khat use and neurobehavioral functions: Suggestions for future studies. Journal of Ethnopharmacology 132, 554–563.

http://www.herbs2000.com/h_menu/tannins.htm accessed on 17,11,2012

Hu, Q., Pan, G., Zhu, J., 2002. Effect of fertilization on selenium content of tea and the nutritional function of Se-enriched tea in rats. Plant and Soil 238, 91–95.

Ishibashi, Y., Matsuo, H., Baba, Y., Nagafuchi, Y., Imato T., Hirata, T., 2004. Association of manganese effluent with the application of fertilizer and manure on tea field. Water Research 38, 2821–2826.

Iskandar, I.K., Kirkham, M.B. (Eds.)., 2001. Trace elements in soil bioavailability, flux, and transfer, Lewis Publishers, New York, USA.

Kabata-Pendias, A. 2001. Trace Elements in Soils and Plants, third ed. Boca Raton, Florida: CRC Press.

Kabata-Pendias, A., 2004. Soil plant transfer of trace elements-an environmental issue. Geoderma 122, 143–149.

Kalix, P., 1990. Pharmacological properties of the stimulant khat. Pharmacological Therapy 48, 397–416.

Kalix, P., 1996. *Catha edulis*, a plant that has amphetamine effects. Pharmacy World & Science 18, 69–73.

Kalix, P., Braenden, O., 1985. Pharmacological aspect of the chewing of khat leaves. Pharmacological Reviews 37,149–164.

Karlsson, S. Reducing farming household vulnerability in connection to khat cultivation; M.Sc. Thesis, Swedish University of Agricultural Sciences Department of Urban and Rural Development Environmental Communication, Oppsala, 2006; pp 67.

Karunamoorthi, K., Udeani, G., Babu, S.M., Mossie, A., 2010. Psychopharmacosocial Aspects of *Catha edulis* Forsk (Fam. Celastraceae). African Journal of Pharmaceutical Sciences and Pharmacy 1, 112–129.

Kassie, F., Darroudi, F., Kundi, M., Schulte-Hermann, R., Knasmuller, S., 2001. Khat (*Catha Edulis*) consumption causes genotoxic effects in humans. International Journal of Cancer 92, 329–332.

Kebede, Y., 2002. Cigarette smoking and khat chewing among university instructors in Ethiopia. East African Medical Journal 79, 274–278.

Kennedy, J.G., Teague, J., Rokaw, W., Cooney, E., 1983. A medical evaluation of the use of qat in North Yemen. Social Science and Medicine 17, 783–793.

Khairiah, J., Ding-Woei, Y., Habibah, J., Ahmad-Mahir, R., Aminah, A., Ismail, B.S., 2009. Concentration of heavy metals in guava plant parts and soil in the sungai

wangi plantation, Perak, Malaysia. International Journal of Agricultural Research 4, 310–316.

Khan, A., Pereira, L., Aspey, S., Bunn, R. Lewis, R., 2009. QuEChERS Dispersive Solid Phase Extraction for the GC/MS Analysis of Pesticides in Grapes. Application note: ANGSC PESTGRAPES 0709, 2009, Thermo Fisher Scientific, Runcorn, UK.

Khanbabaee, K., Ree, T., 2001. Tannins: classification and definition. Natural Products Research 18, 641–649.

Kim, H., Spivak, D.A., 2003. New insight into modeling non-covalently imprinted polymers. Journal of American Chemical Society 125, 11269–11275.

Kim, J-M., Ahn, K-D., Strikovsky, A.G., Wulff, G., 2001. Polymer catalysts by molecular imprinting: A labile covalent bonding approach. Molecular imprinted polymer catalysts. Bulletin of the Korean Chemical Society 22, 689–697.

Kinsella, B., O'Mahony, J., Malone, E., Moloney, M., Cantwell, H., Furey, A., Danaher, M., 2009. Current trends in sample preparation for growth promoter and veterinary drug residue analysis. Journal of Chromtography A 1216, 7977–8015.

Kirby, K.S., 1960. Induction of tumours by tannin extracts. British Journal of Cancer 14, 147–150.

