Investigation of Antioxidant Activities in Relation to the Phytochemical Constituents and Selected Metals in Fresh Fruits and their Health Risk Assessment

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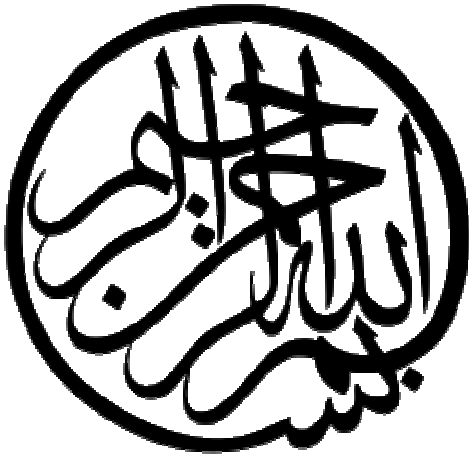

In The Name of Allah, The Most Gracious and The Most Merciful

DECLARATION

This is to certify that this dissertation submitted by ***Ms. Khezina Rafiq*** *is accepted in its present form by the Department of Chemistry, Quaid-i-Azam University, Islamabad, Pakistan as satisfying the dissertation requirements for the degree of* ***Master of Philosophy*** *in* ***Inorganic/Analytical Chemistry****.*

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Khezina Rafiq

Dedicated to My

Beloved Parents

"Every bit of me is a little bit of you"

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ABSTRACT

The present study is based on the measurement of concentrations of selected metals (Na, K, Ca, Mg, Fe, Zn, Cu, Mn, Cr, Co, Sr, Li, Ni, Pb and Cd) as well as quantification of some phytochemical constituents (phenolics, flavonoids, flavonols and ascorbic acid) and various antioxidant activity assays (DPPH radical scavenging assay, hydroxyl radical scavenging activity, Fe^{2+} chelating activity, ferric reducing antioxidant potential assay and phosphormolybdenum assay) in the available commercial fresh fruits. For metal analysis, dried fruit samples were digested in nitric acid, followed by perchloric acid and quantification of selected metals was performed by flame atomic absorption spectrophotometer. For phytochemical and antioxidant analysis, dried samples were extracted in water and acetone for separation of their hydrophilic and lipophilic contents and measurements were performed employing UV-Visible spectrophotometer. Data obtained from the metal analysis, phytochemical constituents and antioxidant assays were subjected to descriptive statistical and correlation study in order to evaluate both the intra and inter-relationships among the selected metals, phytochemicals and antioxidant activities. A number of significant correlations among the phytochemicals in the fruits manifested their mutual distribution in the fruits. Among the metals, Zn showed strong positive association with various phytochemicals and total antioxidant activity assays. Multivariate cluster analysis was used to identify the plausible pollution sources and to find out the multiple relationships among the selected metals in the fruits. Significant anthropogenic intrusions of the metals were noted in the fruits mainly associated with the fertilizers, agriculture sprays, biomass burning and ferrous industries. Individual metal contents, phytochemical constituents and antioxidant activities were also compared in all fruit samples. Among the selected metals, concentrations of Ca, Mg, Na and K were found at the elevated levels in the fruits. Highest antioxidant potential was shown by dates. Other fruits with significant antioxidant potential include round jujube berries, black chaunsa mango, grapes, apple and black mulberry fruit. Risk assessment in terms of health risk index (HRI), target hazard quotient (THQ) and target cancer risk (TCR) was found to be safe and showed non-significant adverse health effect related to the metal contents through lifetime consumption of the fruits.

Chapter 1

INTRODUCTION

1.1 Significance and Classification of Fruits

Agriculture is the mainstay of Pakistan's economy as it accounts for 21% of GDP and along with agro-based products it fetches 80% of the total export earnings of the country. This sector is engaging more than 48% of the labour force. In addition to the wheat, rice, cotton, sugarcane and maize, in which Pakistan has become self sufficient, other important crops are the fruits and vegetables. Among the fruits, Punjab province accounts for more than 95% of citrus fruits, 66% of mangoes, 82% of guava and 34% of dates of the overall national production of these fruits (Agri Punjab, 2010).

Fruit is that part of plant which develops from the ovary along with the seeds. Commonly fruits are grouped into various classes depending primarily upon the chemical composition, botanical structure and climatic requirements; for example, wild fruits, pomaceous fruits, tropical and sub-tropical fruits, grapes, melons, citrus fruits, berries, stone fruits and dry fruits, etc. (Molnar, 2009a). Pomaceous fruits contain many pits and their typical examples are apple and pears. Berries are the type of fruits which are usually small and fragile. Citrus fruits are high in citric acid and grapes are the fruits which are quite fragile and grow in clusters. Drupes or stone fruits contain single pit (e.g. peach, cherries, apricots, plums etc). Melons are large in size and their outer rind is tough. Tropical and sub-tropical fruits include mango, figs, dates, pineapple, banana and certain other fruits that require warm climate excluding citrus fruits (Dauthy, 1995).

1.2 Chemical Composition of Fruits

In a given food, nutritive value and certain other properties are dependent on the nature and quantity of the substances present in it (Molnar, 2009b). Ripeness and variety are the factors that strongly influence the proximate composition of the fruits but their chemical composition is more or less similar in terms of major and minor constituents. Polysaccharides, sugars and organic acids are the major constituents while lipids and N-compounds are present in fewer amounts. Minerals and vitamins of nutritional importance and aroma substances/pigments of sensory importance are the minor constituents (Molnar, 2009a). There are variety of natural antioxidants present in the fruits, such as, vitamin A,

vitamin C, vitamin E and β-carotene. Vitamin C is a hydrophilic compound and it is the major antioxidant that quenches oxidation type of free radicals in blood while, both vitamin E and β-carotene exhibit the antioxidant activity under lipophilic and hydrophilic conditions (Niki et al., 1995; Guo et al., 2001). Fruits not only contain nutritional antioxidants but also possess plenty of non-nutritional antioxidants for example flavones, flavonoids and polyphenolic compounds (Takahama, 1985; Wang et al., 1996).

In fruits, water or moisture is the predominant constituent which supports a large number of chemical reactions and in hydrolytic processes it acts as a direct reactant. Thus, when water is removed from the fruits, the growth of microorganisms is inhibited slowing down the enzyme-catalyzed reactions (involving hydrolyses) and shelf life of the fruits is improved or fruits can be stored for a longer period of time. Most of the fruits contain approximately 80% to 90% of water (Molnar, 2009b). Water is present in three major forms in the vegetal cells; (a) Dilution water or bound water which is present in the cells and with organic/mineral substances it forms solutions, (b) Colloidal bound water which is present in cytoplasm, membrane and nucleus and it acts as a swelling agent, (c) Constitution water which is directly bounded to the chemical component molecules and it is very difficult to remove (Dauthy, 1995).

Carbohydrates are the main component of fruits and they constitute more than 90% of their dry weight. From the energy point of view, carbohydrates are the most valuable among all the food components as their daily adult intake should be about 500 g (Dauthy, 1995). Nanostructural carbohydrates are abundantly present in fruits while fats and proteins levels are typically lower (Ruby et al., 2000). In addition to the fructose and glucose, only trace amounts of monosaccharide are present in the fruits and their ratios differ greatly in different fruits. Among oligosaccharides, sucrose is the dominant constituent. On other hand, among the polysaccharides all fruits contain hemicelluloses, cellulose, pectin and pentosans. The building blocks of these polysaccharides are the glucose, fructose and xylose (Molnar, 2009a). Generally, carbohydrates are produced in green plants through the process of photosynthesis. They serve as structural components, for example, as cellulose; they act as energy reserves, for example, as starch in plants; they function as indispensable components of nucleic acids, for example, as ribose; and they also act as components of vitamins, for example, as ribose and riboflavin (Dauthy, 1995).

Nutritional condition and the yield potential are characterized by the mineral composition of the fruits. Amount of the mineral elements in fruits depends upon the pH of environment, ratios between the mineral elements and several other factors (Jarvan and

Poldma, 2004). In fruits, mineral substances are present in the form of salts of inorganic or organic acids or in the form of complex organic combinations (e.g., lecithin and chlorophyll). Fruits which are rich in minerals include peaches, strawberries, raspberries and cherries. Presence of significant amount of potassium in fruits and absence of sodium chloride give a high dietetic value to the fruits. Some of the fruits are considered to be important for their Ca/P ratio (above 1.0). Such fruits include oranges, lemons, pears and some wild berries. Different mineral salts present in the fruits carry out a basic reaction due to which dietary intake of fruits facilitates the neutralization of harmful and noxious uric acid reactions and also contributes to the acid-base equilibrium in the blood (Dauthy, 1995). The macrominerals are necessary more or less for every aspect of the body's metabolism, e.g., neural transmission, muscle contraction, nucleic acid formation, amino acids and carbohydrate metabolism, etc. (Ruby et al., 2000).

Dietary fibre is not considered a defined chemical group rather it is considered a combination of chemically heterogeneous compounds. There is strong scientific evidence that fundamental characteristic of dietary fibre which is assigned to lignin and non-starch polysaccharides have been extended to other indigestible food constituents. So, as a whole, dietary fibre is defined as indigestible food fraction (Hervert-Hernandez et al., 2011). Dietary fibre is abundantly present in the fruits. It performs many vital functions, for example, it controls blood sugar level, it helps preventing constipation and it lowers the blood cholesterol. There are two types of dietary fibre; (1) soluble fibre; as the name indicates it dissolves in water, and (2) insoluble fibre; it does not dissolve in water. Both types have their own importance as insoluble fibre promotes regular bowel movements while on the other hand when soluble fibre dissolves in water, it forms a gel-like material which helps to control the blood sugar and lowers the blood cholesterol (Oguejiofo, 2010).

Vitamins play a very important role as they facilitate the metabolism of carbohydrates, proteins and fats (Dauthy, 1995). Fruits play an important role in human nutrition, especially they act as sources of vitamins including vitamin C (ascorbic acid), vitamin B_3 (niacin), B_1 (thiamine), B_9 (folacin, also called folic acid or folate), B_6 (pyridoxine) and vitamin E (Craig and Beck, 1999; Wargovich, 2000). Generally, vitamins are classified into two groups, i.e., water-soluble and fat-soluble. Examples of water-soluble vitamins are vitamin C and many members of vitamin B complex. Examples of fat-soluble vitamins are vitamin A, vitamin D, vitamin E and vitamin K. Vitamin C or ascorbic acid is considered to be anti-scurvy vitamin. It is essential for the normal

formation of collagen protein and its deficiency in body causes easy bleeding of gums, fragile capillary walls, bone joint diseases and loosening of teeth. It also aids in maintaining healthy immune system, wound healing and cardiovascular diseases. Citrus fruits are the excellent source of vitamin C (Dauthy, 1995).

Natural acids are present in the fruits; for example, citric acid is present in oranges, tartaric acid in grapes and malic acid in apple. These acids slow down the bacterial spoilage and give fruits specific tartness. Organic acids also have a strong effect on the colour and taste of the fruits. Generally, fresh fruits contain very low fat levels which are below 0.5%. However, significant quantities of fats are present in dry fruits; e.g. in nuts, fat levels are 55% (Dauthy, 1995).

Nitrogen-containing compounds are present in fruits in different combinations; e.g., amino acids, proteins, amines, nitrates, amides, etc. Usually, N-compounds make up 0.1% to 1.5% of the fruits in which 35% to 75% consist of proteins but these proteins are less valuable as compared to the animal proteins because all essential amino acids are not present in their composition. Apart from proteins, other nitrogen compounds are considered to be minor constituents. In various fruits, a large number of aromatic and aliphatic amines are also present which are formed either by amino acid decarboxylation or by transamination of aldehydes (Molnar, 2009a; Dauthy, 1995).

1.3 Nutritional Importance of Fruits

Fruits are considered as natural staple food and they contain a rational proportion of substantial amount of indispensable nutrients. The frequent consumption of fruits in diet promotes the chances of healthy life and generous intake of fruits helps to prevent body from several diseases and keeps the body active and energetic throughout life (Zahir et al., 2009). Fruits are the wonderful medicines of nature packed with antioxidants, vitamins, minerals and many phytochemicals. They contain low calories and fats and they are a source of fibres, vitamins and simple sugars which are essential for optimizing human health. They provide plenty of soluble dietary fibre, which helps to lower the cholesterol level and fats from the body and also helps to get relief from constipation (NAY, 2004). It has been demonstrated in many studies that the diets, which are rich in fruits, play a very important role in the prevention of a lot of diseases and disorders, for example, certain types of cancers, cardiovascular diseases and even aging (Joshipura et al., 2001; Aruoma, 2003; Miller et al., 2000). This evidence is quite convincing that fruits contribute towards

the prevention of many degenerative processes (Ames et al., 1993; Bickford et al., 1997; Joseph et al., 1999). This protection against diseases is due to the presence of various antioxidants in the fruits (Ames et al., 1993; Gey et al., 1991).

Fruits contain many antioxidants like flavonoids, polyphenols, vitamin C and anthocyanins. These compounds possess potent antioxidant properties and remove free radicals from the body thus offering protection against the infections, cancers and aging. These compounds help to protect the human body from oxidant stress and also help to develop ability to fight against various ailments by boosting the immunity level. Many fruits have very high antioxidant properties (NAY, 2004). This fact has been established that the complex mixture of phytochemicals in the fruits provide a better protection as compared with a single phytochemical due to additive and synergistic effects (Liu, 2003). Consequently diet rich in vegetables and fruits are generally recommended. It has been established that these compounds protect human body from minor ailments to major diseases (NAY, 2004). World Health Organization (WHO) and Food and Agricultural Organization (FAO) have recommended the consumers for the daily consumption or intake of at least 400 g of vegetables and fruits in order to prevent the body from cancer, heart disease, diabetes type 2 and obesity (Isabelle et al., 2010).

Nutritional quality of the fruits is strongly affected by the climatic conditions particularly light intensity and temperature. Synthesis of vitamin C and sugars is favoured at low temperatures while glucose is the precursor of ascorbic acid and at the same time, the rate of oxidation of ascorbic acid decreases. Similarly, light intensity also has an effect on the vitamin C because as light intensity increases, vitamin C production increases and at the same time chlorophyll and total carotenoids, which are vitamin A precursors decrease. So, when light intensities are high, more sugars are produced which leads to more production of vitamin C and it also increase plant temperatures thus inhibiting the production of beta-carotene or vitamin A. Fertilization, mulching, soil type, irrigation, rootstock used for fruit trees and other cultural practices also affect the nutrient and water supply to the fruit plants which in turn affect the quality and composition attributes of the harvested fruits (Goldman et al., 1999). Other environmental factors that influence fruit nutritional quality include soil pH, altitude, production practices, soil salinity, insect injury, ozone and plant diseases (Kader et al., 2004).

1.4 Antioxidant Potential of Fruits

Consumers' demand for the functional foods has been increased due to widespread interest in the disease prevention and human nutrition. A food is considered as a functional food if its food ingredients provide a health benefit beyond the traditional nutrients. According to this definition, fruits can be categorized as functional foods (Nikniaz et al., 2009) as many studies have demonstrated that antioxidants, which are present in fruit at higher levels, are the compounds which are responsible for such functionalities (Sugimura, 2002). These antioxidants are considered to exert a potential protective effect against free radical damage (Gursoy et al., 2009) and hence antioxidant activity is the fundamental property that is important for life as there are many biological functions which originate from this property, for example, anti-carcinogenicity, anti-mutagenicity and anti-aging (Cook and Samman, 1996). Antioxidants are the compounds which restrain or in other words delay the oxidation of other molecules through inhibition of the initiation and/or propagation steps of oxidizing chain reactions. Mainly, antioxidants are categorized in synthetic and natural antioxidants. Synthetic antioxidants are those compounds which possess various degrees of alkyl substitution in their phenolic structures while natural antioxidants are considered to be phenolic compounds (phenolic acids, flavonoids and tocopherols), nitrogen compounds (chlorophyll derivatives, amines, alkaloids and amino acids), ascorbic acids and carotenoids (Velioglu et al., 1998). These biomolecules contribute towards the prevention of vascular and coronary diseases and also prevent the tumour formation by inhibiting oxidative chain reactions (Kris-Etherton et al., 2002; Vinson et al., 1998).

Depending upon their origin, antioxidants are classified as endogenous and exogenous; former are actually enzymes, for example, catalase, superoxide dimutase and glutathione peroxidase as well as non-enzymatic compounds, for example, albumin, bilirubin and uric acid. When endogenous antioxidants cannot ensure complete protection against reactive oxygen species (ROS) then exogenous antioxidants are required which can either derived from natural source as flavonoids, anthocyanins and vitamins or they can be synthetic compounds as gallates, butylhydroxy anisoles etc (Pisoschi and Negulescu, 2011). Most of the fruits contain antioxidant compounds and these antioxidant compounds protect the cells against the devastating effects of ROS (singlet oxygen, peroxyl radicals, superoxides, peroxynitrate and hydroxyl radicals) (David et al., 2004; Dasgupta and De, 2004). These pro-oxidants are produced normally by aerobic

metabolism (Lillian et al., 2007). If the balance between ROS and antioxidants is disturbed, it results in oxidative stress which leads to the cellular damage. Many researchers have demonstrated that frequent intake of antioxidants decreases the chances of mortality from coronary heart disease and heart attacks (Hertog et al., 1993; Kris-Etherton and Krummel, 1993; Anderson et al., 1998). It has been reported that phenolic compounds are associated with antioxidative action in biological systems and act as scavengers of free radicals and singlet oxygen (Rice-Evans et al., 1995; Jorgensen et al., 1999; Park et al., 2006).

More awareness about the health promoting effects of natural antioxidants in everyday foods has led mankind to the various investigations in the field of natural antioxidants (Kader et al., 2004; Krishnakumar and Gordon, 1996). In this regard, most attention has been paid to the oral intake or administration of the natural radical scavengers present in diet (Peschel, 2006). Presently; food manufacturers, nutritionists and consumers are much more interested in natural antioxidants because of their potential therapeutic value and presumed safety. Indeed, recent research trends show a great shift towards identifying the non-nutritional functional foods (Takeoka and Dao, 2003). There are more than 5000 phytochemicals in plants which have been identified and still there are many more which need to be identified (Shahidi and Naczk, 1995; Sreeramulu and Raghunath, 2010). Epidemiological studies have shown that frequent use or consumption of natural antioxidants is linked with a lower risk of cancer and cardiovascular disease so natural antioxidants, which are present particularly in the fruits and vegetables, have attained increasing interest among the scientific community and consumers. In fruits and vegetables, the defensive effects of natural antioxidants are associated with three major groups which are (1) vitamins, (2) phenolics, and (3) carotenoids. Among them ascorbic acid and phenolics are considered to be hydrophilic antioxidants, while carotenoids are suggested to be lipophilic antioxidants (Thaipong et al., 2006).

1.5 Phytochemical Constituents of Fruits

Phytochemicals are the natural bioactive compounds found in plants including fruits and vegetables and they form an integrated part of body defence system against stress conditions and various diseases (Koche et al., 2010). These substances are considered to be strong antioxidants and they function to modify the body metabolic activation, detoxification and/or disposition of carcinogens, and they even influence the

processes that change the course of the tumour cells (Wargovich, 2000). The phytochemicals are categorized as primary and secondary and this classification is based on their roles/functions in the metabolism. Primary phytoconstituents consist of proteins, common sugars, chlorophyll and amino acids which are produced within the body by taking proper diet. On the other hand, secondary phytochemicals consist of phenolic compounds, terpenoids, alkaloids, saponins, tannins, flavonoids, etc., which are present in food and should be ingested frequently (Koche et al., 2010). It has been reported that phytochemicals present in the fruits exert beneficial health effects because they maintain balance between oxidants and antioxidants by combating with oxidative stress (Sun, 1990). The phytochemicals protect the cell constituents from destructive oxidative damage, inhibit the oxidative and hydrolytic enzymes which include lipid peroxidation and thus limit the risk of several degenerative diseases which are associated with oxidative stress (Vinary et al., 2010).