Kisser, K.I., 2005. Digestion of solid matrices, Part 1: Digestion with aqua regia report of evaluation study, NUA-Umweltalytik GmbH, Australia, pp1-38

Kite, G.C., Ismail, M., Simmonds, M.S., Houghton, P.J., 2003, Use of doubly protonated molecules in the analysis of cathedulins in crude extracts of khat (*Catha edulis*) by liquid chromatography/serial mass spectrometry. Rapid Communication in Mass Spectrometry 17, 1553–1564.

Kloos, H., Tekle Haimanot R., 1999. Distribution of fluoride and fluorosis in Ethiopia and prospects for control. Tropical Medicine and International Health 4, 355−364.

Korpassy, B., Mosony, M., 1950. The carcinogenic activity of tannic acid. Liver tumors induced in rats by prolonged subcutaneous administration of tannic acid solution. British Journal of Cancer 4, 411–420.

Kristenson, E.M., Ramons, L., Brinkman U.A.T., 2006. Recent advances in matrix solid-phase dispersion. Trends in Analytical Chemistry 2, 96–111.

Krizevski, R., Dudai, N., Bar, E., Dessow, I., Ravid, U., Lewinsohn, E., 2008. Quantitative stereoisomeric determination of phenylpropylamino alkaloids. Israel Journal of Plant Science 56, 207–213.

Krizevski. R., Dudai. N., Bar. E., Lewinsohn.E., 2007. Developmental patterns of phenylpropylamino alkaloids accumulation in khat (*Catha edulis*, Forsk.). Journal of Ethnopharmacology 114, 432–438.

Kubakaddi, A., Bharati, P., Kasturiba, B., 2005. Effect of fluoride rich food adjuncts and prevalence of fluorosis. Journal of Human Ecology 17, 43–45.

Lanza, F., Sellergren, B., 2001. The application of molecular imprinting technology to solid phase extraction. Chromatographia 53, 599–611.

Lasakova, M., Thiebaut, D., Jandera, P., Pichon, V., 2009. Molecularly imprinted polymers and their application in solid phase extraction. Journal of Separation Science 32, 1036–1042.

Laussmann, T., Meier-Giebing S., 2010. Forensic analysis of hallucinogenic mushrooms and khat (*Catha Edulis* Forsk.) using cation-exchange liquid chromatography. Forensic Science International, 195, 160–164.

Lebelle, M.J., Lauriault, G., Lavoie, A. 1993. Gas chromatographic-mass spectrometric identification of chiral derivatives of the alkaloids of khat. Forensic Science International. 61, 53–64.

Lee, M., 1995. The identification of cathinone in khat (*Catha edulis*): a time study. Journal of Forensic Sciences 40, 116–121.

Lee, S.W., Choi, J.H., Cho, S-K., Yu, H-A., El-Aty, A.M.A., Shim, J.H., 2011. Development of a new QuEChERS method based on dry ice for the determination of 168 pesticides in paprika using tandem mass spectrometry. Journal of Chromatography A 1218, 4366–4377.

Lehmann, T., Geisshiisler, S., Brenneisen, R., 1990. Rapid TLC identification test for khat (*Catha edulis*). Forensic Science International 45, 47–51.

Lehotay, S.J., Mastovska, K., Lightfield, A.R., 2005. Use of buffering and other means to improve results of problematic pesticides in a fast and easy method for residue analysis of fruits and vegetables. The Journal of AOAC International 88, 615–629.

Lehotay, S.J., 2007. Determination of pesticide residues in foods by acetonitrile extraction and partitioning with magnesium sulfate: collaborative study. The Journal of AOAC International 90, 485–520.

Lemessa, D., 2001. Khat (*Catha edulis*): Botany, distribution, cultivation, usage and economics in Ethiopia. United Nations Development Programme UNDP – Emergencies Unit for Ethiopia (EUE), pp 1–66.

Li, Z-Y., Zhang, Z-C., Zhou, Q-L., Gao, R-Y., Wang, Q-S., 2002. Fast and precise determination of phenthoate and its enantiomeric ratio in soil by the matrix solid-phase dispersion method and Liquid Chromatography. Journal of Chromatography A 977, 17–25.

Lizcano, L.J., Bakkali, F., Ruiz-Larrea, B., Ruiz-Sanz, J., 2010. Antioxidant activity and polyphenol content of aqueous extracts from Colombian Amazonian plants with medicinal use. Food Chemistry 119, 1566–1570.

Llaudy, M.C., Canals, R., Canals, J., Rozeas N., Arola, L., Zamora, F., 2004. New Method for Evaluating Astringency in Red Wine. Journal of Agricultural and Food Chemistry 52, 742–746.

Loftleidir, H., Celand, R., 2005. NJF Essential trace elements for plants, animals and humans, NJF (Nordic , Association of Agricultureal science) Seminar no. 370, Islands..

López, M.M.C., Perez, M.C.C., García, M.S.D., Vilarino, J.M.L., Rodriguez, M.V.G., Losada, L.F.B., 2012. Preparation, evaluation and characterization of quercetin-molecularly imprinted polymer for preconcentration and clean-up of catechins. Analytical Chimica Acta 721, 68–78.

Lu, Z., Liu, Y., Barreto, V., Pohl, C., Avdalovic, N., Joyce, R. Newton, B., 2002. Determination of anions at trace levels in power plant water samples by ion chromatography with electrolytic eluent generation and suppression. Journal of Chromatography A 956, 129–138.

Lubke, C., Lubke, M.,Whitcombe, M.J., Vulfson, E., 2000. Imprinted polymers prepared with stoichiometric template–monomer complexes: efficient binding of ampicillin from aqueous solutions. Macromolecules 33, 5098–5105.

Lukandu, O.M., Costea, D.E., Neppelberg, E., Johannessen, A.C., Vintermyr, O.K., 2008. Khat (*Catha edulis*) induces reactive oxygen species and apoptosis in normal human oral keratinocytes and fibroblasts. Toxicological Sciences 103, 311–324.

Luthria, D.L., 2006. Perspective significance of sample preparation in developing analytical methodologies for accurate estimation of bioactive compounds in functional foods. Journal of the Science of Food and Agriculture 86, 2266–2272.

Makkar, H.P.S., Siddhuraju, P., Backer, K., 2007. Plant secondary metabolities. Humana press, Totowa, New Jersey, pp 1–251.

Malde, M.K., Bjorvatn, K., Julshamn. K., 2001. Determination of fluoride in food by the use of alkali fusion and fluoride ion-selective electrode. Food Chemistry, 73, 373–379.

Malde, M.K., Maage, A., Macha, E., Julshamn, K., Bjorvatn, K. 1997. Fluoride content in selected food items from five areas in East Africa. Journal of Food Composition and Analysis 10, 233–245.

Manach, C., Scalbert, A., Morand, C., Remesy, C., Jime'nez, L. 2004. Polyphenols: Food sources and bioavailability. American Journal of Clinical Nutrition, 79, 727–747.

Marschner, H., 1995. Mineral Nutrients of Higher Plants. 2nd Edn. Acadamic Press, London, pp 106–130.

Mathys, K., Brenneisen, R., 1993. HPLC and TLC profiles of phenylalkylamines of khat (*Catha edulis* Forsk.) Confiscated in Switzerland. Pharmaceutica Acta Helvetiae 68, 121–128.

Matloob, M.H., 2003. Determination of cadmium, lead, copper and zinc in Yemeni khat by anodic stripping voltammetry. Eastern Mediterranean Health Journal 9, 28–36.

Mayes, A.G., Whitcombe, M.J., 2005. Synthetic strategies for the generation of molecularly imprinted organic polymers. Advanced Drug Delivery Reviews 57, 1742–1778.

McLaughlin, M.J., Stevens, D.P., Keerthisinghe, D.G., Cayley, J.W.D., Ridley, A.M., 2001. Contamination of soil with fluoride by long-term application of superphosphates to pastures and risk to grazing animals. Australian Journal of Soil Research 39, 627–640.

Medical Research Council., 2002. Water fluoridation and health. Working group report. Accessed at: http://www.mrc.ac.uk/Utilities/Documentrecord/index.htm?d=MRC002482. Accessed: 20 October 2012.