The health enhancing effects and properties of fruits are due to the presence of various phytochemicals especially phenolics and it is widely accepted that their beneficial action is linked with their antioxidant activity. Polyphenols include more than 8000 compounds which are composed of one or more benzene rings and one or more hydroxyl groups. These compounds mainly exist in plants as esters or in glycosylated forms. There are many variations in sugar moiety and its binding position (Huang et al., 2007). Based on their chemical structure, these are divided into 10 classes ranging from the simple phenols (C_6) to highly cross linked aromatic polymer (Garcia-Salas et al., 2010).

Polyphenols are the common constituents present in the human diet and fruits are the major source of these bioactive compounds (Kumaraswamy and Satish, 2008). In the prevention of degenerative diseases, evidences for the role of polyphenols are emerging as many experimental studies carried out on animals and human cell lines have shown that polyphenols can play an important role in the prevention of cancer and cardiovascular diseases (Wijngaard et al., 2009). It has been reported that polyphenols are located specifically in peels of the fruits (Wolfe et al., 2003) but, in our daily life, some fractions of fruits are often discarded due to their unpleasant taste or sanitary reasons and as a result, important nutrients in these fractions are lost (Ji et al., 2011). Polyphenols constitute a wide variety of substances and these are divided into several classes which are hydroxycinnamic acids, hydroxybenzoic acids, flavonols, flavanones, flavones, isoflavones, anthocyanins, proanthocyanindins, stilbenes and lignans which are present in plants (Pantelidis et al., 2007; Lattanzio et al., 2006). The antioxidative potential of the

phenolic compounds is supposed to come from their strong capability to transfer electrons to reactive oxygen species or free radicals, to activate inhibitory oxidases and antioxidant enzymes and to chelate metal ions (Cos et al., 1998). This antioxidant activity of phenolics permits them to act as hydrogen donors, reducing agents, metal chelators and singlet oxygen quenchers (Rice-Evans et al., 1995).

Flavonoids are three-ring phenolic compounds which consist of a double ring that is attached to a third ring by a single bond. Flavonoids make up a large family of low molecular weight plant secondary metabolites (Stewart et al., 2000) which is sub-divided into 6 classes based upon their structural characteristics which are flavanols, flavonols, flavones, flavonones, isoflavones and anthocyanins (Wang and Helliwell, 2000). More than 4000 different flavonoids have been described in literature. In addition to antioxidant activities, they also inhibit the production of enzymes such as lipoxygenase, prostaglandin synthase and cycloxygenase which are closely related to tumourigenesis and they also induce detoxifying enzymes in the body, for example, glutathione S-transferase (Lee et al., 1995). Flavonoids exhibit a broad spectrum of biological effects which include antiviral, antibacterial, anti-inflammatory, antithrombotic, anti-allergic and vasodilatory actions (Cook and Sammon, 1996). It has been reported that flavonoids have 2 to 5 times greater antioxidant potential as compared to vitamins C and E on an equimolar basis (Toit et al., 2001). In some past years, public and scientific interest in flavones has grown enormously due to their beneficial effects against diabetes mellitus, osteoporosis, atherosclerosis and certain cancers (Sava and Sirbu, 2010).

Vitamin C or ascorbic acid is a monosaccharide redox catalyst present abundantly in plants especially in the fruits and its concentration can reach 20 millimolar in chloroplasts (Smirnoff and Wheeler, 2000). It has been established that during primitive evolution, one of the enzymes has been lost which is needed for the production of ascorbic acid, so humans must have to obtain it from their diet (Smirnoff, 2001). The requirement of ascorbic acid is due to the reason that through oxidation of proline residues to hydroxyproline it converts the procollagen into collagen while, in some other cells, by reacting with glutathione, it is maintained in its reduced state (Meister, 1994; Wells et al., 1990). Ascorbic acid is a redox catalyst thus it can reduce and neutralize ROS (Padayatty et al., 2003). In addition to acting as direct antioxidant, ascorbic acid also acts as a substrate for the redox enzyme ascorbate peroxidase. This function is considered to be particularly important in stress resistance in plants (Shigeoka et al., 2002).

Gallic acid is a type of phenolic acid which is also known as 3,4,5-

trihydroxybenzoic acid. Its chemical formula is $C_6H_2(OH)_3COOH$. Its salts and esters are known as gallates. For the determination of polyphenolic contents by the Folin-Ciocalteu assay, gallic acid is used as a standard and results are expressed in Gallic acid equivalents (Barros et al., 2007). It acts as an antioxidant and protects the human cells against oxidative damage. It shows cytotoxicity against cancerous cells without even harming the healthy cells. It is also used to treat diabetes and albuminuria.

Rutin is also called rutoside, sophorin or quercetin-3-O-rutinoside. Its chemical formula is $C_{27}H_{30}O_{16}$. It is commonly found in the fruits especially citrus fruits including lime, orange, lemon and grapefruit. Though it is not strictly a vitamin, but it is sometimes referred to as vitamin P. In human body, rutin attaches to the Fe^{2+} ion and prevent its binding to hydrogen peroxide which otherwise creates a highly-reactive free radical which may damage the cells. Rutin is one of the most abundantly present flavonoid compounds in plants. It has ability to control the permeability of capillaries. It is commonly used as standard in the determination of flavonoid contents in various plant extracts and results are represented as rutin equivalent (Smirnova et al., 2000).

1.6 Reactive Oxygen Species (ROS)

Initially, free radicals were considered species of the major interest for early radiologists and physicists but much later they were found to be the product of normal metabolism (Demir et al., 2009). The molecule of oxygen is very stable in its ground state but when its supply is in excess or its reduction is insufficient then reactive oxygen species are formed (Aruoma, 1998). These are also formed by radiolysis, environmental pollutants, UV radiations, pharmaceuticals, smoke, toxic chemicals, alcohol, cooked food and oxidized polyunsaturated fats. During the reduction pathway to H_2O in living tissues, oxygen molecule is changed into hydroxyl radical, superoxide radical and hydrogen peroxide which are called as ROS and they induce oxidative damage to biomolecules (Wiseman and Halliwell, 1996). Among these species, hydroxyl radical is most reactive and causes severe damage to adjacent biomolecules (Dasgupta and De, 2004). Generally, ROS damage various parts of the cells, particularly, DNA, proteins and cell membranes by gaining their electrons through the process of oxidation. These free radicals may results in cancer, heart damage, and a weak immune system (Feinman, 1988; Esterbauer et al., 1991; Maharaj et al., 2006).

All the living organisms possess the enzymatic as well as non-enzymatic defence

systems against the excess production of ROS. Enzymatic defence system include key metabolic steps as catalase (CAT), superoxide dimutase (SOD) and glutathione peroxidase (GPX) while non-enzymatic molecules include thiols, thioredoxin and disulfide bonding (Kumaraswamy and Satish, 2008). The evidence, which indicate that the free radicals are the reason behind the oxidative damage to proteins, lipids and nucleic acids, is overwhelming. Therefore, it is considered that free radicals lie at the heart of the natural history of a number of diseases, such as, heart attack, diabetes, cancer and neurodegenerative diseases (Halliwell and Gutteridge, 1999; Nagai et al., 2005).

There are many disorders which are partly due to the presence of reactive nitrogen species (RNS) and ROS. Although there are intrinsic processes in the cells which can deal with these reactive species but when excessive formation of ROS and other free radicals occurs, it results in serious pathological consequences and this may overcome the inside activity of the endogenous antioxidant system. So, the consumption of fruits is recommended as antioxidant supplement (Knekt et al., 2002). Main targets of free radical attack are erythrocytes due to oxygen transport that is associated with haemoglobin molecules which are redox active and thus promote the formation of ROS as well as due to the abundance of polyunsaturated fatty acids in the membranes of erythrocytes (Youdim et al., 2000; Tedesco et al., 2001).

1.7 Mechanism of Antioxidation

On the basis of the chemical reactions involved, major antioxidant activity assays are divided into two broad categories; (1) hydrogen atom transfer (HAT) reaction based assays and (2) single electron transfer (ET) reaction based assays. In ET-based assays, redox reaction is involved and oxidant act as indicator of the reaction end point while in HAT-based assays, an oxidiseable molecule, a synthetic free radical generator and an antioxidant is involved (Huang et al., 2005). Generally, the mechanism of antioxidation of a phenolic antioxidant is divided into two stages, as shown by the following schemes:

(1) Radical trapping stage [S-OO (radical) + AH → S-OOH + AH (radical)]

(2) Radical termination stage [A (radical) → non-radical materials]

Where, S is the substrate for oxidation, S-OO is the peroxyl radical of S, A is the antioxidant radical and AH is the antioxidant. The first process is reversible and the second stage is irreversible and it produces stable radical termination products. The structure of the products determines the antioxidation mechanism for each phenolic

antioxidant (Masuda et al., 2010).

There are number of physical and chemical factors that contribute towards the initiation of oxidation which continues in the presence of a suitable substrate. These target substrates may be oxygen, DNA, cholesterol, phospholipids and polyunsaturated fatty acids, etc. (Ming-Hua and Schaich, 1996). The essential steps of an oxidation chain reaction by the free radical mechanisms are initiation, propagation and termination (Antolovic et al., 2002). Initiators may be either physical, for example, light, heat or ionizing radiation or may be chemical in origin, for example, metal ion or metalloprotein (Kanner et al., 1987).

$$LH + R^{\bullet} \rightarrow L^{\bullet} + RH$$

In this case, LH represents a substrate molecule, for example, a lipid; $R^{\bullet}$ represents the initiating radical. When lipid is being oxidized, it generates a highly reactive allyl radical represented by $L^{\bullet}$ with the production of a neutral species RH.

In propagation step, $L^{\bullet}$ rapidly reacts with oxygen in order to form a lipid peroxyl radical ($LOO^{\bullet}$).

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH$$

These peroxyl radicals act as chain carriers for the reaction which further oxidize the lipid molecules thus producing lipid hydroperoxides (LOOH). In turn, these lipid hydroperoxides break down to a broad range of other compounds (Cheeseman and Slater, 1993) which include aldehydes, ketones, alcohols, alkyl formates, hydrocarbons as well as radicals which include alkoxyl radical ($LO^{\bullet}$).

Branching occur when lipid hydroperoxides (LOOH) are broken down to other compounds. Mostly, this breakdown involves transition metal ion catalysis producing lipid peroxyl ($LOO^{\bullet}$) and lipid alkoxyl radicals ($LO^{\bullet}$).

$$LOOH \rightarrow LO^{\bullet} + HO^{\bullet}$$

$$2\ LOOH \rightarrow LOO^{\bullet} + LO^{\bullet} + H_2O$$

Termination step involves the combination of radicals in order to form neutral or non-radical products.

$$LO^{\bullet} + LO^{\bullet}$$

$$LOO^{\bullet} + LOO^{\bullet}$$

$$LO^{\bullet} + LOO^{\bullet}$$

When primary antioxidants for example; AH are present in trace amounts, then

they either inhibit the initiation step by reacting with a lipid radical or inhibit the propagation step by reacting with peroxyl or alkoxyl radicals (Pisoschi and Negulescu, 2011).

$$L^{\bullet} + AH \rightarrow LH + A^{\bullet}$$

$$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet}$$

$$LO^{\bullet} + AH \rightarrow LOH + A^{\bullet}$$

1.8 Food Safety and Role of Trace Metals

Safety of the food is major public concern worldwide; especially during the last few decades. Increase in the demand of food safety has prompted many researchers to carry out research related to the risk linked with consumption of trace metals, pesticides and/or toxins contaminated foodstuffs (D'Mello, 2003). Among the above mentioned food contaminants, trace metals are considered to be the major contaminants of foodstuffs and they are supposed to be the most important problem to our environment (Zaidi et al., 2005). There are a number of trace metals, such as, Fe, Co, Mn, Ni, Cu, Cr and Zn which are considered micronutrients and their presence is essential for the normal growth as they take part in electron transfers, many redox reactions and other important metabolic processes while there are some metals which are considered nonessential (Cd, As, Pb and Hg) and potentially toxic (Michalak, 2006).

The trace metals are present as a part of metalloenzymes and these metals participate in several important biological functions, such as, free-radical scavenging, oxygen transport, hormonal activity and structural organization of macromolecules (Pasha et al., 2010). Trace metals play a vital role in a wide range of essential processes related to health which range from muscle contraction, nerve conduction, structural support, hormones or enzyme production and maintenance of mineral balance in the body. There is a delicate balance between other nutrients and trace metals (Hashmi and Shah, 2012) which is responsible for various physiological and metabolic processes. Any disorder in trace metal balance may cause instability in the system and it may lead to some serious pathologic conditions thus resulting in various ailments (Pasha et al., 2010). There are some metals which are considered as micronutrients and if their intake is not sufficient, it may lead to the various diseases and result in increased rates of ailments. However, if their intake is much higher than the body demands, they may exert harmful effects on the human health (Hashmi and Shah, 2012). Trace metals exert detrimental effects due to their

non-biodegradable and non-thermodegradable nature, their potential of accumulation in different body parts as well as their long biological half-lives. The extreme toxicity of the metals is attributed to their water solubility. There is no good enough mechanism for their elimination from the body; hence they may cause damage even in their low concentration (Arora et al., 2008).

1.9 Contamination of Metals in Fruits and Oxidative Stress

In the recent years, contamination of food chain by metals has become a burning issue because of their potential to accumulate in the biosystems through the air, soil, water and diet (Lokeshwari and Chandrappa, 2006). Similarly, fruits are important food stuffs in human diet so their metal contamination cannot be underestimated which is one of the most important factors of fruit quality assurance (Sharma et al., 2009). Due to increase in awareness of the risk of metals to the contamination of food chain, national and international regulations on food quality have decreased the maximum permissible values of the toxic metals in the food items (Radwan and Salama, 2006).

According to numerous studies, the pollution sources of various trace metals in environment are mainly derived from anthropogenic sources (Sakan et al., 2011). Most important pathway is dietary uptake of the metals through soil that is irrigated with contaminated wastewater. When, due to repeated use of wastewater, soil capacity of retaining the metals is reduced; then soil releases the metals into groundwater available for plant uptake (Sharma et al., 2007). Major contributor of the metal contents in soil is considered to be wastewater irrigation and important sources of the metals in wastewater include industrial and urban effluents, deterioration of treatment works and sewerage pipes and wear of household plumbing fixtures (Mapanda et al., 2005; Nan et al., 2002; Singh et al., 2004).

The increasing trend of fruit contamination especially in the urban areas is largely because of contaminated food transport, polluted environment in urban agriculture, use of contaminated water for irrigation and poor market sanitary conditions (Mahdavian and Somashekar, 2008). Emissions from metallurgical industries, during the smelting of non-ferrous metals, in different chemical forms are partly responsible for the metal accumulation in food chain (Wei-Xin et al., 2008). During the recent years, many other factors, such as, industry wastes, exhaustion gases and waste water have contributed towards polluting the plants and they are affecting the metal contents of fruits. There is a

broad spectrum of sources that contribute towards the fruit contamination with metals including metal-based fertilizer, pesticides, irrigation with contaminated water, industrial emissions, harvesting process, storage and transportation. The fruits which are grown at contaminated sites can accumulate the metals at toxic concentrations (Ozcan et al., 2012).

There are a number of factors which influence the uptake and bioaccumulation of the metals in fruits which are climate, chemical form and concentration of a particular metal in soil, atmospheric depositions, soil nature on which the fruits are grown as well as the degree of maturity of the plants at the time of harvest, soil pH, aeration, moisture, temperature, presence or absence of competing ions, phosphate, organic matter contents, plant species, age, rooting depth and seasonal growth effects (Scott et al., 1996; Voutsa et al., 1996; Tokalioglu and Kartal, 2010). Air pollution is also an important factor that threatens the fruits during their transportation and marketing and elevates the levels of some metals in fruits (Sharma et al., 2008). Usually, fruits are contaminated by metals through different modes such as deposition of metals on their surface or plants may take up the metals through their roots and incorporate into the fruit tissues (Ozcan et al., 2012).

It has been reported that metals may induce oxidative stress in the living cells and tissues by the following ways (Michalak, 2006):

a) In single-electron reactions, metals transfer the electrons directly and produce free radicals. Different transition metals, for example, Cu, Fe and Mn which possess unpaired electrons in their orbital, can accept and donate single electrons and thus promote the single-electron transfer to the O_2 and promote ROS inter conversions.

b) Metals can also disturb various metabolic pathways, especially those carrying out in the thyroid membranes. It also contributes towards the increased formation of reactive oxygen species and free radicals.

c) Some metals can trigger the formation of free radicals by inactivating the antioxidant enzymes, for example, catalases, peroxidases and superoxide dismutases, etc. which are responsible for the detoxification of the free radicals.

d) Accumulation of the metal can deplete the some antioxidants, for example, glutathione; which is utilized due to phytochelate formation.

1.10 Health Risks of the Metals

Essential metals are considered to be those metals which must be present in the human diet in order to maintain the normal physiological functions of the body. On the basis of risk assessment of trace metals, there are two ends of this toxicity spectrum: First is associated with high intakes of the metals resulting in toxicity and; second is associated with low intakes of these trace metals thus resulting in nutritional problems (Goldhaber, 2003). Depending upon the physical and chemical properties, the toxic metals exhibit detrimental health effects either by production of ROS or by blocking the essential functional groups present in the biomolecules or by substituting the essential metal ions by other ions (Michalak, 2006). The accumulation of toxic metals in living tissues may result in serious health issues such as, kidney damage, carcinogenicity and developmental toxicity (Akesson et al., 2008; Ergon et al., 1999; Thomas et al., 2009). When the intake of toxic metals is of chronic level, then the harmful impacts appear usually after several years of exposure (Bahemuka and Mubofu, 1999; Ikeda et al., 2000). However, when the metal contaminated food is frequently ingested, it causes serious depletion of some essential nutrients which is further responsible for intrauterine growth retardation, decreasing immunological defences, disabilities associated with malnutrition and high prevalence of upper gastrointestinal cancer rates (Khan et al., 2008).

Some metals and metalloids, which are supposed to possess the highest toxic potential, when present in food are considered to be the main threats to the human health. In most countries, their presence in the food is legally restricted (Jarup, 2003). Cadmium is a non-essential metal and it causes damage to the cells even if it is present at very low concentration because it is readily taken up by the plants (Wagner, 1993). The main source of Cd in fruits is through the soil and soil is contaminated with Cd mainly due to the irrigation with sewage effluent, disposal of industrial and municipal wastes, atmospheric deposition and application of phosphorous fertilizers. The plant tissue accumulation of Cd is dependent on the Cd availability and Zn–Cd interaction (Hart et al., 2002). Toxicity of intake of Cd via diet is very important issue for the human health because about 70% of dietary intake of Cd is through consumption of fruits and vegetables (Nabulo et al., 2010; Yang et al., 2009). Long- term Cd exposure may exert adverse health effects which include bone fractures, kidney damage, prostate, renal and ovarian cancers (Cao et al., 2010; Hartwig, 1998). Long-term Pb exposure leads towards prolonged reaction time, memory deterioration and reduced ability to understand. Specially, it causes learning and

concentration difficulties and behavioural disturbances in children (Jarup, 2003). Both Pb and Cd are potential carcinogens and it has been reported that they are associated with a number of diseases including kidney, cardiovascular, bone, blood related and nervous system diseases (Zhuang et al., 2009).

There are other metals (Fe, Cu, Zn, Mn, Cr, etc.) which act as micronutrients when present in trace amounts in human diet but they become toxic either under specific conditions or when their amount exceeds a particular level (Bakkali et al., 2009). Chromium in 3+ oxidation state is inevitable for the maintenance and normal metabolism of glucose. Its deficiency in the body leads to impaired glucose tolerance, fasting hyperglycemia, glycosuria, elevated glucagon and insulin level, while severe symptoms include brain and nerve disorders (Anderson, 1994a). Chromium toxicity is quite low compared to other trace metals. Its adverse effects are kidney and liver problems after high dose ingestion (Loubieres et al., 1999) while chronic exposure to Cr in 6+ oxidation state is associated with lung cancer (Anderson, 1994a). Similarly, deficiency of Cu causes neutropenia, degeneration of nervous system, anemia, skeletal defects and abnormalities (Linder and Azam, 1996). Very high copper levels may cause acute toxicity as its excess in body leads to Wilson's disease (Olivares and Uauy, 1996; Pandit and Bhave, 1996).