Mengel, K., Kirkby, E. A., 1978. Principles of plant nutrition, Vol 11, International Potash Institute, Berne, Switzerland.

Miller, G.W., Shupe, J.L., Vedina, O.T., 1999. Accumulation of fluoride in plants exposed to geothermal and industrial water. Fluoride 32, 74–83.

Molyneux, P., 2004. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Journal of Science and Technology 26, 211-219.

Müller, J., Heindl, A., 2006. Drying of medicinal plants. 2006. Medicinal and Aromatic Plants 237-252, http://edepot.wur.nl/137179. Accessed on 10, September, 2012.

Mullett, W.M., 2000. Investigation of selective binding interactions for analytical separations and determination of pharmaceuticals and toxins. Doctoral Thesis, Ottawa, Ontario, Canada.

National Research Council, 1989. Recommended Dietary Allowance. 10th Edn., National Academy Press, Washington, DC.

Nemade, P.D., Vasudeva Rao, A., Alappat, B.J., 2002. Removal of fluorides from water using low cost adsorbents. Water Science and Technology: Water Supply 2, 311–317.

Nencini, P., Amiconi, G., Befani, O., Abdullahi, M.A., Anania, M.C., 1984. Possible involvement of amine oxidase inhibition in the sympathetic activation by khat (*Catha edulis*) chewing in humans. Journal of Ethnopharmacology 11, 78–86.

Nencini, P., Grassi, M.C., Botan, A.A., Asseyr, A.F., Paroli, E., 1989. Khat consumption: a pharmacological review. Drug and Alcohol Dependence 23, 255–258.

Nieuwenhuize, J., Poley-vos, C.H. 1991. Comparison of microwave and conventional extraction techniques for the determination of metals in soil, sediments and sludge samples by Atomic spectrometry. Analyst 116,347-351

Nouri, J., Khorasani, N., Lorestani, B., Karami, M., Hassani, A.H., Yousefi, Y., 2009. Accumulation of heavy metals in soil and uptake by plant species with phytoremediation potential. Environmental Earth Science 59, 315–323.

Numan, N., 2004. Exploration of adverse psychological symptoms in Yemeni khat users by the Symptom Checklist-90 (SCL-90). Addiction 99, 61–65.

Nwachukwu, O.I., 2008. Contaminant source as factor of soil heavy metal toxicity and bioavailability to plants. Environmental Research Journal 2, 322–326.

O'Mahony, J., Molinelli, A., Nolan, K., Smyth, M.R., Mizaikoff, B., 2005. Towards the rational development of molecularly imprinted polymers: ^{1}H NMR studies on hydrophobicity and ion-pair interactions as driving forces for selectivity. Biosensors and Bioelectronics 20, 1884–1893.

O'Brien, T.B., 2005. Insight into the Selectivity of molecular imprinted polymers. PhD Dissertation, Seton Hall University, Chemistry department, South Orange, New Jersey, USA.

Odenwald, M., 2007. Chronic khat use and psychotic disorders: A review of the literature and future prospects. Sucht 53, 9–22.

Odenwald, M., Neuner, F., Schauer, M., Elbert, T., Catani, C., Lingenfelter, B., Hinkel, H., Hafner, H., Rockstroh, B., 2005. Khat use as risk factor for psychotic disorders: a cross-sectional and case-control study in Somalia. BMC Medicine 3, 5–15.

Ophaug R.H., Singer, L., Harland, B.F., 1985. Dietary fluoride intake of 6-month and 2-year-old children in four dietary regions of the United States. American Journal of Clinical Nutrition 42, 701–707.

Padda, M. S., Picha, D.H., 2007. Methodology optimization for quantification of total phenolics and individual phenolic acids in sweetpotato (*Ipomoea batatas* L.) Roots. Journal of Food Science 72, C412–C416.

Pansu, M., Gautheyrou, J., 2006. Handbook of Soil Analysis Mineralogical, Organic and Inorganic Methods, Springer-Verlag Berlin Heidelberg, The Netherlands, pp 1–995.

Paris, M.R., Moyse, H., 1958. Abyssinian tea (*Catha edulis* Forsk., *Celastraceae*). Bulletin on Narcotics 10, 29–34.

Payá P, Anastassiades M, Mack D, Sigalova I, Tasdelen B, Oliva J., 2007. Analysis of pesticide residues using the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) pesticide multiresidue method in combination with gas and liquid

chromatography and tandem mass spectrometric detection. Analytical Bioanalytical Chemistry 389, 1697–1714.

Pérez-Burgos, R., Grzelak, E.M., Gokce, G., Saurina, J., Barbosa, J., Barrón, D., 2012. Quechers methodologies as an alternative to solid phase extraction (SPE) for the determination and characterization of residues of cephalosporins in beef muscle using LC–MS/MS. Journal of Chromatography B 899, 57– 65.

Pichon, V., 2007. Selective sample treatment using molecularly imprinted polymers. Journal of Chromatography A 1152, 41–53.

Pichon, V., Chapuis-Hugon, F., 2008. Role of molecularly imprinted polymers for selective determination of environmental pollutants—a review. Analytical Chimica Acta 622, 48–61.

Pires, M.A.F., Dantas, E.S.K., Munita, C.S., 1996. Fluoride content of some teas consumed in Sao Paulo. Fluoride 29, 144−146.

Porębska, G., Ostrowska, A., 1999. Heavy metal accumulation in wild plants: implications for phytoremediation. Polish Journal of Environmental Studies 8, 1999, 433–442.

Prabu, P.C., 2009. Impact of heavy metal concentrations of Akaki river of Ethiopia on soil and metal toxicity on cultivated vegetable crop. Electronic Journal of Environmental, Agricultural and Food Chemistry 8, 818–827.

Qian, J-Y., Liu, D., Huang, A-G., 2004. The efficiency of flavonoids in polar extracts of Lycium chinense Mill fruits as free radical scavenger. Food Chemistry 87, 283–288.

Qiao, F., Sun, H., Hongyuan, Y. Row, K.H., 2006**.** Molecularly imprinted polymers for solid phase extraction. Chromatographia 64, 625–634.

Ramadan, M.A.E., Al-Ashkar, E.A., 2007. The effect of different fertilizers on the heavy metals in soil and tomato plant. Australin Journal of Basic and Applied Science 1, 300–306.

Rao, Nagendra, C.R., 2003. “Fluoride and environment- a review” in Bunch, M.J., Madha, V., Kumaran, T.V., (Eds.), Proceedings of the third international conference on environment and health, Chennai, India, pp 386–399.

Record, I.R., Dreosti, I.E., McInerney, J.K., 2001. Changes in plasma antioxidant status following consumption of diets high or low in fruit and vegetables or following dietary supplements with an antioxidant mixture. British Journal of Nutrition 85, 459–464.

Reda, A.A., Moges, A., Biadgilign, S., Wondmagegn, B.Y., 2012. Prevalence and determinants of khat (*Catha edulis*) chewing among high school students in Eastern Ethiopia: A cross-sectional study. PLoS ONE 7, 1–5.

Rice-Evans, C., Miller, N., Paganga, G., 1996. Structure–antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine 20, 933–956.

Rice-Evans, C., Miller, N.J., Bolwell, P.G., Bramley, P.M. Pridham, J.B., 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radical Research 22, 375–383.

Rickman, J.C., Barrett, D.M., Barrett, D.M., 2007. Nutritional comparison of fresh, frozen and canned fruits and vegetables. Part 1. Vitamins C and B and phenolic compounds. Journal of the Science of Food and Agriculture 87, 930–944.

Ripani, L., Schiavone, S., Garofano, L., 1996. GC/MS identification of Catha edulis stimulant-active principles. Forensic Science International 78, 39–46.

Robards, K., 2003. Strategies for the determination of bioactive phenols in plants, fruit and vegetables. Journal of Chromatography A 1000, 657–691.

Robards, K., Prenzler, P.D., Tucker, G., Swatsitang, P., Glover, W., 1999. Phenolic compounds and their role in oxidative processes in fruits. Food Chemistry 66, 401–436.