Iron is an essential metal for the human body as it is a constituent of myoglobin, haemoglobin and a number of enzymes. Its deficiency leads to anaemia, while the toxicity causes diarrhoea, vomiting and affect the liver, kidney, cardiovascular system, central nervous systems and blood (Anderson, 1994b). A genetic disease called "hereditary hemochromatosis" may results due to its slow and long accumulation in the tissues (Bacon et al., 1999). Manganese is also considered to be an essential metal for human body. Its deficiency leads to growth retardation, poor bone formation, poor reproductive performance, congenital malformations in offspring, impaired glucose tolerance and abnormal function of cartilage and bone. Long-term exposure of Mn may cause prominent neurological and psychological disturbances which leads to an irreversible brain disease; called manganism which resembles the Parkinson's disease (Aschner et al., 2006). Likewise, higher concentration of Ni in the body may cause skin rashes, headache, fatigue, dizziness, heart problems, respiratory illness and even cancer (Khan et al., 2010). Zinc is present in a wide variety of enzymes. Symptoms of Zn deficiency include loss of appetite, skin changes, growth retardation and immunological abnormalities (Goldhaber, 2003), while its toxicity is manifested by vomiting, gastrointestinal irritation and functional impairment of the immune system (Chandra, 1984).

1.11 Aims and Objectives

Assessment and monitoring of the trace metal levels in the fruits collected from the markets has been reported worldwide (Milacic and Kralj, 2003; Parveen et al., 2003; Jassir et al., 2005; Radwan and Salama, 2006) but in Pakistan very limited data are available on the metals concentrations in the marketed fruits. On the basis of description in the foregoing sections, the present study is conducted with the following aims and objectives:

- To quantify the levels of selected metals (Na, K, Ca, Mg, Fe, Zn, Li, Sr, Cr, Mn, Cu, Ni, Co, Cd and Pb) in the edible portions of fruits from local market.
- To carry out the quantitative analysis of major phytochemical constituents (polyphenols, flavonoids, flavonols and ascorbic acid) in the edible portion of the fruits.
- To evaluate the antioxidant activities (DPPH radical scavenging activity, hydroxyl radical scavenging activity, Fe^{2+} chelating activity, ferric reducing antioxidant power and phosphormolybdenium assay) in the edible portion of the fruits by employing different activity assays.
- To establish a relationship between the phytochemical constituents and the antioxidant activities in the fruits.
- To explore the correlation between phytochemical constituents, antioxidant activity and selected metal levels in the fruits.
- To define natural and anthropogenic sources of the metals using multivariate cluster analysis (CA).
- To assess the health effects via consumption of the commercially available fruits.

Chapter 2

EXPERIMENTAL METHODOLOGY

2.1 Collection and Processing of the Fruit Samples

In the present study, fruit samples were collected from the local markets of Islamabad and Rawalpindi. The region is located in the Pothohar plateau, north-eastern Pakistan. The climate of the region features three distinct seasons: a very hot and long humid subtropical summer, a monsoon and a short, wet and mild winter with temperature ranging from the maximum of 47°C (117°F) in summer to the minimum of −4°C (25°F) in the winter. The average annual rainfall of the area is 990 mm. The sampling period was ranging from September 2012 to March 2013. Good quality fresh fruit samples; with elimination of defective fruits, were collected in the clean plastic bags from the different fruit markets of Islamabad and Rawalpindi, Pakistan (Andarwulan et al., 2010; Mahdavian and Somashekar, 2008). All collected fruits were at commercial maturity stage while unripe and overripe fruits were discarded (Goulas and Manganaris, 2012). Sampling was done randomly from the different vendors and retailers within the market areas and samples were brought to the lab. All the experiments were conducted at the Environmental Chemistry Laboratory, Department of Chemistry, Quaid-i-Azam University, Islamabad, Pakistan. The description of fruits, including their local name, English name, scientific name, family, variety, growing season and part consumed are shown below in Table 1. Overall, 32 fruits commonly consumed by the local population were included in this study.

Every fruit sample was washed in fresh running water in order to eliminate dirt, dust, possible parasites or their eggs and then they were again rinsed with distilled water (Yusuf et al., 2003). After drying them at room temperature, the uneatable portions of all fruit samples were removed and their edible portions were chopped into thin slices (Sharma et al., 2009). Fruits which are eaten both peeled-off and non-peeled were separated into two sections; one, with peels and the other, without peels. Finally, the samples were dried in an electric oven at 80°C for 48 hours. The dried samples were ground into the fine powder using mortar and pestle and passed through a sieve. These powdered samples were placed in clean glass vials and stored at room temperature in desiccators for further analysis (Cao et al., 2010; Sharma et al., 2008).

Table 1. Description of the fruit samples used in the present study

S. #	Scientific Name	Family	English Name	Local Name	Variety	Season	Part Consumed
1	*Prunus persica*	Rosaceae	Peach	Aaroo	Freestone	Monsoon	a. Whole (with skin) without pit b. Only flesh without skin and pit
2	*Malus domestica*	Rosaceae	Apple	Saeb	Golden delicious	Monsoon	a. Whole (with skin) without seeds b. Only flesh without skin and seeds
					Red delicious	Autumn	a. Whole (with skin) without seeds b. Only flesh without skin and seeds
3	*Pyrus communis*	Rosaceae	Pear	Nashpaati	Bartlett	Monsoon	Whole fruit is eaten without seeds
4	*Mangifera indica*	Anacardiaceae	Mango	Aam	White Chaunsa	Monsoon	Flesh without skin and pit
					Black Chaunsa	Monsoon	Flesh without skin and pit
5	*Musa acuminata*	Musaceae	Banana	Kela	Cavendish	Monsoon	Flesh without peels
6	*Citrus latifolia*	Rutaceae	Persian lime	Meetha	-	Monsoon	Pulp without skin and seeds
7	*Punica granatum*	Lythraceae	Pomegranate	Anaar	White Muscat	Monsoon	Arils without peels
8	*Cucumis melo*	Cucurbitaceae	Canary melon	Kharbooza	Juan Canary	Monsoon	Flesh without skin and seeds
9	*Citrullus lanatus*	Cucurbitaceae	Watermelon	Tarbooz	Seedless/ Icebox	Monsoon	Flesh without skin and seeds
10	*Cucumis melo var. reticulatus*	Cucurbitaceae	Galia melon	Sarda	-	Autumn	Flesh without skin and seeds
11	*Diospyros kaki*	Ebenaceae	Winter Persimmon	Amlok	Fuyu or Japanese	Autumn	Flesh without skin
12	*Vitis vinifera*	Vitaceae	Grapes	Angoor	White table	Autumn	Whole berries are eaten
					Toffee	Autumn	Whole berries are eaten
13	*Carica papaya*	Caricaceae	Papaya	Papita	Sunrise solo	Autumn	Flesh without skin and seeds
14	*Citrus paradisi*	Rutaceae	Grapefruit	Chakotara	Foster	Autumn	Pulp without skin and seeds
15	*Psidium guajava*	Myrtaceae	Guava	Amrood	White	Autumn	Whole fruit is eaten

S. #	Scientific Name	Family	English Name	Local Name	Variety	Season	Part Consumed
16	*Ananas comosus*	Bromeliaceae	Pineapple	Ananaas	Kew	Autumn	Only flesh without skin and core
17	*Actinidia deliciosa*	Actinidiaceae	Kiwifruit	Kiwi	Hayward	Autumn	Flesh without skin
18	*Annona squamosa*	Annonaceae	Sugar apple	Sharifa	-	Autumn	Flesh without skin and seeds
19	*Citrus limettiodes* Tan.	Rutaceae	Sweet lime	Mosambi	Indian or Palestine	Autumn	Pulp without skin and seeds
20	*Citrus sinensis*	Rutaceae	Fruiter Orange	Fruiter	-	Winter	Pulp without skin and seeds
21	*Phoenix dactylifera*	Arecaceae	Date	Khajoor	Aseel	Winter	Whole fruit is eaten without stone
22	*Cocos nucifera*	Arecaceae	Coconut	Nariyal	-	Winter	Inner meat without shell
23	*Manilkara zapota*	Sapotaceae	Sapodilla	Chikoo	Brown sugar	Winter	Flesh without skin and seeds
24	*Citrus sinensis*	Rutaceae	Orange	Maalta	Sweet (Shakri)	Winter	Pulp without skin and seeds
25	*Physalis peruviana*	Solanaceae	Cape gooseberry	Rusbharri	Golden berry	Winter	Whole berry without outer calyx
26	*Ziziphus mauritiana*	Rhamnaceae	Indian Jujube	Baer	Round Oval	Spring Spring	Berry without seed Berry without stone
27	*Fragaria ananassa*	Rosaceae	Strawberry	Toot farangi	Chandler	Spring	Whole fruit is eaten
28	*Citrus sinensis* cultivar	Rutaceae	Blood Orange	Surkh maalta	Khanpur	Winter	Pulp without skin and seeds
29	*Citrus reticulata*	Rutaceae	Clementine	Kinnow	-	Winter	Pulp without skin and seeds
30	*Citrus tangerina*	Rutaceae	Tangerine	Sangtara	-	Winter	Pulp without skin and seeds
31	*Opuntia ficus-indica*	Cactaceae	Non-prickly pear fruit	Samarkand	Spineless	Spring	Flesh without spiny skin
32	*Morus nigra*	Moraceae	Black Mulberry fruit	Shehtoot	Black elongated	Spring	Whole fruit is eaten

2.2 Determination of Moisture Contents

For the solid samples mostly, analytical results are expressed on dry weight basis. In the present study, fresh fruit samples were first dried because the presence of moisture may cause variability in the composition (Patnaik, 2004). Fresh fruit samples were weighed to record the fresh weight (FW) of their edible portions and weighed again to record their dry weight (DW) after complete drying at 80°C for 48 hours (Li et al., 2010). Moisture contents (MC) of the fruit samples were calculated using following relationship (Roy et al., 2007):

$$MC(\%) = \frac{FW - DW}{FW} \times 100 \qquad (01)$$

2.3 Sample Preparation for Metal Analysis

Generally the fruit samples are digested in appropriate solvents/mixtures for the determination of their metal contents. Various methods used for the digestion of fruit samples in recent literature are briefly discussed below:

1. In a Pyrex beaker, 2 g of powdered sample was taken and 10 mL of concentrated HNO_3 was added to the sample and kept overnight without any heating. Then, sample was heated on a hotplate and after evaporating it near to the dryness, it was cooled and 5 mL of $HClO_4$ was added to it and sample was heated again on the hotplate. After completion of digestion, the sample was filtered into a 50 mL volumetric flask and the final volume was adjusted with de-ionized water (Khan et al., 2010).
2. Powdered sample (2 g) was weighed in a beaker and the sample was digested on a hotplate in 10 mL mixture of concentrated $HClO_4$ and HNO_3 (1:3 ratio), until a clear solution was obtained. After cooling to the room temperature, the samples were acidified with 10 mL mixture of HCl and H_2O (1:1 ratio) and then filtered through 0.45 μm filter paper and the volume was made up to 50 mL with distilled water (Zahir et al., 2009).
3. Digestion of fruit samples for the metals analysis involved a high performance microwave assisted digestion. Dried sample (0.5 g) was weighed and taken in a clean conical flask to which 4 mL of concentrated HNO_3 and 0.2 mL H_2O_2 was added. The mixture was put in a reference reaction vessel which was then inserted into a rotor unit ready for digestion. Then, system was pre-programmed; first for 5 minutes of microwave digestion at 250 W power and then for another 5 minutes at 500 W power. Digested sample solution was left for 10 minutes for automatic ventilation. After cooling, the

samples were filtered and the volume was made up to 100 mL with distilled water (Mahadavian and Somashekar, 2008).

4. Powdered fruit sample (0.5 g) was taken in a cup and 5 mL of concentrated HNO_3 and 2 mL of H_2O_2 (30%) was added in it. Then, the sample was incinerated at 210°C in the microwave oven. After digestion, the samples were filtered through Whatmann filter paper No. 42 into the clean 50 mL volumetric flasks (Ozcan and Harmankaya, 2012).

5. Dried fruit sample (1.0 g) was weighed into a conical flask and digested in a mixture of 20 mL of concentrated HNO_3, 5 mL of HCl and 2 mL of H_2SO_4 on the hotplate through gentle heating at 180-220°C for about 30 minutes until the appearance of dense white fumes. Finally, the sample was heated strongly for about 30 minutes. After digestion, sample was allowed to cool and volume was made up to the mark with distilled water in 50 mL volumetric flask (Sobukola et al., 2010).

6. Dried powdered sample (1 g) was weighed into a 50 mL beaker to which 10 mL tri-acid mixture of HNO_3, H_2SO_4 and $HCIO_4$ was added in the ratio of 1:1:1. The beaker containing the sample was covered with watch glass and left overnight. Then, the sample was digested at 96°C until about 4 mL was left in the beaker. Afterward, a further 10 mL of tri-acid mixture was added into the sample and the mixture was heated to evaporate to about 4 mL. After cooling it to room temperature, the solution was filtered into a 50 mL volumetric flask and made up to a final volume with distilled water (Yusuf et al., 2003).

7. Dried powdered sample (1 g) was taken into a beaker and 15 mL of tri-acid mixture (70% HNO_3, 65% $HClO_4$, 70% H_2SO_4) in ratio of 5:1:1 was added into it. Then, mixture was digested at 80°C until solution became transparent. After cooling it to room temperature, digested samples were filtered through Whatman filter paper No. 42 and volume was made up to 50 mL with deionised water (Sharma et al., 2008).

8. Dried sample was accurately weighed (0.5 g) and placed in a crucible. The sample was digested in $HClO_4$ and HNO_3 in the ratio of 1:4 and left to cool and then contents were filtered through Whatman filter paper No. 42 into a 25 mL volumetric flask and the mixture was made up to a final volume with distilled water (Arora et al., 2008).

9. Ground and oven-dried sample was weighed (2 g) into a crucible and ashed in a muffle furnace at 450°C for 24 hours until a whitish-to-grey ash was achieved. Then, crucible was cooled in a desiccator and 5 mL of aqua-regia was added to the ash and the mixture was digested on a hot plate at 105°C covering with the watch glass. The digested mixture was filtered into 50 mL volumetric flask and volume was made up to the mark with 2 M HNO_3 (Mapanda et al., 2007).

In the present study, digestion of the fruit samples was carried out following the method reported by Khan et al., (2010) with some modifications: Accurately weighed (1.000 g) powdered fruit sample was taken in a 50 mL conical flask to which 10 mL of concentrated HNO_3 was added and covered with the watch glass and left overnight without heating. Then, the flask was heated on a hotplate at 70°C until evaporation near to dryness. After cooling the sample contents to room temperature, 5.0 mL of $HClO_4$ was added to it and mixture was heated again at 70°C until the appearance of white dense fumes which was indication of the complete digestion. The digested samples were filtered into the clean 25 mL volumetric flasks and made up to the mark with 0.1 N HNO_3.

Experimental work related to the digestion of fruit samples was done using 65% nitric acid and 71% perchloric acid (analytical grade). Samples were dried in an electric oven (LDO-031SF, LabTech, Korea) and an electronic balance (BL-320H, Shimadzu, Japan) with readability of 0.001 g was used to weigh the samples.

2.4 Metal Analysis by Atomic Absorption Spectroscopy

Atomic absorption spectroscopy (AAS) is defined as a method for determining the concentration of an element (particularly a metal) present in a sample by measuring the intensity of external radiation absorbed by atoms produced from a sample at a wavelength characteristic for that element. In AAS, the analytical signal is produced due to the absorption of resonant radiation by ground state atoms of the analyte. This absorption of resonance radiation is highly selective and very sensitive. Therefore, AAS is considered as a very powerful method of analysis, which is nowadays used for trace and major elemental determinations for a wide variety of applications in analytical laboratories (Broekaert, 2002). In the present study, Flame Atomic Absorption Spectrophotometer (Shimadzu, AA670, Japan) was employed for the determination of selected metals in the fruit samples. In atomic absorption spectrophotometer the radiation source is line source particularly hollow cathode lamp (HCL) although for more volatile elements an electrodeless discharge lamp (EDL) may be used. The function of light source is to provide radiation of a specific wavelength for excitation of ground state atoms.

An atomizer is used to generate ground state atoms. Most commonly a flame is used for this purpose in which sample is introduced as aerosol or fine mist. Most prevalent flame in AAS is air-acetylene flame with the temperature of 2300-2500 K and it can be employed for about 40-50 elements in periodic table. However, for refractory elements, a

hotter N_2O-acetylene flame with temperature 3200-3500 K is recommended. Sample introduction system should be very efficient and reproducible in transferring the analyte to the atomizer. For the introduction of solution samples, pneumatic nebulizers are most widely used in which sample solution is sucked through the capillary tube. AAS is a selective method of analysis which may encounter spectral interferences thus a very highly efficient monochromator is required having a typical resolution of 0.02 nm. The main function of monochromator is to isolate or separate the wavelength of interest from the polychromatic light. The transmitted/isolated photons are detected by most frequently used detection system which is photomultiplier tube (PMT). Its basic function is to convert the light signal into an electrical signal. Its sensitivity depends upon the material coating of the cathode. The electrical signals coming from the PMT are fed into the amplifier, which amplifies these signals. In modern instrumentation, graphic presentations or digital displays on external computers or video display units are used as readout devices (Sneddon, 1997).

In this technique, a sample is heated in the flame to form dissociated atoms, which absorb light from the radiation source. There is a direct relationship between the quantity of light absorbed or absorbance (A) and the concentration of the element as expressed by Beer-Lambert's law. This linear relationship is used to calibrate the instrument thus quantification of the elements is based on calibration lines of standard solutions of elements, particularly salts of the metals, which are determined several times during the period of analysis. In AAS, linearity of the calibration curve is typically 3 orders higher than the detection limit (Lavinson, 2001; Reilly, 2002). AAS measures the concentration of metals down to part per billion (ppb or µg/g). The reason for the continuing popularity of AAS is primarily based on the fact that the instrument is relatively inexpensive; and in its different mode, it can be used to determine more than 60 elements in different matrix and in a wide range of concentration (Reilly, 2002).

In the present study, the quantification of fruit samples was performed for Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, Pb, Sr and Zn on Flame Atomic Absorption Spectrophotometer (Shimadzu AA-670, Japan) under optimum analytical conditions as shown in Table 2. Standard solutions of the metals (1000 mg/L) procured from Merck were used in this study. The stock solution of standards was prepared from the individual 1000 mg/L standards in 0.1 N HNO_3. Low and high concentration working standards were prepared by appropriate dilution of the stock solution with doubly distilled water.