Romero-González, R., Frenich, A.G., Vidal, J.L.M., Prestes, O.D., Grio, S.L., 2011. Simultaneous determination of pesticides, biopesticides and mycotoxins in organic products applying a quick, easy, cheap, effective, rugged and safe extraction procedure and ultra-high performance liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 1218, 1477–1485.

Sass-Kiss, A., Kiss, J., Milotay, P., Kerek, M. M. and Toth-Markus, M., 2005. Differences in anthocyanin and carotenoid content of fruits and vegetables. Food Research International 38, 1023–1029.

Satong-aun, W., Assawarachan, R., Noomhorm, A., 2011. The influence of drying temperature and extraction methods on α-mangostin in mangosteen pericarp. Journal of Food Science and Engineering 1, 85–92.

Schechter, M.D., 1990. Dopaminergic nature of acute cathine tolerance. Pharmacology Biochemistry and Behavior 36, 817–820.

Selassie, S.G., Gebre, A., 1996. Rapid assessment of drug abuse in Ethiopia. Bulletin on Narcotics 48, 53–63.

Shi, X., Jin, F., Huang, Y., Du, X., Li, C., Wang, M., Shao, H., Jin, M., Wang, J., 2012. Simultaneous determination of five plant growth regulators in fruits by modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction and liquid chromatography-tandem mass spectrometry. Journal of Agricultural Food Chemistry 60, 60−65.

Shi, X., Li, X., Liu, J., Zhou, H., Zhang, H., Jin, Y., 2010. Lignan extraction from the roots of sinopodophyllum emodi wall by matrix solid-phase dispersion. Chromatographia 72, 713–717.

Simon R. 2005. Molecular recognition and its underlying mechanisms in molecularly imprinted polymers. Doctoral thesis, department of Chemistry, Louisiana State University.

Singh, V., Gupta, M.K., Rajwanshi, P., Mishra, S., Srivastava, S., Srivastava, R., Srivastava, M.M., Prakash, S., Dass, S., 1995. Plant uptake of fluoride in irrigation water by Ladyfinger (*Abelmorchus esculentus*). Food Chemistry and Toxicology 33, 399–402.

Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in Enzymology 299, 152–178.

Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. American Journal of Enology and Viticulture 16, 144–158.

Sirhan, A.Y., Tan, G.H., Wong, R.C.S., 2011. Method validation in the determination of aflatoxins in noodle samples using the QuEChERS method (Quick, Easy, Cheap, Effective, Rugged and Safe) and high performance liquid chromatography coupled to a fluorescence detector (HPLC-FLD). Food Control 22, 1807–1813.

Smith, M.L., Vorce, S.P., Holler, J.M., Shimomura, E., Magluilo, J., Jacobs, A., Huestis, M., 2007. Modern instrumental methods in forensic toxicology. Journal of Analytical toxicology 31, 257–251.

Solomon, D., Lehmann, J., Tekalign, M., Fritzsche, F., Zech, W., 2001. Sulfur fractions in particle-size separates of the sub-humid Ethiopian highlands as influenced by land use changes. Geoderma, 102, 41–59.

Spigno, G., Tramelli, L., De Faveri, D.M., 2007. Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. Journal of Food Engineering 81, 200–208.

Stevens, D.P., McLaughlin, M.J., Alston, A.M., 1995. Limitation of acid digestion techniques for determination of fluoride in plant material. Communications in Soil Science and Plant analysis 26, 1823–1842.

Sudjaroen, Y., 2009. Plant-derived phenolic antioxidants and cancer prevention. Thai Cancer Journal 29, 126–134.

Szendrei, K., 1980. Chemistry of khat. Bulletin on Narcotics 32, 5–35.

Tan, K.H., 1996. Soil Sampling Preparation and Analysis. Marcel Dekker, New York.

Tesfaye, F., Byass, P., Wall, S., Birhane, Y., Bonita, R., 2008. Association of smoking and khat (*Catha edulis* Forsk) use with high blood pressure among adults in Addis Ababa, Ethiopia, Preventing Chronic Disease 5, 1–11.