Table 2. Optimum analytical conditions maintained on AAS for the analysis of selected metals using air-acetylene flame (Shimadzu AA-670, Japan)

Metal	Wavelength (nm)	HC lamp current (mA)	Slit width (nm)	Fuel-gas flow rate (L/min.)	1% Absorption concentration (ppm)
Ca	422.7	6.0	0.5	2.0	0.08
Cd	228.8	4.0	0.3	1.8	0.02
Co	240.7	6.0	0.2	2.2	0.20
Cr	357.9	5.0	0.5	2.6	0.09
Cu	324.8	3.0	0.5	1.8	0.09
Fe	248.3	8.0	0.2	2.0	0.10
K	766.5	5.0	0.5	1.9	0.04
Li	670.7	4.0	0.5	1.6	0.05
Mg	285.2	4.0	0.5	1.6	0.007
Mn	279.5	5.0	0.4	1.9	0.05
Na	589.0	6.0	0.5	1.6	0.02
Ni	232.0	4.0	0.15	1.7	0.10
Pb	217.0	7.0	0.3	1.8	0.20
Sr	460.7	4.0	0.5	1.6	0.10
Zn	213.9	4.0	0.5	2.0	0.02

2.5 Determination of Phytochemical Constituents

2.5.1 Reagents and Chemical Standards

Gallic acid ($C_7H_6O_5.H_2O$), rutin trihydrate ($C_{27}H_{30}O_{16}.3H_2O$) and ascorbic acid ($C_6H_8O_6$) were used as standards for the analysis of different phytochemicals. Other reagents and chemicals used were Folin-Ciocalteu reagent, sodium carbonate, 98% pure anhydrous aluminium trichloride (Merck), 99-100% pure sodium nitrite (Sigma-Aldrich), sodium hydroxide, sodium acetate, 85% phosphoric acid and 2,6-dichloroindophenol sodium salt hydrate. All the chemicals and reagents were obtained commercially and used without further purification. All solutions and dilutions were made in doubly-distilled water unless otherwise stated.

2.5.2 Preparation of the Standards

Four millimolar (mM) stock solution of gallic acid (3,4,5-trihydroxybenzoic acid) was prepared by dissolving 75 mg of dry gallic acid in 100 mL of distilled water. Stock solution was stored at 4°C. Working standards of concentrations ranging from 0.2 mM to 0.8 mM were prepared fresh by diluting the stock solution with distilled water.

Two mM stock solution of rutin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyloxy]-4*H*-chromen-4-one) was prepared by dissolving 133 mg of dry rutin in 100 mL of methanol. Stock solution was stored at 4°C. Working standards of concentrations ranging from 0.1 mM to 0.8 mM were prepared fresh by diluting the stock solution.

One mM stock solution of ascorbic acid ((5*R*)-[(1*S*)-1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5*H*)-one) was prepared by dissolving 17.6 mg of dry ascorbic acid in 100 mL of double distilled water. Stock solution was stored at 4°C. Working standards of concentrations ranging from 0.1 mM to 0.5 mM were prepared fresh by diluting the stock solution with distilled water.

2.5.3 Extraction of the Samples

Water and acetone extracts were prepared using Herolab centrifuge (Model Unicen FR, Germany). Accurately weighed 1.000 g of dried powdered fruit sample was taken in a centrifuge tube and soaked in 10 mL of distilled water and kept overnight. After this, homogenate was shaken and centrifuged at speed of 3800 rpm for 15 minutes and supernatant was filtered and collected in labelled clean plastic bottles. This extraction procedure was repeated four to five times to obtain 40-50 mL of the water extract and each time supernatants were filtered and collected in the plastic bottles. The precipitates left were further extracted four to five times in acetone (1:10 w/v) to obtain 40-50 mL of the acetone extracts and supernatants were again filtered and collected in separate bottles preferably of glass. Both water and acetone extracts were stored in the freezer before further processing (Ji et al., 2011). The water extract contain hydrophilic part while the acetone extract contain the lipophilic part of the phytochemical constituents.

2.5.4 Determination of Total Phenolic Contents (TPC assay)

Total phenolic content in water and acetone extract was measured according to the method reported by the Ji et al., (2011). Aliquots of 1.0 mL of water or acetone extract was mixed with 5.0 mL of 10 fold diluted Folin-Ciocalteu reagent and 4.0 mL of 7.5%

sodium carbonate solution. The mixture was allowed to stand at room temperature for 90 minutes. Subsequently the absorbance was measured at 765 nm spectrophotometrically. Colour of the mixture was deep blue at the time of absorbance measurement. Same procedure was repeated for the working standards containing dilutions of gallic acid and blank containing water or acetone instead of sample extract. The total phenolic contents were calculated and expressed as gallic acid equivalents (GAE).

2.5.5 Determination of Total Flavonoids

Total flavonoids content in water and acetone extract was determined according to the procedure described by Ji et al., (2011). A volume of 5.0 mL of water or acetone extract was transferred to the test tube to which 0.3 mL of 5% sodium nitrite solution was added and the tube was left for 5 minutes. Then, 0.3 mL of 10% aluminium trichloride solution was added to the tube. After 6 minutes, reaction was stopped by addition of 2 mL of 4% sodium hydroxide solution. Then, mixture was further diluted with distilled water up to 10 mL and left for 15 minutes at room temperature. The absorbance of the mixture was measured spectrophotometrically at 510 nm. Same procedure was repeated for the working standards containing rutin dilutions and blank containing water or acetone instead of sample extract. The flavonoid contents were calculated and expressed as rutin equivalents (RE).

2.5.6 Determination of Total Flavonols

Total flavonols content in water and acetone extract was determined following the method reported by Kumaran and Karunakaran, (2006). In this protocol, to 2.2 mL of water or acetone extract, 2.0 mL of 2% aluminium trichloride and 3.0 mL (50 g/L) sodium acetate solutions were added and the mixture was left for 2.5 hours. Then, absorbance of the mixture was measured at 440 nm at 20°C. Same procedure was repeated for the working standards containing rutin dilutions and blank containing water or acetone. The flavonols content was calculated and expressed as rutin equivalent (RE).

2.5.7 Determination of Ascorbic Acid

Ascorbic acid content was determined according to the method reported by Klein and Perry, (1982) with certain modifications. Aliquot of 5.0 mL of water or acetone extract was re-extracted with 10 mL of 1% Meta phosphoric acid for 45 minutes at room temperature. Then, 1.0 mL of the extract was mixed with 9.0 mL of 20 μM 2,6-

dichloroindophenol and the absorbance was measured spectrophotometrically within 30 minutes at 515 nm against blank. Results were expressed as ascorbic acid equivalent (AAE).

2.6 Determination of Antioxidant Activities

2.6.1 Reagents and Chemical Standards

Sample extraction was carried out using distilled water and acetone as described in the previous section. Gallic acid, rutin trihydrate and ascorbic acid were used as standards for performing different antioxidant activities. Other reagents and chemicals used were 96% sulphuric acid, 98-100% sodium phosphate monobasic, 99% ammonium molybdate tetrahydrate, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 98-100% sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium hexacyanoferrate (III), 99.5% trichloroacetic acid (TCA), 99% ferric chloride hexahydrate, 99.5% ferrous sulphate heptahydrate, 97% 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p*-disulfonic acid monosodium salt hydrate (ferrozine), 35% hydrogen peroxide and 99.5% 1,10-phenanthroline monohydrate. All the chemicals and reagents were obtained commercially and used without further purification. All solutions and dilutions were made in doubly distilled water unless otherwise stated.

2.6.2 DPPH Radical Scavenging Activity

The DPPH radical scavenging activity was estimated according to the method reported by Yu et al., (2002) and Aoshima et al., (2004) with slight modification**.** The basis of this method is the capability of antioxidants to scavenge the DPPH cation radical. In this assay, 2.0 mL of water or acetone extract was added to the 5.0 mL of 0.1 mM DPPH (prepared in methanol) and mixture was shaken vigorously. Then, it was incubated in the dark for 30 minutes at room temperature and the decolourization of DPPH was measured spectrophotometrically at 517 nm. Results were expressed as gallic acid equivalents (GAE) and % inhibition was calculated using the following relationship:

$$\%Inhibition = \frac{A_o - A_S}{A_o} \times 100 \qquad (02)$$

Where; A_o is the absorbance of the blank and A_s is the absorbance of the sample.

2.6.3 Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity of water and acetone extract was determined using the method described by Yu et al., (2004) with slight modification. This assay is based on Fenton reaction. Briefly 2.0 mL of 0.2 M phosphate buffer of pH 7.2, 0.04 mL of 0.02 M ferrous sulphate solution, 2.0 mL of water or acetone extract and 1.0 mL of 0.04 M 1,10 phenanthroline solution were delivered in to the test tube. The Fenton reaction was initiated by the addition of 0.1 mL of 7 mM H_2O_2. Absorbance was measured spectrophotometrically at 560 nm after 5 minutes incubation at room temperature. The percentage hydroxyl radical scavenging activity (SA) was calculated using the following relationship:

$$SA = \frac{A_o - A_S}{A_o} \times 100 \qquad (03)$$

Where, A_o is the absorbance of the blank and A_S is the absorbance of the sample.

2.6.4 Fe^{2+} Chelating Activity

Water and acetone extracts of the fruits were assessed for their ability to compete with ferrozine for ferrous ions in the solution. The chelating ability of ferrous ion of various fractions was estimated using method described by Dinis et al., (1994). Water or acetone extract (2.0 mL) was added to the 2.0 mL of 0.125 mM ferrous sulphate solution. The reaction was initiated by addition of 2.0 mL of 0.3125 mM ferrozine solution. Then, mixture was shaken vigorously and left to stand at room temperature for 10 minutes. Absorbance of the solution was measured spectrophotometrically at 562 nm against the blank. The percentage chelating activity (CA) of ferrozine-Fe (II) complex was calculated using the relationship:

$$CA = \frac{A_o - A_S}{A_o} \times 100 \qquad (04)$$

Where, A_o is the absorbance of the blank and A_S is the absorbance of the sample.

2.6.5 Ferric Reducing Antioxidant Power (FRAP Assay)

The ferric reducing powers of the water and acetone extracts were determined by the method reported by Hazra et al., (2008) with slight modification. Aliquot of 2.0 mL of water or acetone extract was mixed with the 2.0 mL of 0.2 M of phosphate buffer of pH 6.6 and 2.0 mL of 0.1% potassium ferricyanide solution. Then, flask was covered and heated in water bath at 50ºC for 20 minutes after which the reaction was stopped by the

addition of 2.0 mL of 10% trichloroacetic acid (TCA) solution. Then, 2.0 mL of upper portion of sample solution was mixed with 2.0 mL of distilled water and 2.0 mL of 0.01% of ferric chloride solution and it was left for 20 minutes at room temperature and absorbance was measured spectrophotometrically at 700 nm against the blank. A higher absorbance of reaction mixture indicated greater reducing power. Rutin, gallic acid or ascorbic acid can be used as positive control.

2.6.6 Phosphomolybdenium Complex Assay (PMA)

The total antioxidant activity of the water and acetone extracts was measured spectrophotometrically as reported by Prieto et al., (2006). Reagent solution was prepared by mixing equal volumes of 0.6 mol/L sulphuric acid, 28 mol/L sodium phosphate and 4 mol/L ammonium molybdate. Then, 2.0 mL of water or acetone extract was added to the 6.6 mL of the reagent solution in a tube. It was capped and heated in water bath at 95ºC for 90 minutes. After cooling it to the room temperature, absorbance was measured spectrophotometrically at 695 nm against the blank. The results were expressed as ascorbic acid equivalents (AAE).

In the present study, all measurement realted to the phytochemical analysis and the antioxidant activities of the fruit samples were performed on UV-VIS spectrophotometer (U2020, IRMECO-Gmbh, Geesthacht, Germany).

2.7 Statistical Analysis

Descriptive statistical data including minimum, maximum, mean, median, standard deviation (SD), standard error (SE), skewness and kurtosis were computed using MS-Excel software. These statistical variables reveal the spread, central tendency, dispersion, uncertainty and frequency distribution of the measured levels in the fruit samples (Dragovic and Mihailovi, 2009). Correlation study was also carried out in order to determine the extent of the relationship between the variables investigated in this study (Gong et al, 2010). Along with the basic statistical parameters and correlation study, multivariate method consisting of cluster analysis (CA) was also performed on the data-set using the STATISTICA software (StatSoft, 1999). There are varied opinions on the use of multivariate statistical methods in environmental studies, varying from sceptical, such as “complex and to some extent formal” to very optimistic like “the best tool for interpretation of environmental data and understanding the status of the environment”.

Multivariate statistical methods have many applications in environmental studies. They may be particularly useful when there is a large volume of experimental results and sometimes they provide imminent into the multidimensional patterns in the data that would be overlooked with univariate analysis. The purpose of such a statistical interpretation of the data is to try to make some assumptions on experiential variations in the data and the processes controlling the changes in concentrations, thus raising the knowledge on the environmental behaviour of different variables (Shtangeeva et al, 2009).

2.7.1 Cluster Analysis (CA)

Clustering is the statistical operation of grouping objects (individuals or variables) into a limited numeral of groups known as clusters or segments, which have two properties; they are not defined in advance by the analyst, but are discovered through the operation and the clusters are combination of objects having similar characteristics, which are separated from objects having dissimilar characteristics, thus resulting in internal homogeneity and external heterogeneity. The cluster to which each object belongs is not known in advance and the number of clusters is not fixed in advance. This is because there is no dependent variable, so clustering is basically descriptive not predictive (Toffery, 2011). The cluster analysis is normally used for non supervised classification of observations. It searches for patterns in a data set by grouping the observations into cluster. There are two general approaches to classify the observations, hierarchial clustering and partitioning. In hierarchial clustering we start with the number of clusters equal to the number of observations and end with one cluster. Partitioning is the reverse this process. One of the most popular hierarchial clustering methods is Ward's method, which is very efficient and uses analysis of variance approach to evaluate the distance between clusters and attempts to reduce the sum of the squares of any two clusters that can be formed at each step. A significant step in any clustering is to select a distance measure which determines how the similarity of two observations is calculated (Chwiej, 2010). Typical output is a dendrogram where the objects are set in a hierarchy reflecting their resemblance. Objects with similar patterns of attentiveness values are fused to a cluster on a low level in the dendrogram (Gottelt et al, 1997).

2.8 Health Risk Assessment

There are numerous pathways for exposure of the metals to humans, but food chain is the most important and noteworthy route. Assessment of health risk to human by the metals in food is most commonly gauged by the measurement of health risk index (HRI). Generally, the index value less than unity (1.0) shows safe levels while the values higher than 1 are associated with adverse health effects to the consumers. HRI depends on daily intake of the metals through consumption of the fruits and then comparing them to the prescribed reference oral dose (Cui et al., 2004; Li et al., 2012; Luo et al., 2011; Pandey et al., 2012; Singh et al., 2010). This index was calculated using following relationship:

$$HRI = \sum_{n}(C_n \times D_n) \Big/ RfD \times B_w \quad (05)$$

Where, 'C_n' represented the mean metal concentration in a specific fruit on fresh weight basis (mg/kg), 'D_n' denoted average daily intake rate of the fruit (100 g/day), 'RfD' showed safe level of exposure by oral for lifetime, and 'B_w' is the average body weight (70 kg for adult). In this study, the dietary reference intakes (DRI) of the elements were taken as RfD (FNB, 2004), except Cd and Pb, for which maximum allowed levels (ML) were considered (EC, 2006).

Non-carcinogenic health risks for humans associated with the consumption of these fruits were also assessed by calculating target hazard quotient (THQ) (Storelli, 2008; Yang et al., 2011). The method to estimate THQ was provided in USEPA Region III Risk-Based Concentration Table (USEPA, 2006):

$$THQ = (C \times I \times 10^{-3} \times EF_r \times ED_{tot}) \Big/ (RfD \times BW_a \times AT_n) \quad (06)$$

where, C is the mean metal level in fruit (mg/kg, fresh weight); I is the ingestion rate (100 g/day/person); EF_r is the exposure frequency; ED_{tot} is the total exposure duration (30 years); BW_a is the average body weight (70 kg); and AT_n is the averaging time, non-carcinogens (ED_{tot} x 365 day/year).

Target cancer risk (TCR) is used to assess the carcinogenic risks to the consumers from food consumption. The method to estimate TCR was also provided in USEPA Region III Risk-Based Concentration Table (USEPA, 2006):

$$TCR = (C_b \times I \times 10^{-3} \times CPS_o \times EF_r \times ED_{tot}) \Big/ (BW_a \times AT_c) \quad (07)$$

Where, CPS_o is the carcinogenic potency slope, oral $(\mu g/g/day)^{-1}$; AT_c is the averaging

time, carcinogens (70 x 365 days). Since CPS_o values were not available for all selected metals, so the TCR of Cd, Cr and Pb were calculated only to indicate the lifetime carcinogenic risk to the consumers (USEPA, 2010).

Chapter 3

RESULTS AND DISCUSSION

3.1 Layout of Data

Experimental data related to the distribution and covariation of selected essential and toxic trace metals, major phytochemical constituents and antioxidant activities in seasonally collected commercial fruits in the local market are presented in this chapter in various figures, graphs and tabular form. First part of this chapter deals with the distribution and correlations among selected metals (Na, K, Ca, Mg, Fe, Zn, Li, Sr, Cr, Mn, Cu, Ni, Co, Cd and Pb) in commercially available seasonal fruits. Distribution of the metals is discussed in terms of basic statistical parameters (minimum, maximum, mean, median, standard deviation (SD), standard error (SE), skewness and kurtosis) and quartile distribution, whereas, their interrelationships are envisaged in terms of the Pearson correlation coefficients. Variations in the metal concentrations in each fruit sample are also presented and discussed. Second part of the chapter deals with the quantitative variations of phytochemical constituents, including total phenolics, flavonoids, flavonols and ascorbic acid, followed by the measurements of antioxidant activities including DPPH radical scavenging activity, hydroxyl radical scavenging activity, Fe^{2+} chelating activity, ferric ion reducing antioxidant potential (FRAP) assay, and phosphomolybdenum assay (PMA). Statistical summery of the phytochemicals and antioxidant activities are discussed for their hydrophilic (water extract) and lipophilic (acetone extract) fractions and their relative variations in each fruit sample are also compared. Correlation coefficients were measured among the phytochemicals and antioxidant activities. Plausible associations between the metal contents and phytochemical constituents/antioxidant activity are also investigated. In the last part of the chapter, multivariate cluster analysis (CA) is employed for the source apportionment of the metals in the fruits, followed by the health risk assessment of the metals in the fruits in terms of health risk index (HRI), target hazard quotient (THQ) and target cancer risk (TCR). Salient findings emerging from the present study are then presented at the end.

The above outlined layout of the entire data forms the basis of an orderly sequence of discussion which now follows.

3.2 Distribution of Selected Metals in Fruits

Basic statistical parameters related to the distribution of selected metal levels (mg/kg, fresh weight) in seasonally available commercial fruits are presented in Table 3. Most of the metals exhibited broad range in their concentrations as specified by their minimum and maximum levels. Minimum concentration of as low as 0.006 mg/kg was found for Li and maximum concentration of as high as 6735 mg/kg for K. On the average basis, dominant levels were shown by Ca (1518 mg/kg), K (901.6 mg/kg) and Mg (140.0 mg/kg), followed by Na (75.66 mg/kg), Fe (5.229 mg/kg) and Mn (5.160 mg/kg). Relatively lower mean levels were observed for Zn (1.626 mg/kg), Sr (1.472 mg/kg), Ni (1.361 mg/kg), Pb (1.308 mg/kg), Cu (0.663 mg/kg), Co (0.374 mg/kg), Cr (0.312 mg/kg), Cd (0.166 mg/kg) and Li (0.107 mg/kg). The decreasing trend of average metal levels in the fruits revealed following order; Ca > K > Mg > Na > Fe > Mn > Zn > Sr > Ni > Pb > Cu > Co > Cr > Cd > Li.

Table 3. Statistical distribution parameters for selected metal levels (mg/kg, fresh weight) in the fruit samples

	Min	Max	Mean	Median	SD	SE	Skewness	Kurtosis
Na	1.173	748.1	75.66	22.74	146.8	23.50	3.201	11.60
K	277.6	6735	901.6	572.1	1180	188.9	3.954	16.93
Ca	134.5	5332	1518	1269	1139	182.5	1.452	2.365
Mg	26.42	496.4	140.0	104.5	113.1	18.11	1.907	3.536
Fe	0.236	24.63	5.229	3.263	5.547	0.888	2.403	5.984
Zn	0.038	13.74	1.626	0.819	2.463	0.394	3.567	15.41
Li	0.006	0.808	0.107	0.072	0.141	0.025	4.042	19.64
Sr	0.016	10.79	1.472	0.946	1.978	0.317	3.476	14.01
Cr	0.015	1.378	0.312	0.194	0.321	0.054	2.223	5.487
Mn	0.139	23.98	5.160	0.580	7.802	2.053	4.228	18.85
Cu	0.025	4.389	0.663	0.384	0.806	0.129	3.080	11.89
Ni	0.010	4.696	1.361	1.005	1.362	0.257	1.308	0.921
Co	0.015	2.049	0.374	0.190	0.448	0.075	2.162	5.162
Cd	0.004	1.211	0.166	0.128	0.205	0.034	3.964	19.28
Pb	0.162	7.525	1.308	1.125	1.189	0.190	3.944	20.05

The mean and median levels for the most of the metals were considerably different indicating their predominant non-Gaussian distribution in the fruits. Random distribution pattern of the metals in the fruits was also manifested by large range and considerably higher SD and SE values, particularly in the case of K, Ca, Na and Mg. Higher skewness and kurtosis indicated asymmetrical distribution for most of the metals, especially Mn, Li, Pb, Cd and K in the fruits. Among the selected metals, Pb, Cd, and Ni revealed more or less comparable mean and median levels and Ni showed lower skewness and kurtosis values thus reflecting somewhat normal distribution in the fruits.

The quartile distribution of selected metals in the fruits is also investigated and presented as a box and whisker plot in Figure 1. Relatively broad and random distribution in quartile levels was shown by Mn, Na, Co, Cu, Sr and Zn while somewhat narrow distribution was revealed by Cd, Ni and Li where upper and lower quartiles overlapped and their levels were lying between 25^{th} to 75^{th} percentiles. Among the metals, relatively symmetric distribution was observed for Mg. Overall; most of the metals showed random variation in the fruits or in other words quartile distribution of the metals in fruits samples under study were considerably divergent.

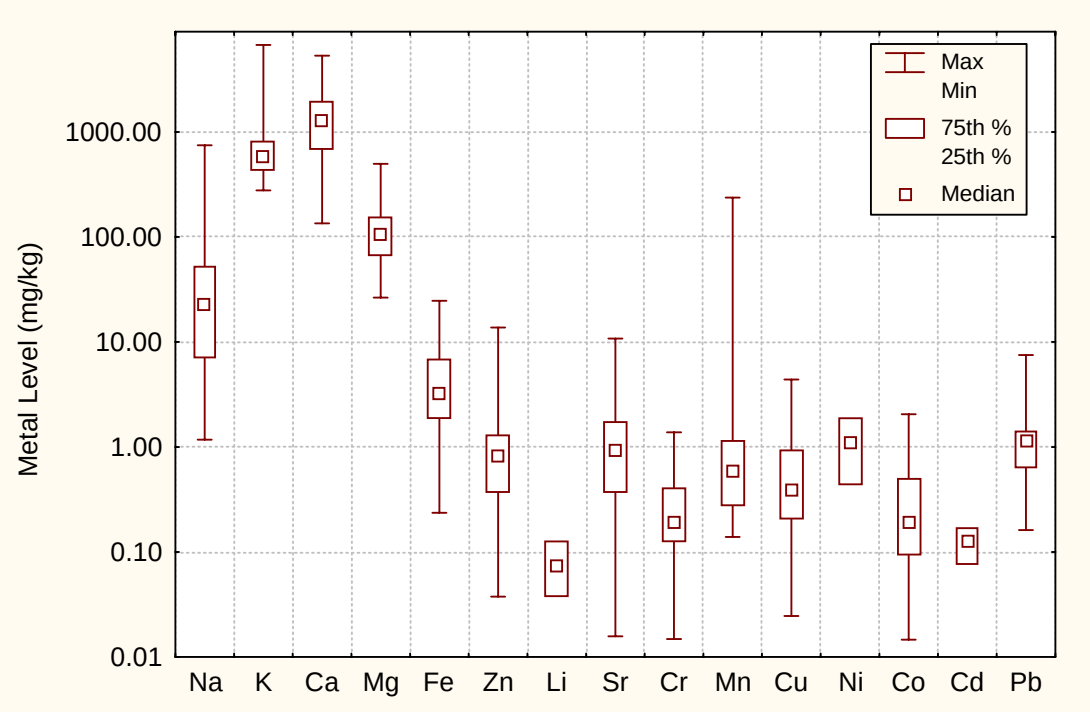

Figure 1. Box & Whisker plot showing the quartile distribution of selected metals in the fruit samples

Comparison of average metals levels in various commercially available market fruits is also made by categorizing the metals in different categories including macronutrients (Na, K, Ca and Mg), essential trace metals (Fe, Zn, Cu and Co) and other trace and toxic metals (Sr, Mn & Li, and Cr, Ni, Cd & Pb). Comparative evaluation of Na, K, Ca and Mg in various fruit samples is shown in Figure 2. All these metals were present in the fruits at highest concentration thus acting as macronutrients and carrying out essential biological processes (Stef et al., 2010). Among these macronutrients, lowest concentrations were found for Na in the fruits. However, relatively higher concentrations of Na were noted in canary melon (748.1 mg/kg), followed by galia melon (401.0 mg/kg), sugar apple (320.6 mg/kg), papaya (317.4 mg/kg), coconut (208.0 mg/kg) and prickly pear (203.9 mg/kg). On other hand, relatively low Na concentrations were found in peeled-off red apple (2.044 mg/kg), blood orange (1.173 mg/kg), peeled-off peach (3.356 mg/kg), pineapple (2.190 mg/kg), guava (3.306 mg/kg) and tangerine (1.648 mg/kg).

Measured concentrations of K were found to be exceptionally high in the fruit samples (Figure 2). Elevated K concentration was found in round jujube berries (6735 mg/kg), which indicated the uptake and enrichment of the metal in these fruits. Hence, the fruits may be used as a food supplement to overcome the K-deficiency (Mutalik et al., 2011). Other significant concentrations of K were observed in coconut (1765 mg/kg), Date (2654 mg/kg) and black mulberry fruit (4082 mg/kg). All fruit samples showed relatively higher concentrations of K compared to those of Na and Mg.

In the present study, Ca levels were found to be significantly high in most of the fruit samples (Figure 2). Significantly higher concentrations of Ca were noted in black mulberry fruit (5332 mg/kg), pear (3790 mg/kg), date (3175 mg/kg), kiwi (3677 mg/kg), sugar apple (2774 mg/kg) and blood orange (3596 mg/kg). Consequently, the consumption of these fruits may be recommended to address the Ca-deficiency particularly in women and children (Pettifor, 2008). In most of the fruits included in the present study, Mg concentrations were found to be lower than those of Ca and K but higher than that of Na (Figure 2). Comparatively higher concentration of Mg was present in date (496.4 mg/kg) and coconut (492.6 mg/kg), followed by sugar apple (358.5 mg/kg), black chaunsa mango (309.0 mg/kg), canary melon (258.5 mg/kg), banana (253.6 mg/kg), prickly pear (236.0 mg/kg) and cape gooseberry (223.2 mg/kg). All other fruit samples showed relatively lower Mg contents in the present study. Overall, most of the fruits were found to be good source of these macronutrients.

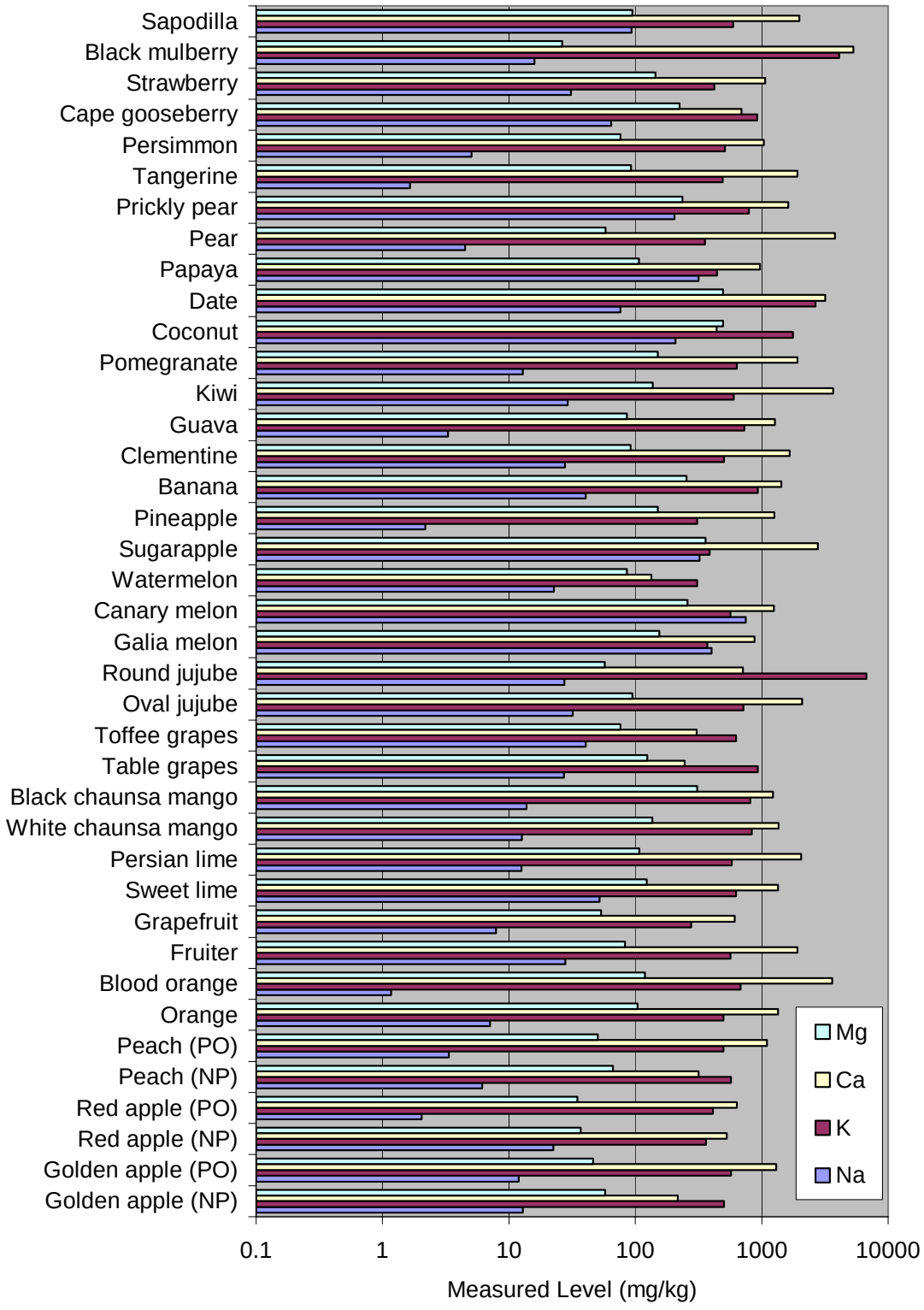

Figure 2. Comparison of the macronutrients (mg/kg, FW) in the fruits

Comparative levels of essential trace metals (Fe, Zn, Cu and Co) in various fruit samples are shown in Figure 3. Generally, these metals were present at intermediate to low concentrations in the fruit samples with some exceptions. Among these essential trace metals, Fe is considered as an important micronutrient for plants (Conte and Walker, 2011) and its concentrations were considerably higher as shown in Figure 3. Highest concentration of Fe was found in strawberry (24.63 mg/kg) and black mulberry fruit (23.79 mg/kg), followed by, date (16.93 mg/kg), sweet lime (10.57 mg/kg), round jujube berries (9.200 mg/kg), coconut (8.697 mg/kg), blood orange (8.132 mg/kg) and oval jujube berries (7.421 mg/kg). As a whole, most of the fruits revealed almost comparable concentration of Fe; however, relatively lower levels were noted in grapefruit (0.236 mg/kg).

Zinc belongs to a group of trace metals, which are essential for the growth and sustainability of humans, animals and plants but at very high concentrations, it is potentially dangerous for the biosphere (Gul et al., 2009). Comparative levels of Zn measured in various fruits are shown in Figure 3. Highest concentration of Zn was observed in black chaunsa mango (13.74 mg/kg), while lowest in non-peeled golden apple (0.038 mg/kg). Other fruits exhibiting significant concentration of Zn included non-peeled peach (5.762 mg/kg), round jujube berries (3.597 mg/kg), coconut (5.818 mg/kg), date (3.483 mg/kg), prickly pear (2.563 mg/kg), cape-gooseberry (2.380 mg/kg), black mulberry fruit (4.457 mg/kg) and pomegranate (1.753 mg/kg).

Copper has critical biological functions in plant evolution as a micronutrient; nonetheless, increased concentration in plant tissues can be exhibited by its phytotoxic influences (Xiong et al., 2006). Comparative levels of Cu measured in various fruits in the present study are shown in Figure 3. Highest concentration of Cu was observed in coconut (4.389 mg/kg), followed by strawberry (2.494 mg/kg), black chaunsa mango (1.652 mg/kg), date (1.602 mg/kg), table grapes (1.274 mg/kg) and white chaunsa mango (1.217 mg/kg) while grapefruit (0.155 mg/kg) exhibited lowest Cu contents among all the fruits.

Concentration of Co in most of the fruits was found to be the lowest among all four essential trace metals in the present study as shown in Figure 3. Highest concentration of Co was found in date (2.049 mg/kg), followed by coconut (1.481 mg/kg), round jujube berries (1,127 mg/kg) and black chaunsa mango (1.000 mg/kg), while, in case of kiwi and pomegranate, its concentration was below the detection limit of the instrument. Generally, appreciable levels of the essential metals were observed in most of the fruits in the present study.

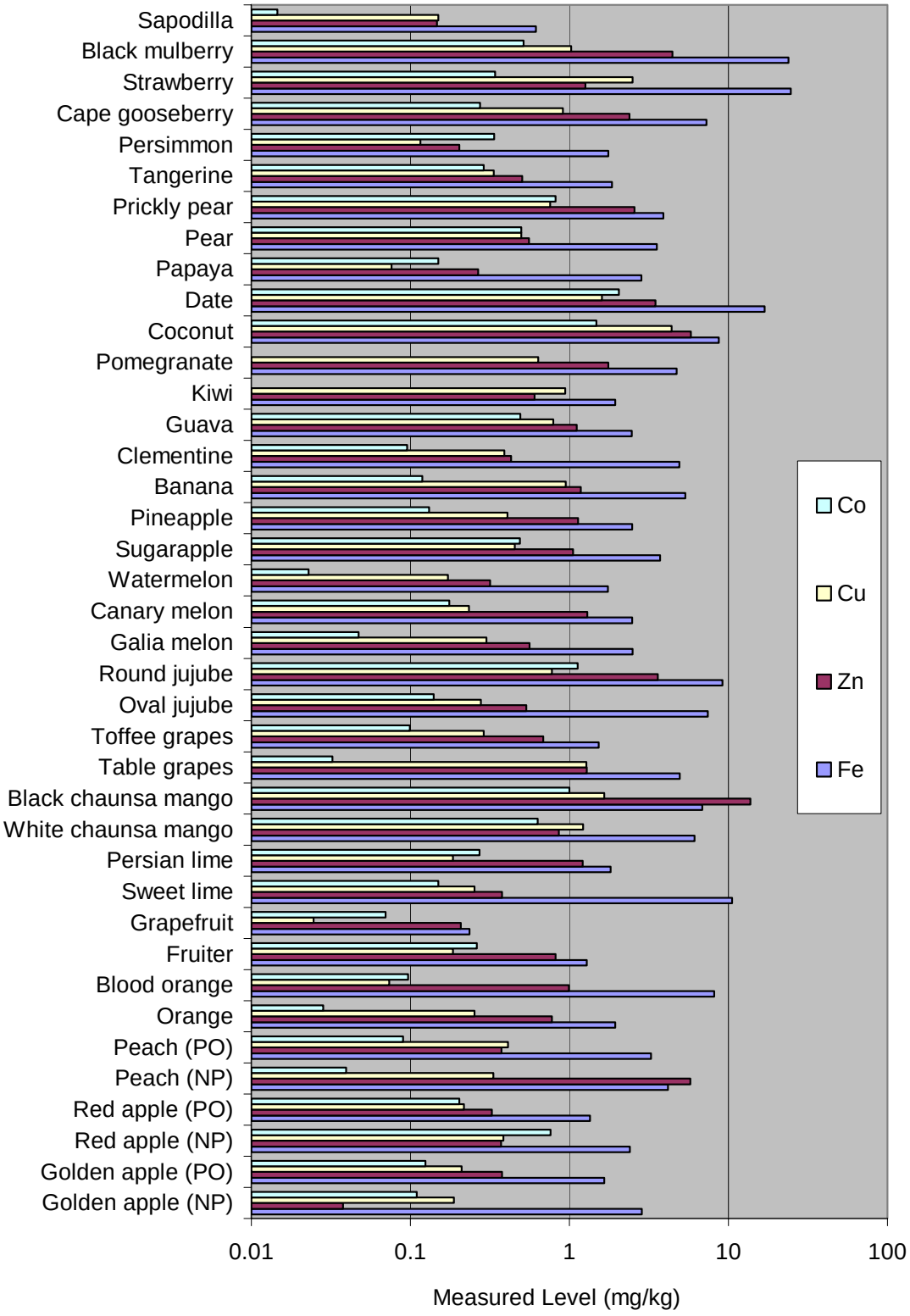

Figure 3. Comparison of the essential trace metals (mg/kg, FW) in the fruits

Comparative levels of Sr, Mn and Li in various fruit samples are shown in Figure 4. These metals were also recorded at intermediate to low concentration in the fruit samples with few exceptions. Among these metals, measured levels of Sr in most of the fruit samples were intermediate between Mn and Li. Highest concentration of Sr was found in prickly pear (10.79 mg/kg), followed by round jujube berries (7.094 mg/kg), sugar apple (3.235 mg/kg), papaya (3.135 mg/kg), sweet lime (2.254 mg/kg) and orange (1.921 mg/kg), whereas, its lowest concentration was present in pineapple (0.016 mg/kg). Concentration of Mn in the fruits were relatively higher among these trace metals in the present study. Highest concentration of Mn was shown by prickly pear (23.98 mg/kg), while other fruits showing significant concentration of Mn included date (7.728 mg/kg), coconut (6.316 mg/kg), strawberry (3.568 mg/kg), black mulberry fruit (2.702 mg/kg), banana (2.434 mg/kg) and round jujube berries (1.970 mg/kg). All other fruits depicted more or less lower and comparable Mn concentration.

Among the above mentioned three trace metals, Li concentration was the lowest and in some fruits, the concentration is too low to be detected by the instrument. Relatively higher concentration of Li was found in non-peeled red apple (0.808 mg/kg) while, other fruits with significant concentrations of Li included white chaunsa mango (0.149 mg/kg), black chaunsa mango (0.186 mg/kg), canary melon (0.225 mg/kg), coconut (0.231 mg/kg), date (0.205 mg/kg), prickly pear (0.175 mg/kg) and cape-gooseberry (0.163 mg/kg). Nonetheless, in peeled-off peach, blood orange, toffee grapes, round jujube berries and persimmon, Li concentration was below the detection limit of the instrument.

Comparative distribution of trace/toxic metals (Cr, Ni, Cd and Pb) in various fruit samples is shown in Figure 5. Comparison of average levels of Cr in various fruits revealed higher concentrations in date (1.378 mg/kg) and coconut (1.372 mg/kg), followed by banana (0.737 mg/kg), table grapes (0.626 mg/kg), blood orange (0.554 mg/kg) and white chaunsa mango (0.518 mg/kg). This showed significant bio-accumulation of Cr by these fruits. Nickel showed a wide range of concentration with some fruits exhibiting very high Ni levels and other showing Ni levels below the detection limits of the instrument. Relatively higher Ni concentration was recorded in kiwi (4.696 mg/kg), banana (4.358 mg/kg), sugar apple (3.946 mg/kg) and oval jujube berries (4.002 mg/kg) while, in non-peeled golden apple, orange, blood orange, black chaunsa mango, round jujube berries, pomegranate, date, pear, prickly pear, strawberry and black mulberry fruit, the metal levels were below the detection limit.