Thaiponga, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L., Hawkins Byrne, D., 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. Journal of Food Composition and Analysis. 19, 669–675.

Theodoridis, G., Kantifesa, A., Manesiotis, P., Raikos, N., Tsoukali-Papadopoulou, H., 2003. Preparation of a molecularly imprinted polymer for the solid-phase extraction of scopolamine with hyoscyamine as a dummy template molecule. Journal of Chromatography A 987, 103–109.

Thieme, D., Sachs, H., 2003. Improved screening capabilities in forensic toxicology by application of liquid chromatography–tandem mass spectrometry. Analytica Chimica Acta 492, 171–186.

Toennes, S.W., Harder, S., Schramm, M., Niess, C., Kauert, G.F., 2003. Pharmacokinetics of cathinone, cathine and norephedrine after the chewing of khat leaves. British Journal of Clinical Pharmacology, 56, 125–130.

Tomasini, D., Sampaio, M.R.F., Caldas, S.S., Buffon, J.G., Duarte, F.A., Primel, E.G., 2012. Simultaneous determination of pesticides and 5-hydroxymethylfurfural in honey by the modified QuEChERS method and liquid chromatography coupled to tandem mass spectrometry. Talanta 99, 380–386.

Vermerris, W., Nicholson, R., 2006. Phenolic Compound Biochemistry. Springer, Dordrecht, The Netherlands, pp 1–285.

Vinokur, Y., Levi, A., Feygenberg, O., Rodov, V., 2008. Hydrophilic and lipophilic antioxidant capacity and content of phenolic compounds in fresh khat leaves (*Catha edulis* Forsk). Ethnobotanical Leaflets 12, 557-64.

Walsh, R., 2010. Development and characterization of molecularly imprinted suspension polymers. Doctoral Thesis, Waterford Institute of Technology, Ireland.

Wang, A.S., Angle, J.S., Chaney, R.L., Delorme, T.A. Reeves, R.D., 2006. Soil pH effects on uptake of Cd and Zn by *Thlaspi caerulescens*. Plant and Soil 281, 325–337.

Wang, Y., Cai, C., Xiao, L., 2011. Determination of phenylureas herbicides in foodstuffs based on matrix solid-phase dispersion extraction and RP-LC with UV Detection. Chromatographia 73, 403–406.

Warfa, N., Klein, A., Bhui, K., Leavey, G., Craig, T., Stansfeld, S., 2007. Khat use and mental illness: a critical review. Social Science and Medicine 65, 309–318.

WHO (World Health Organization)., 2002. Environmental Health Criteria 227: Fluorides, World Health Organization, Geneva, http://www.inchem.org/documents/ehc/ehc/ehc227.htm#2.2

WHO (World Health Organization)., 2004. Fluoride in Drinking-water: Background document for development of WHO Guidelines for Drinking-water Quality. World Health Organization, Geneva. http://www.who.int/water_sanitation_health/dwq/chemicals/fluoride.pdf

WHO (World Health Organization)., 1984. Fluorine and fluorides: In environmental health criteria 36**,** Geneva. www.inchem.org/documents/ehc/ehc/ehc36.htm.

Widler, P., Mathys, K., Brenneisen, R., Kalix P., Fisch, H., 1994. Pharmacodynamics and pharmacokinetics of khat: a controlled study. Clinical Pharmacology and Therapeutics 55, 556–562.

Wille S.M., Lambert, W.E., 2007. Recent developments in extraction procedures relevant to analytical toxicology. Analytical Bioanalytical Chemistry 388, 1381–1391.

Williams, C.A., Grayer, R.J., 2004. Anthocyanins and other flavonoids. Natural Products Reports 21, 539–573.

Wollgast, J. Anklam, E., 2000. Review of polyphenols in *Theobroma cacao:* changes in composition during the manufacture of chocolate and methodology for identification and quantification. Food Research International 33, 423–447.

Wong, M.H., Fung, K.F., Carr, H.P., 2003. Aluminium and fluoride contents of tea, with emphasis on brick tea and their health implications. Toxicology Letters. 137, 111–120.

www.quechers.com, QuEChERS, 2012-11-10.