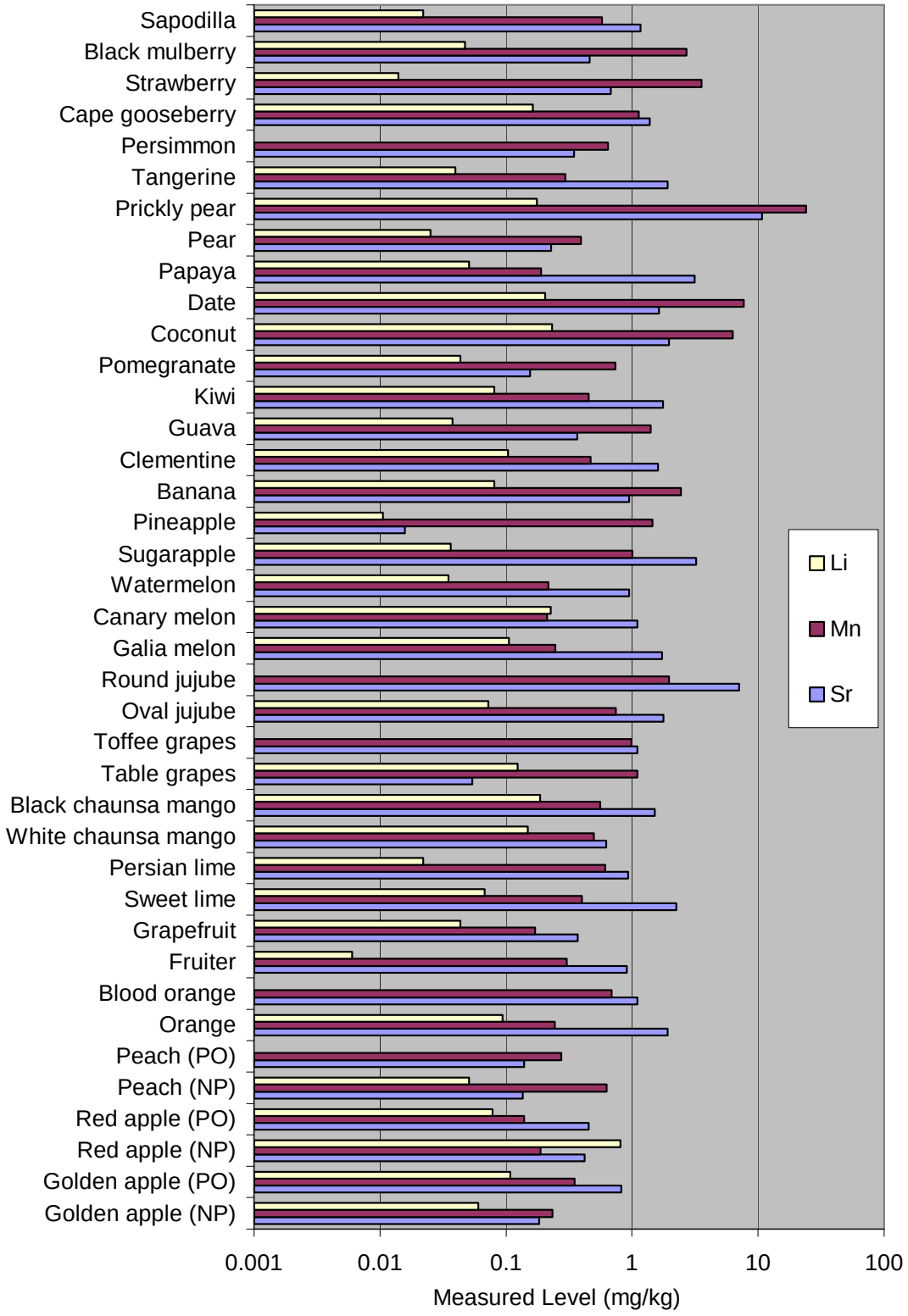

Figure 4. Comparison of the trace metals (mg/kg, FW) in the fruits

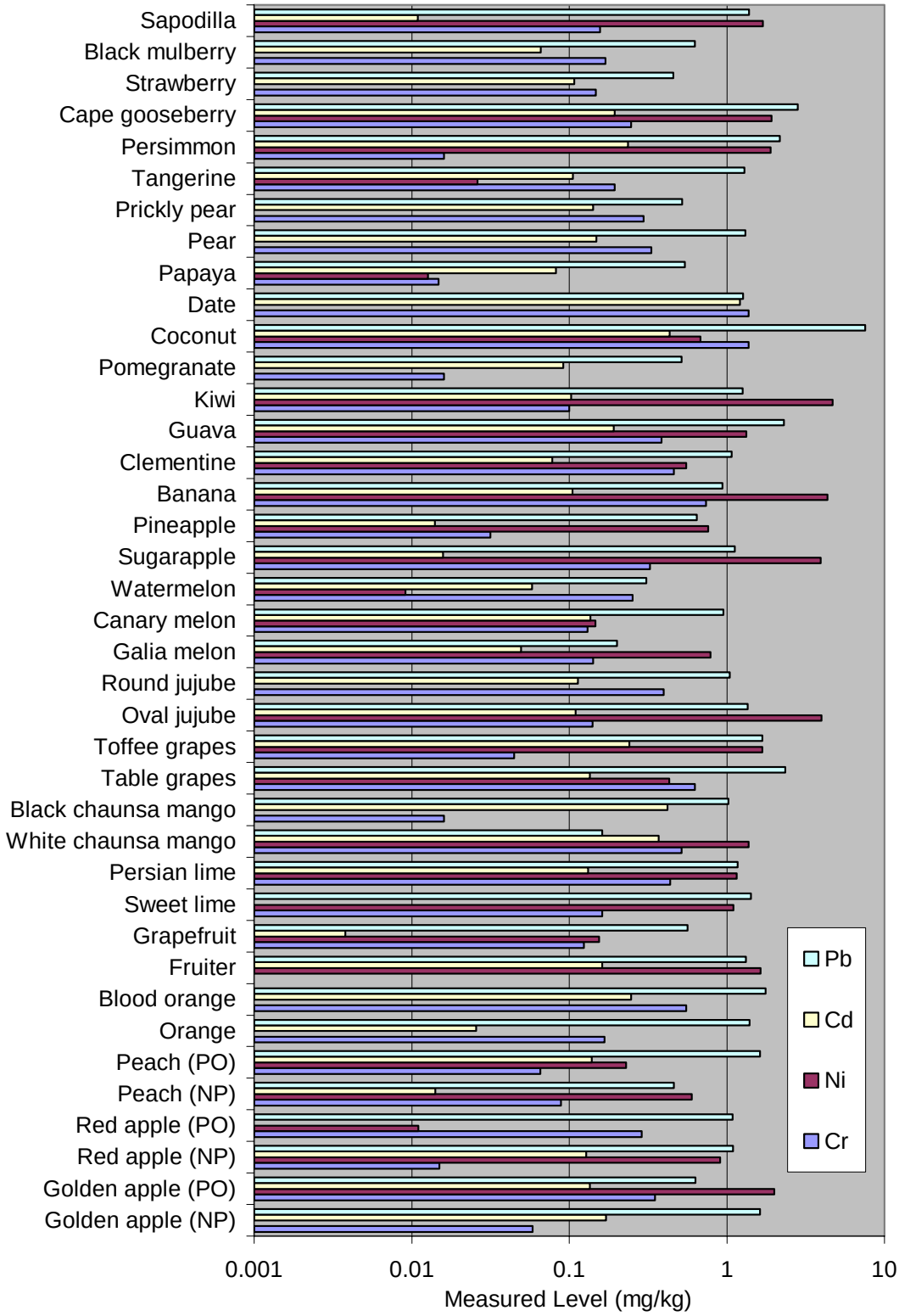

Figure 5. Comparison of trace/toxic metals (mg/kg, FW) in the fruit

Cadmium is one of the most toxic metals and its uptake and accumulation in the fruit is of major concern. Its input to the plants can proceed through the leaves (atmospheric deposition) or by root system (intake from the soil solution) (Grant et al., 1998). Comparative distribution of Cd in various fruits is also depicted in Figure 5. Highest Cd concentration was noted in date (1.211 mg/kg), followed by coconut (0.435 mg/kg), black chaunsa mango (0.419 mg/kg), white chaunsa mango (0.369 mg/kg), blood orange (0.248 mg/kg), persimmon (0.236 mg/kg), guava (0.192 mg/kg) and table grapes (0.135 mg/kg), while, in peeled-off red apple and sweet lime its concentration was found to be below the detection limit of the instrument.

Lead is also considered as a toxic metal which inhibits many enzymatic activities that regulate a normal biological functioning in plants and animals (Goering, 1993). Average concentrations of Pb measured in various fruit samples are also represented in Figure 5. Significantly higher concentration of Pb was noted in all fruit samples except white chaunsa mango which exhibited the lowest Pb contents (0.162 mg/kg). Highest level of Pb was observed in coconut (7.525 mg/kg), followed by cape-gooseberry (2.832 mg/kg), table grapes (2.349 mg/kg), guava (2.307 mg/kg) and persimmon (2.166 mg/kg). Other fruits also exhibited relatively higher concentrations of Pb compared to other toxic metals. Such appreciably higher contents of Pb in the fruits may be associated with anthropogenic intrusion of Pb in the local environment which would be further explored by multivariate statistics in latter section.

3.3 Correlation Study of Selected Metals in the Fruits

Correlation coefficients between selected metal pairs were calculated to investigate their inter-relationships in the seasonally available commercial fruit samples as shown in the correlation coefficient matrix for selected metals (Table 4) wherein the bold r-values are significant at $p < 0.05$. Very strong positive correlations were noted for following pairs; Cd-Co (r = 0.788), Mn-Sr (r = 0.777), Co-Cr (r = 0.742), Cd-Cr (r = 0.727), Cr-Mg (r = 0.725), Cu-Cr (r = 0.671), Co-Mg (r = 0.654), Cu-Mg (r = 0.645), Ni-Ca (r = 0.641), Pb-Cu (r = 0.636), Co-Cu (r = 0.632), Cd-Mg (r = 0.629), Cr-Li (r = 0.581), Cu-Fe (r = 0.533), Co-K (r = 0.533), Pb-Cr (r = 0.531), Co-Zn (r = 0.513) and Cu-Zn (r = 0.509). Some significantly positive correlations were observed among Mg-Na (r = 0.414), Fe-K (r = 0.495), Fe-Ca (r = 0.384), Zn-Mg (r = 0.454), Sr-K (r = 0.385), Cr-Zn (r = 0.478), Co-Fe (r = 0.417), Co-Li (r = 0.396), Co-Sr (r = 0.335), Cd-Fe (r = 0.360), Cd-Zn (r = 0.343),

Cd-Cu (r = 0.441), Pb-Mg (r = 0.436) and Pb-Co (r = 0.352). Correlation study indicated diverse and mutual relationship among the essential and trace metals in the fruit samples. Among various metals, Mn and Sr showed strong associations in the fruits while Cd, Co, Cr, Cu, Mg, Pb and Zn indicated strong relationships and mutual variations which may be attributed to their common origin in the fruits. This aspect requires further investigation by multivariate statistics to explore the complex nature of the relationships and apportionment among the metals in the fruits samples. Only weak negative correlations, indicating inverse relationships and opposing distribution were exhibited by Li-Ca (r = -0.230), Na-Ni (r = -0.122), Ni-Li (r = -0.107) and Pb-Ca (r = -0.147), thus reflecting somewhat divergent/dissimilar sources of these metals in the fruit samples. However, there were no significant inverse relationships among the metals.

Table 4. Correlation coefficient (r)* matrix for selected metals in the fruit samples

	Na	K	Ca	Mg	Fe	Zn	Li	Sr	Cr	Mn	Cu	Ni	Co	Cd	Pb
Na	1.000														
K	-0.066	1.000													
Ca	-0.058	0.210	1.000												
Mg	**0.414**	0.086	0.078	1.000											
Fe	-0.088	**0.495**	**0.384**	0.259	1.000										
Zn	-0.021	0.326	0.026	**0.454**	0.321	1.000									
Li	0.127	0.046	-0.230	0.145	-0.026	0.118	1.000								
Sr	0.259	**0.385**	0.014	0.232	0.040	0.135	0.054	1.000							
Cr	-0.015	0.302	0.116	**0.725**	0.295	**0.478**	**0.581**	0.067	1.000						
Mn	0.144	0.004	0.021	0.169	-0.012	0.076	0.090	**0.777**	0.024	1.000					
Cu	0.026	0.268	-0.029	**0.645**	**0.533**	**0.509**	0.157	0.061	**0.671**	0.051	1.000				
Ni	-0.122	0.148	**0.641**	0.232	0.149	-0.093	-0.107	0.258	0.055	0.111	0.034	1.000			
Co	0.023	**0.533**	0.186	**0.654**	**0.417**	**0.513**	**0.396**	**0.335**	**0.742**	0.203	**0.632**	0.032	1.000		
Cd	-0.035	0.239	0.145	**0.629**	**0.360**	**0.343**	0.241	0.004	**0.727**	0.010	**0.441**	-0.013	**0.788**	1.000	
Pb	0.021	0.092	-0.147	**0.436**	0.028	0.190	0.166	-0.048	**0.531**	-0.089	**0.636**	-0.013	**0.352**	0.270	1.000

*r-values shown in bold are significant at $p < 0.05$

3.4 Distribution of Phytochemical Constituents in the Fruits

Phytochemical constituents present in the fruits are considered very effective in the prevention of certain chronic diseases. Due to the prevalence of chronic diseases worldwide, the availability of information related to the phytochemical constituents and antioxidant rich foods may help individuals make informed choices in the consumption of foods that could help to protect them from such chronic diseases (Willcox et al., 2004). The experimental data based on fresh weight showed large variations in total phenolics, flavonoids, flavonols and ascorbic acid contents of the fruits estimated in this study. Basic statistical parameters related to the distribution of the phytochemical constituents (mg/100 g, fresh weight) in the available market fruits are presented in Table 5. All the phytochemicals exhibited broad range in their concentrations as specified by their minimum and maximum levels with minimum concentration of as low as 0.019 mg/100g, FW for ascorbic acid in the acetone extract (AE) and maximum concentration of as high as 879.7 mg/100 g, FW for flavonoids in the water extract (WE). On average basis, dominant levels were observed in the water extracts of the fruits, such as, flavonoids (195.1 mg RE/100 g, FW), followed by phenolics (137.1 mg GAE/100 g, FW), flavonols (102.1 mg RE/100 g, FW) and ascorbic acid (2.174 mg AAE/100 g, FW), while relatively lower mean levels were observed in the acetone extracts of the fruits; flavonoids (27.81 mg RE/100 g, FW), followed by flavonols (26.14 mg RE/100 g, FW), phenolics (19.86 mg GAE/100 g, FW) and ascorbic acid (1.308 mg AAE/100 g, FW). The decreasing trend of average levels in the fruits revealed following order; flavonoids (WE) > phenolics (WE) > flavonols (WE) > flavonoids (AE) > flavonols (AE) > phenolics (AE) > ascorbic acid (WE) > ascorbic acid (AE). Measured mean and median levels of the phytochemical constituents were considerably divergent indicating their random distribution in the fruit samples. The random distribution for most of the phytochemical constituents in the fruit samples were also manifested by large range and considerably higher SD and SE values, particularly in the case of water extracts of flavonoids, phenolics and flavonols. Relatively higher skewness and kurtosis values indicated asymmetrical and random distribution for the phytochemicals especially water and acetone extract of flavonoids and of ascorbic acid. Among the selected phytochemical constituents, water extract of flavonols reflected low skewness and kurtosis values thus reflecting somewhat normal distribution in the fruit samples. Overall, most of the fruit samples were found to be enriched source of the phytochemicals which are beneficial for the health of consumers.

Table 5. Statistical distribution parameters for phytochemical constituents in the fruit samples

		Min	Max	Mean	Median	SD	SE	Skew	Kurtosis
Phenolics (mg	WE	20.22	516.0	137.1	113.7	97.14	15.55	1.916	5.230
GAE/100 g, FW)	AE	0.535	94.17	19.86	9.724	22.70	3.784	1.940	3.488
Flavonoids (mg	WE	12.53	879.7	195.1	131.7	170.2	27.26	2.339	6.612
RE/100 g, FW)	AE	0.498	214.3	27.81	14.12	39.83	6.831	3.474	14.70
Flavonols (mg	WE	0.685	346.8	102.1	81.19	80.86	12.95	1.213	1.381
RE/100 g, FW)	AE	0.804	110.3	26.14	14.71	26.38	4.224	1.609	2.083
Ascorbic Acid (mg	WE	0.534	10.39	2.174	1.642	1.845	0.295	2.827	10.03
AAE/100g, FW)	AE	0.019	7.256	1.308	0.915	1.503	0.274	2.487	7.852

Average concentrations of the phytochemicals in the available fruits were also compared on individual basis as shown in Figures 6 to 9. Generally higher levels of the phytochemical constituents were noted in the water extracts of the fruits. Comparative evaluation of total phenolic contents in the fruits is shown in Figure 6. Significantly higher phenolic contents were recorded in water extracts of date (516.1 mg GAE/100 g), followed by table grapes (342.5 mg GAE/100 g), banana (298.2 mg GAE/100 g) and black chaunsa mango (282.1 mg GAE/100g), while canary melon (20.22 mg GAE/100 g) and prickly pear (20.73 mg GAE/100 g) showed the lowest phenolic contents among the water extracts of the fruits. On other hand, acetone extracts showed comparatively lower phenolic levels than their water extracts. Among them, relatively higher phenolic contents were found in black chaunsa mango (94.16 mg GAE/100 g), followed by pomegranate (83.04 mg GAE/100 g), toffee grapes (65.40 mg GAE/100 g) and strawberry (50.40 mg GAE/100 g) while acetone extracts of canary Melon, coconut and date depicted the lowest quantities of total phenolics which were below the detection limit of the instrument.

Total flavonoid contents in the collected fruit samples exhibited diverse variations and highest quantities, since these pigments are responsible for the red and blue colour (Grlesbach, 1984). The comparison of flavonoid contents in different fruits is shown in Figure 7. Among the water extracts of the fruits, date contained the highest flavonoid contents (879.7 mg RE/100 g), followed by table grapes (597.4 mg RE/100 g), peeled-off golden apple (552.0 mg RE/100 g) and banana (398.4 mg RE/100 g) whereas, coconut

(12.53 mg RE/100 g), sapodilla (24.41 mg RE/100 g) and canary melon (35.42 mg RE/100 g) showed lower amounts of the flavonoid in their water extracts. Acetone extracts of the fruits showed lower flavonoid levels than the counterpart water extracts. In acetone extracts, highest flavonoid contents were noted in pomegranate (214.3 mg RE/100 g), followed by black chaunsa mango (96.58 mg RE/100 g), guava (81.37 mg RE/100 g) and toffee grapes (55.19 mg RE/100 g). Flavonoid contents in the acetone extract of date, coconut, prickly pear, peeled-off golden apple and galia melon were below the detection limit of the instrument. However, smallest detected flavonoid level was observed in acetone extract of sapodilla (0.498 mg RE/100 g).

Total flavonol contents in the fruits (expressed as mg rutin equivalent/100 g, fresh weight) are shown in Figure 8. Among water extracts of the fruits, highest contents were shown by the table grapes (346.8 mg RE/100 g), followed by non-peeled red apple (285.1 mg RE/100 g), peeled-off golden apple (262.2 mg RE/100 g), date (230.2 mg RE/100 g) and banana (222.6 mg RE/100 g). However, among water extracts of the fruits, lowest levels were shown by the coconut in which concentration of total flavonols is too low to be detected by the instrument. Smallest detected total flavonol level was noted in sapodilla (0.685 mg RE/100 g). In case of acetone extracts, highest contents were found in black chaunsa mango (110.3 mg RE/100 g), followed by toffee grapes (84.01 mg RE/100 g), pomegranate (80.53 mg RE/100 g) and kiwi (79.81 mg RE/100 g), whereas, the lowest contents were observed in canary melon (0.804 mg RE/100 g), galia melon (2.501 mg RE/100 g) and sapodilla (1.979 mg RE/100 g). Nevertheless, total flavonol contents of toffee grapes were more or less comparable in water (81.19 mg RE/100 g) and acetone (84.01 mg RE/100 g) extracts.

Ascorbic acid contents were found to be the lowest among selected phytochemical constituents in most of the fruit samples (Figure 9). In water extracts, highest ascorbic acid contents were found in date (10.39 mg AAE/100 g), followed by banana (6.264 mg AAE/100 g), table grapes (5.074 mg AAE/100 g), peeled-off golden apple (3.648 mg AAE/100 g) and black mulberry (3.809 mg AAE/100 g). Most of the fruits showed relatively lower ascorbic acid contents which may be partially due to the fact that during the extraction process ascorbic acid is highly unstable and may be oxidized to deascorbate ion which is not detected under experimental conditions (Maria et al., 2006). Lowest ascorbic acid contents were observed in persimmon (0.534 mg AAE/100 g), peeled-off red apple (0.632 mg AAE/100 g) and non-peeled red apple (0.698 mg AAE/100 g).

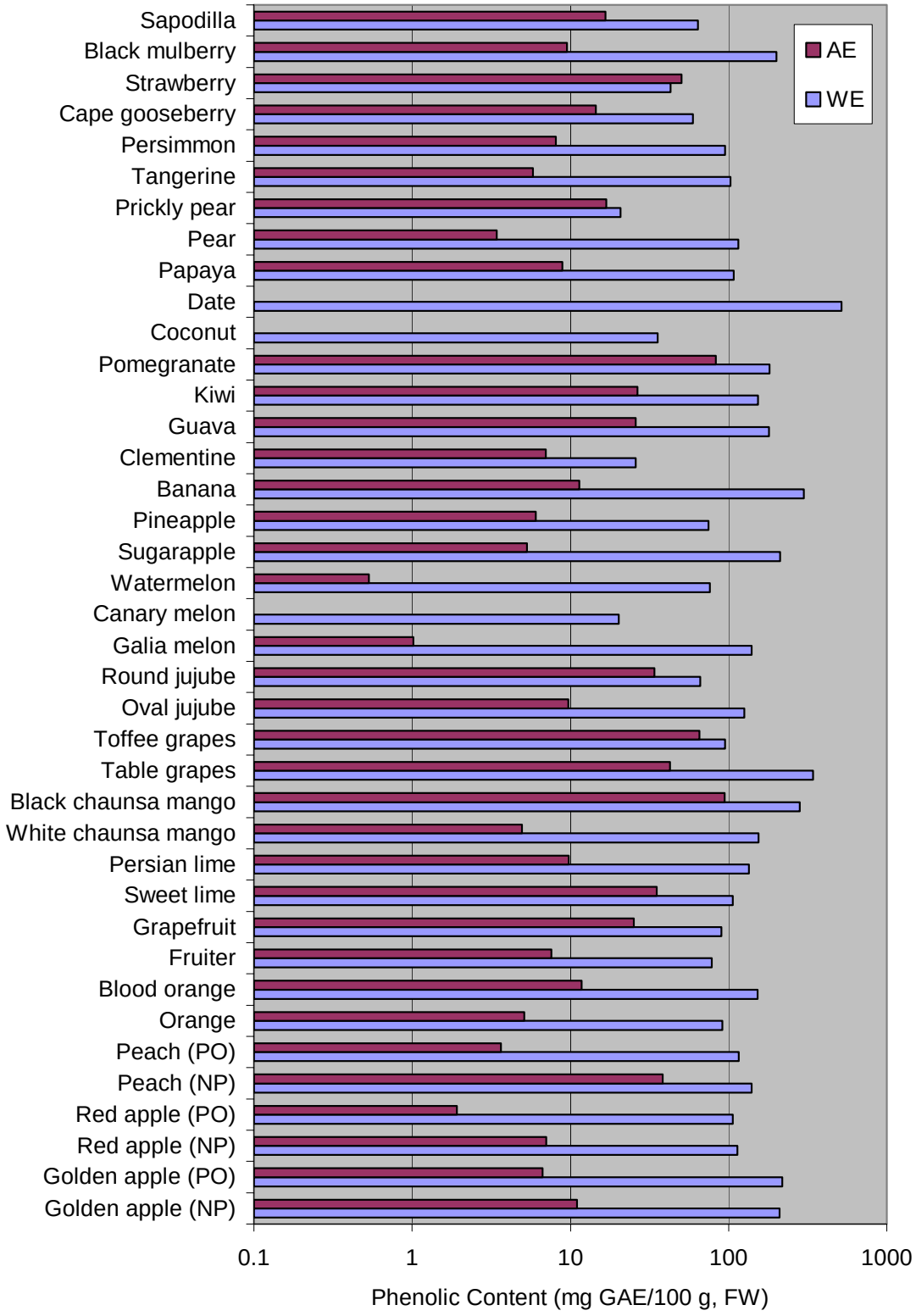

Figure 6. Comparison of total phenolic contents (mg GAE/100 g, FW) in the fruits

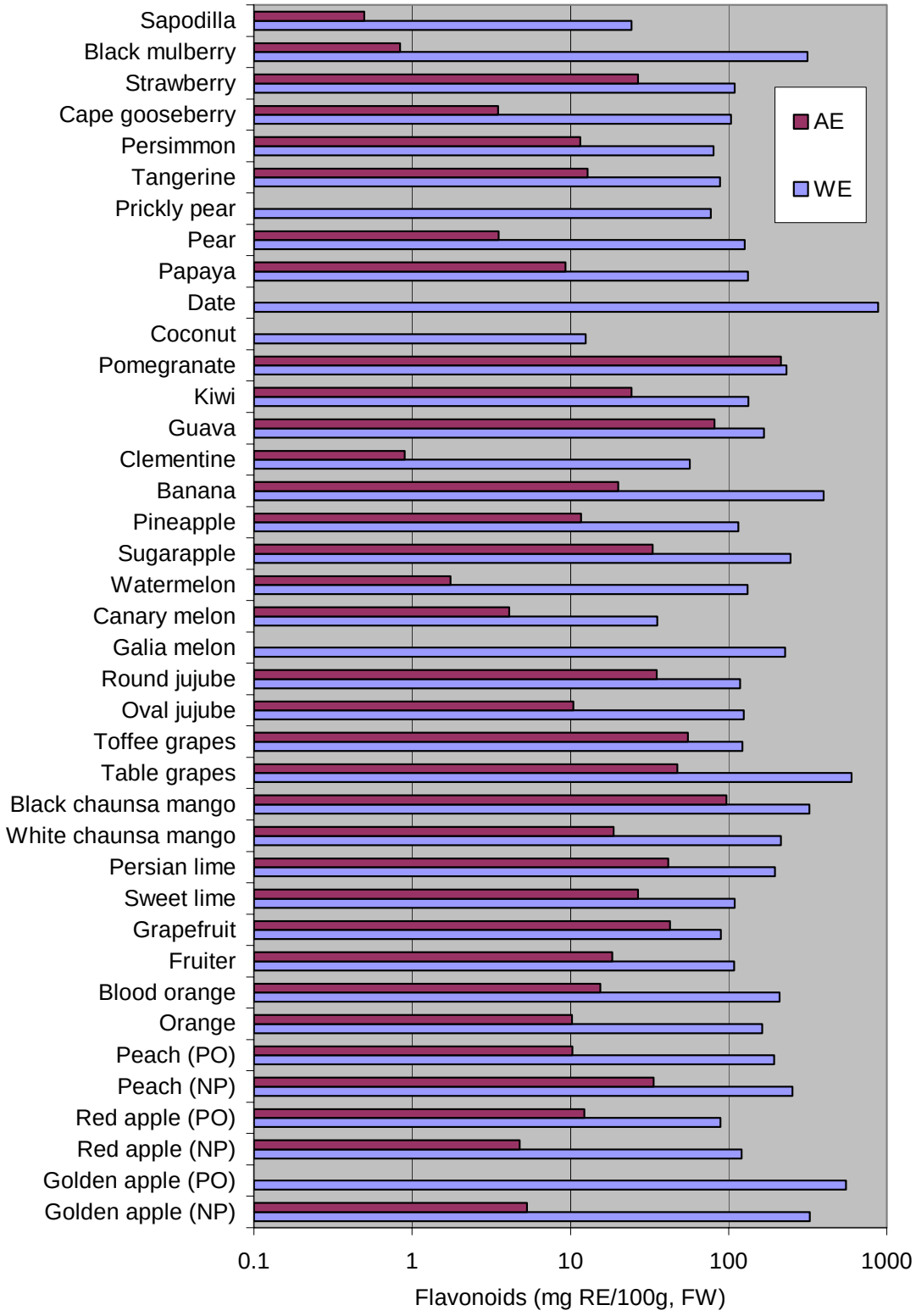

Figure 7. Comparison of total flavonoid contents (mg RE/100 g, FW) in the fruits

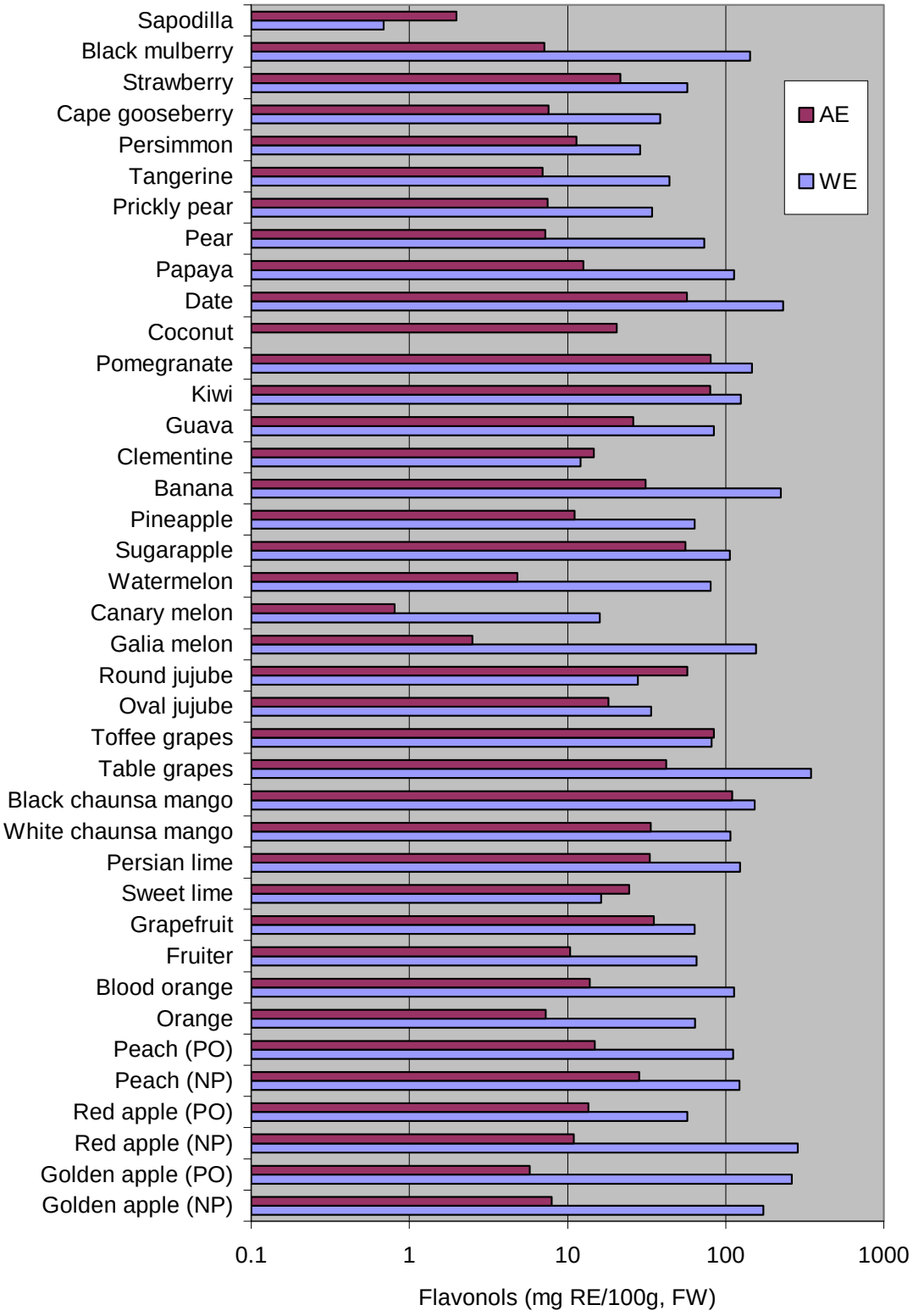

Figure 8. Comparison of total flavonol contents (mg RE/100 g, FW) in the fruits

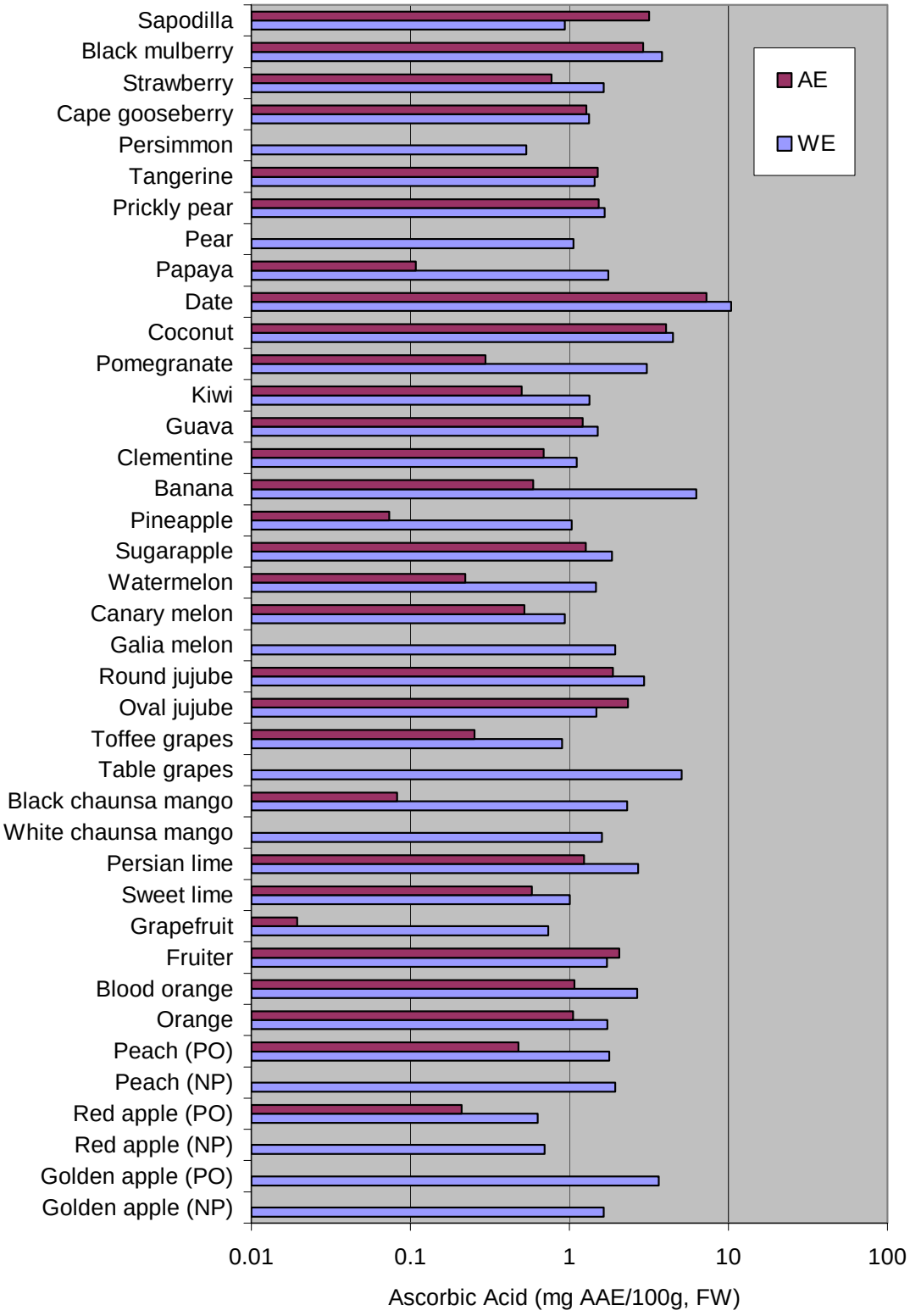

Figure 9. Comparison of total ascorbic acid (mg AAE/100 g, FW) in the fruits

In case of acetone extracts of the fruits (Figure 9), relatively lower concentrations were noted in most of the fruits while in galia melon, white chaunsa mango, non-peeled peach, non-peeled golden apple, peeled-off golden apple, table grapes, pear, persimmon and non-peeled red apple ascorbic acid contents were below the detection limit of the instrument. Overall, acetone extract contained small fraction of the ascorbic acid in the fruits. As shown in Figure 9, in case of acetone extracts of the fruits, highest ascorbic acid contents were observed in the date (7.256 mg AAE/100 g), followed by coconut (4.069 mg AAE/100 g), sapodilla (3.163 mg AAE/100 g), black mulberry fruit (2.919 mg AAE/100 g), oval jujube berries (2.339 mg AAE/100 g) and fruiter (2.059 mg AAE/100 g). Smallest detected ascorbic acid contents were observed in case of grapefruit (0.019 mg AAE/100 g) and pineapple (0.074 mg AAE/100 g). More or less equivalent ascorbic acid contents were noted in cape-gooseberry (1.326 mg AAE/100 g in WE, and 1.281 mg AAE/100 g in AE) and tangerine (1.436 mg AAE/100 g in WE, and 1.506 mg AAE/100 g in AE). Generally, acetone extract only contained small fraction of the ascorbic acid contents as most of the ascorbic acid was extracted in the water extract of the fruits. Overall, most of the phytochemical constituents showed predominantly hydrophilic character and consequently most of the ingested contents in food are bioavailable to the consumers.

3.5 Correlation Study of Phytochemical Constituents in the Fruits

Correlation coefficients among the phytochemical constituents were calculated to investigate their inter-relationships in the fruit samples as shown in the correlation coefficient matrix for the phytochemical constituents (Table 6), wherein the bold r-values are significant at $p < 0.05$. Very strong positive correlations were noted in the water extract of following pairs; phenolics and flavonoids ($r = 0.934$), phenolics and flavonols ($r = 0.744$), phenolics and ascorbic acid ($r = 0.783$), flavonoids and flavonols ($r = 0.779$), flavonoids and ascorbic acid ($r = 0.825$) and flavonols and ascorbic acid ($r = 0.599$). This showed the simultaneous accumulation and inter-conversion of these phytochemiacls in the fruit samples. Nevertheless, phytochemicals in the acetone extract also showed some strong positive correlations as depicted by following pairs; phenolics and flavonoids in AE ($r = 0.777$), phenolics and flavonols ($r = 0.803$) and flavonoids and flavonols ($r = 0.694$). A few strong correlations were also observed among the phytochmicals in water and acetone extracts as shown by flavonoids in WE with ascorbic acid in AE ($r = 0.547$) and ascorbic acid in WE with ascorbic acid in AE ($r = 0.726$). Importantly, there were no

significant inverse relationships among the phytochemicals; hence they showed similar behaviour in the fruit samples.