Xiang, G., Yang, L., Zhang, X., Yang, H., Ren, Z., Miao, M., 2009. Comparison of three methods of extraction for the determination of polyphenols and organic acids in tobacco by UPLC–MS–MS Chromatographia 70, 1007–1010.

Yadav, A.K., Kaushik, C.P., Haritash, A.K., Singh, B., Raghuvanshi, S.P., Kansal, A., 2007. Determination of exposure and probable ingestion of fluoride through tea, toothpaste, tobacco and pan masala. Journal of Hazardous Materials 142, 77−80.

Yan, H., Row, K.H., 2006. Characteristic and synthetic approach of molecularly imprinted polymer. International Journal of Molecular Science 7, 155–178.

Yang, B., Kotani, A., Arai, K., Kusu, F., 2001. Estimation of the antioxidant activities of flavonoids from their oxidation potentials. Analytical Sciences 17, 599–604.

Yang, T., Li, Y-H., Wei, S., Li, Y., Deng, A. 2008. Development of a selective molecularly imprinted polymer-based solid-phase extraction for indomethacin from water samples. Analytical Bioanalyitcal Chemistry 391, 2905–2914.

Yousef, G., Huq, Z., Lambert, T., 1995. Khat chewing as a cause of psychosis. British Journal of Hospital Medicine 54, 322–326.

Zein, Z.A., 1988. Polydrug abuse among Ethiopian university students with particular reference to khat (*Catha edulis*). Journal of Tropical Medicine and hygiene 91, 71–75.

Zhang, Z-S., Wang, L.J., Ozkan, N., Chen, X.D., Mao, Z-H., Yang, H-Z., 2007. Optimization of ethanol-water extraction of lignans from flaxseed. Journal of Separation and Purification Technology 57, 17–24.

Zhao, M., Wielen, F., Voogt, P., 1999. Optimization of a matrix solid phase dispersion method with sequential clean-up for the determination of alkylphenol ethoxylates in biological tissues. Journal of Chromatography A, 837, 129–138.

Ziakova, A., Brandsteterova, E., Blahova, E., 2003. Matrix solid-phase dispersion for the liquid chromatographic determination of phenolic acids in *Melissa officinalis.* Journal of Chromatography A 983, 271–275.

8. APPENDEX

A. List of publications fundamentally related to this thesis

1. **Atlabachew, M.,** Chandravanshi, B.S., Redi, M., **2010.** Concentration level of essential and non essential minerals in Ethiopian khat, (Catha *Edulis* Forsk). Biological trace element research 138, 316-325.
2. **Atlabachew, M.,** Chandravanshi, B.S., Redi, M., **2011.** Profile of Major, Minor and Toxic metals in soil and Khat (Catha edulis Forsk) Cultivars in Ethiopia, Trends in Applied science Research 6, 640-655.
3. **Atlabachew, M.,** Chandravanshi, B.S., Zewge, F., Redi, M., **2011**. Fluoride content of Ethiopian khat. Toxicological and Environmental chemistry 93, 32-43.
4. **Atlabachew, M.,** Chandravanshi, B.S., Redi, M., **2012**. Selected secondary metabolites and antioxidant activity of Khat (*Catha edulis Forsk*) chewing leaves extract. In press in the international Journal of Food properties.
5. **Atlabachew, M.,** Torto, N.**,** Chandravanshi, B.S., Redi, M. Matrix solid-phase dispersion for the liquid chromatographic determination of alkaloids of khat (*Catha edulis* Forsk) chewing leaves. Submitted for publication to Chromatographia and it is under review

B. Oral presentation

1. Level of fluoride and selected minerals in Khat grown in Ethiopia on the international symposium entitled as "The 40th South African Chemical Institute (SACI) National Convention and Federation of African Chemical Societies (FaCS) Meeting: A Prelude Event to the International Year of Chemistry" which was held in Johhanesburg, South Africa, January 16th – 21st, **2011**

2. Composition of selected inorganic nutrients in Ethiopian khat on the occasion of the 27th annual anniversary of the Chemical Society of Ethiopia, February 21-24**, 2011**