Table 6. Correlation coefficient matrix for phytochemical constituents in the fruit samples

		Phenolics		Flavonoids		Flavonols		Ascorbic Acid	
		WE	AE	WE	AE	WE	AE	WE	AE
Phenolics	WE	1.000							
	AE	0.269	1.000						
Flavonoids	WE	**0.934**	0.171	1.000					
	AE	**0.360**	**0.777**	0.267	1.000				
Flavonols	WE	**0.744**	0.113	**0.779**	0.217	1.000			
	AE	**0.431**	**0.803**	0.282	**0.694**	0.223	1.000		
Ascorbic Acid	WE	**0.783**	0.155	**0.825**	0.249	**0.599**	0.252	1.000	
	AE	**0.460**	-0.288	**0.547**	-0.271	0.257	-0.062	**0.726**	1.000

*r-values shown in bold are significant at $p < 0.05$

3.6 Statistical Summary of Antioxidant Activities in the Fruits

Five antioxidant assays were performed during the present study which included DPPH (2,2–diphenyl–1–picrylhydrazyl) radical scavenging activity, hydroxyl radical ($OH^{\bullet}$) scavenging activity, Fe (II) chelating activity, ferric reducing antioxidant power (FRAP) and Phosphomolybdenium assay (PMA). The details regarding experimental protocols of these assays have already been described in Chapter 2. The bleaching action is mainly attributed to the presence of polyphenols and ascorbic acid extracted into the solution (Lamien-Meda et al., 2008). Basic statistical parameters related to the distribution of the antioxidant activities in the fruits are presented in Table 7. All the antioxidant assays exhibited broad range as specified by their minimum and maximum values. In case of DPPH radical scavenging activity, minimum activity was as low as 13.16% in water extract of galia melon and maximum activity of as high as 95.38% in acetone extract of round jujube berries. In, hydroxyl radical scavenging activity, minimum value was as low as 4.930% in water extract of table grapes and maximum as high as 96.88% in acetone extract of peeled-off golden apple. In case of iron (II) chelating activity, minimum activity

was as low as 1.623% in water extract of peeled-off golden apple and maximum activity of as high as 95.26% in acetone extract of white chaunsa mango. On the other hand, among the total antioxidant activity assays, FRAP assay showed minimum value of 0.616 mg GAE/100 g, FW in acetone extract of galia melon and maximum value of 295.6 mg GAE/100 g, FW in water extract of date. Similarly, for Phosphomolybdenum assay, minimum measured value was 0.742 mg AAE/100 g, FW in acetone extract of canary melon and maximum value of 392.5 mg AAE/100 g, FW in water extract of date.

Table 7. Statistical distribution parameters for antioxidant activities in the fruit samples

		Min	Max	Mean	Median	SD	SE	Skew	Kurtosis
DPPH radical scavenging	WE	13.16	93.98	64.569	71.05	20.72	3.406	-0.833	-0.013
activity (%)	AE	32.17	95.39	52.72	47.95	17.98	2.879	0.849	-0.297
Hydroxyl radical	WE	4.930	85.54	38.57	42.64	19.84	3.307	0.049	-0.556
scavenging activity (%)	AE	27.94	96.88	81.9	83.89	13.14	2.105	-2.089	6.590
Iron (II) chelating activity	WE	1.623	57.73	32.20	32.11	11.02	1.788	0.029	0.934
(%)	AE	13.44	95.26	37.92	39.74	12.85	2.058	2.123	9.790
FRAP assay (mg GAE/100	WE	5.761	295.6	57.01	44.72	45.67	7.314	4.050	20.15
g, FW)	AE	0.616	78.91	17.18	10.88	17.39	2.785	1.981	4.505
Phosphomolybdenium	WE	3.146	392.5	41.16	34.62	61.64	9.870	5.123	29.35
assay (mg AAE/100g, FW)	AE	0.742	143.0	26.10	17.59	25.95	4.156	2.665	10.04

On the average basis, among % radical scavenging activities, higher mean value was shown by the hydroxyl radical scavenging activity in acetone extract of the fruits (81.9%), followed by DPPH radical scavenging activity in water extracts (64.56%) and in acetone extract (52.72%) of the fruits, while relatively lower mean values were observed for Iron (II) chelating activity in water (32.20%) and acetone (37.92%) extracts of the fruits. In case of total antioxidant activity assays, highest mean value was shown by FRAP assay in water extract of the fruits (57.01 mg GAE/100 g, FW) while lowest mean value was observed for the same assay in acetone extract of the fruits (17.18 mg GAE/100 g, FW). In case of % scavenging activities, the difference of median from the mean values was relatively low, especially in the case of hydroxyl radical scavenging activity in

acetone extract (mean = 81.90 & median = 83.89) and in case of Iron (II) chelating activity in both water (mean = 32.20 & median = 32.11) and acetone (mean = 37.92 & median = 39.74) extracts of the fruits, indicating that statistical distribution of the data were relatively normal/Gaussian and symmetrical. On other hand, for total antioxidant activities including FRAP assay and Phosphomolybdenum assay, distribution of data were relatively random and asymmetrical as shown by divergent mean and median values. In most of the antioxidant assays, relatively low SD and SE values suggested low dispersion and moderately normal distribution pattern particularly in the case of Iron (II) chelating activity of the fruits. Similarly, comparatively lower skewness and kurtosis values indicated fairly symmetrical distribution in the fruit samples.

Antioxidant activities measured in the available commercial fruits were also compared on individual basis as shown in Figures 10 to 14. The DPPH radical scavenging activity of the fruits is compared as shown in Figure 10. Among the water extracts of the fruits, highest activity was shown by the sapodilla (93.98%), followed by persimmon (91.19%), canary melon (86.49%) and date (86.57%) while lowest detected activity was shown by the galia melon (13.16%) and watermelon (16.83%). DPPH radical scavenging activity in peeled-off golden apple and table grapes was too low to be detected by the instrument. Overall, among the water extracts of the fruits, date, round jujube berries, clementine, kiwi, canary melon, persimmon, non-peeled and peeled-off red apple, tangerine, prickly pear and sapodilla revealed >80% DPPH radical scavenging activity. Nonetheless, in case of acetone extract, highest activity was shown by round jujube berries (95.38%), followed by strawberry (92.68%) and guava (88.75%) while; canary melon (32.17%) and watermelon (32.87%) showed the lowest activity in acetone extract of the fruits. Overall, guava, round jujube berries and strawberry revealed >80% DPPH radical scavenging activity in their acetone extracts. More or less comparable DPPH scavenging activity was found in the water and acetone extracts of peeled-off peach (40.45% in WE and 40.02% in AE) and papaya (43.16% in WE and 42.81% in AE).

Hydroxyl radicals ($OH^{\bullet}$) are extremely reactive and may be generated in the human body under physiological conditions, where they can react with non-selective compounds such as proteins, DNA, unsaturated fatty acids and almost every biological membrane (Halliwell, 1994). Figure 11 shows the comparative evaluation of OH radical scavenging activity estimated in the fruits. Acetone extracts of most of the fruits were very good OH radical scavengers. Among the water extract of the fruits, highest activity was manifested by coconut (85.54%), followed by persimmon (68.45%), peeled-off red apple (63.60%)

and sapodilla (58.50%). In galia melon, strawberry and peeled-off golden apple the activity was below the detection limit of the instrument while very low activity was observed in the water extract of table grapes (4.930%), watermelon (7.724%), pineapple (8.135%) and banana (9.532%). Among the fruits, only coconut in its water extract depicted >80% OH radical scavenging activity. Nevertheless, in case of acetone extract, highest scavenging activity was shown by the peeled-off (96.88%) and non-peeled golden apple (96.47%), non-peeled red apple (96.71%), pear (96.06%), peeled-off peach (94.33%) and sugar apple (93.34%) while lowest scavenging activity was shown by strawberry (27.94%). Many fruits, such as white and black chaunsa mango, peeled-off and non-peeled peach, peeled-off and non-peeled golden apple, table grapes, sweet lime, pear, peeled-off and non-peeled red apple, sugar apple, banana, galia melon, guava, clementine, canary melon, pineapple, persimmon, cape-gooseberry, black mulberry fruit, persian lime, sapodilla, toffee grapes and watermelon in their acetone extract revealed >80% scavenging activity.

Iron (II) chelating ability of the extract measures the effectiveness of the extract to compete with ferrozine for ferrous ion (Fe^{2+}). The iron–ferrozine complex has maximum absorbance at 562 nm and a large decrease in absorbance indicates strong chelating power of the extract. By forming a stable iron (II) chelate, an extract with high chelating power reduces the free ferrous ion concentration thus decreasing the extent of Fenton reaction which is implicated in many diseases (Mates et al., 1999). In the present study, both water and acetone extracts of most of the fruits manifested somewhat comparable chelating activity, which was comparatively lower than the DPPH and hydroxyl radical scavenging activity as shown in Figure 12. It was found that among water extracts of the fruits, round jujube berries showed highest chelating power (57.73%), followed by grapefruit (53.18%), prickly pear (51.10%) and non-peeled red apple (49.61%). Lowest detected activity was observed in water extract of peeled-off golden apple (1.623%) and table grapes have very low chelating power which was not detected. None of the fruit sample showed >80% chelating activity in its water extract. In case of acetone extract of the fruits, best chelating power was shown by the white chaunsa mango (95.26%), followed by sapodilla (54.48%) and sweet lime (51.36%), while cape-gooseberry (13.44%) showed the lowest value. Only white chaunsa mango showed >80 % chelating activity in its acetone extract. More or less similar Fe^{2+} chelating activity was observed in both the water and acetone extracts of fruiter (25.13% in WE & 25.65% in AE), guava (32.79% in WE & 33.44% in AE), clementine (30.39% in WE & 30.13% in AE) and date (25.58% in WE & 25.06% in AE).

Ferric reducing antioxidant power (FRAP) of the fruits was also measured and the results are shown in Figure 13. In case of water extracts of the fruits, date showed the highest antioxidant power (295.7 mg GAE/100 g, FW), followed by drastic decrease in antioxidant power as depicted by round jujube berries (138.1 mg GAE/100 g, FW) and black chaunsa mango (102.7 mg GAE/100 g, FW) while coconut (5.761 mg GAE/100 g, FW) showed the lowest FRAP values. Among the acetone extract of the fruits, the highest ferric reducing antioxidant power was recorded for black chaunsa mango (78.99 mg GAE/100 g, FW), followed by round jujube berries (68.80 mg GAE/100 g, FW), while antioxidant power for all the fruits in their acetone extracts is lower than the water extracts of the same fruits. Non-peeled golden apple (7.858 mg GAE/100 g, FW), galia melon (0.616 mg GAE/100 g, FW), watermelon (1.251 mg GAE/100 g, FW), clementine (3.526 mg GAE/100 g, FW), kiwi (1.567 mg GAE/100 g, FW) and pear (2.817 mg GAE/100 g, FW) showed the lowest FRAP values in acetone extracts.

Comparison of Phosphomolybdenium assay in the fruits is expressed as mg AAE/100 g, FW (Figure 14). Among the water extract of the fruits, overwhelmingly higher value was recorded for date (392.5 mg AAE/100 g, FW). Round jujube berries showed the second highest content in water extract (102.6 mg AAE/100 g, FW) which was significantly lower than that of date; followed by cape-gooseberry (74.49 mg AAE/100 g, FW), black chaunsa mango (69.90 mg AAE/100 g, FW) and prickly pear (56.96 mg AAE/100 g, FW). Lowest contents in water extract of the fruits were observed in galia melon (3.146 MG AAE/100 g, FW) and watermelon (3.372 mg AAE/100 g, FW). In case of acetone extract of the fruits, phosphomolybdenium assay showed relatively higher values for black chaunsa mango (143.0 mg AAE/100 g, FW), followed by strawberry (67.25 mg AAE/100 g, FW), pomegranate (62.19 mg AAE/100 g, FW) and toffee grapes (46.34 mg AAE/100 g, FW), while peeled-off golden apple (3.142 mg AAE/100 g, FW), orange (4.613 mg AAE/100 g, FW), galia melon (3.547 mg AAE/100 g, FW) and watermelon (2.907 mg AAE/100 g, FW) showed the lowest values in their acetone extracts. More or less similar values of Phosphomlybdenium assay were noted in water and acetone extracts of the persian lime (11.75 mg AAE/100 g in WE & 9.749 mg AAE/100 g in AE), galia melon (3.146 mg AAE/100 g in WE & 3.547 mg AAE/100 g in AE), watermelon (3.372 mg AAE/100 g in WE & 2.907 mg AAE/100 g in AE) and pineapple (9.408 mg AAE/100 g in WE & 9.272 mg AEE/100 g in AE).

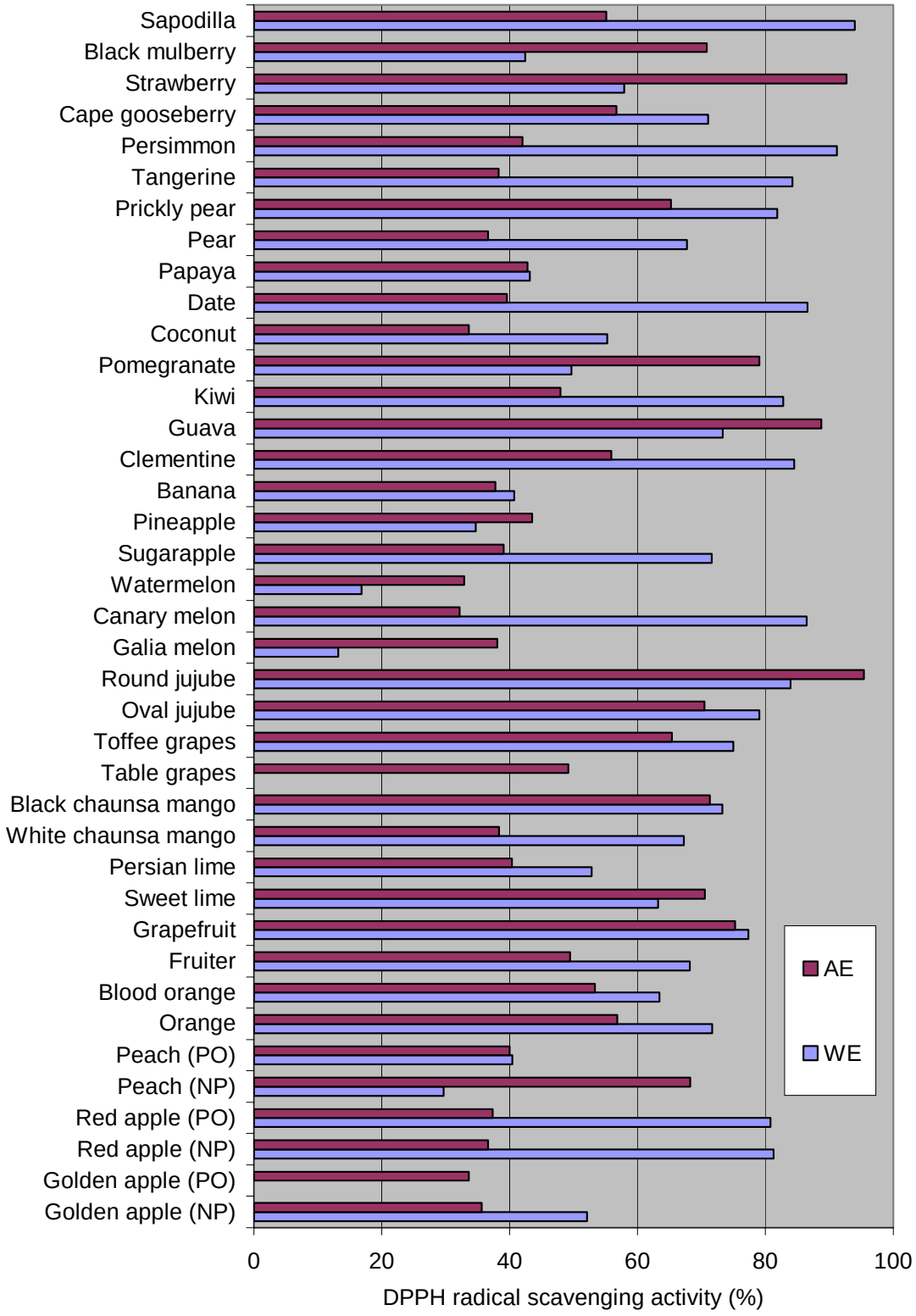

Figure 10. Comparison of DPPH scavenging activity (%) in the fruits

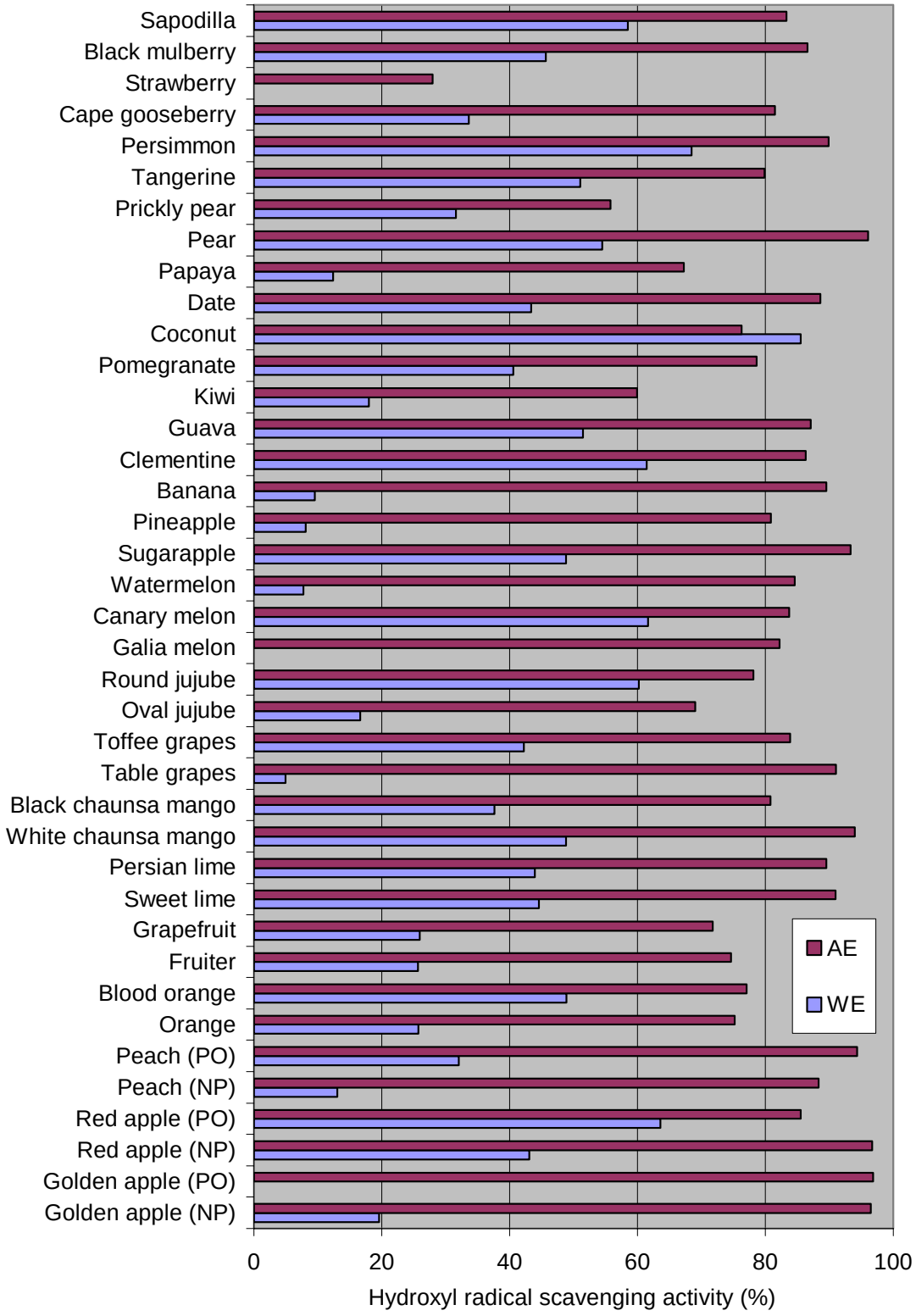

Figure 11. Comparison of OH radical scavenging activity (%) in the fruits

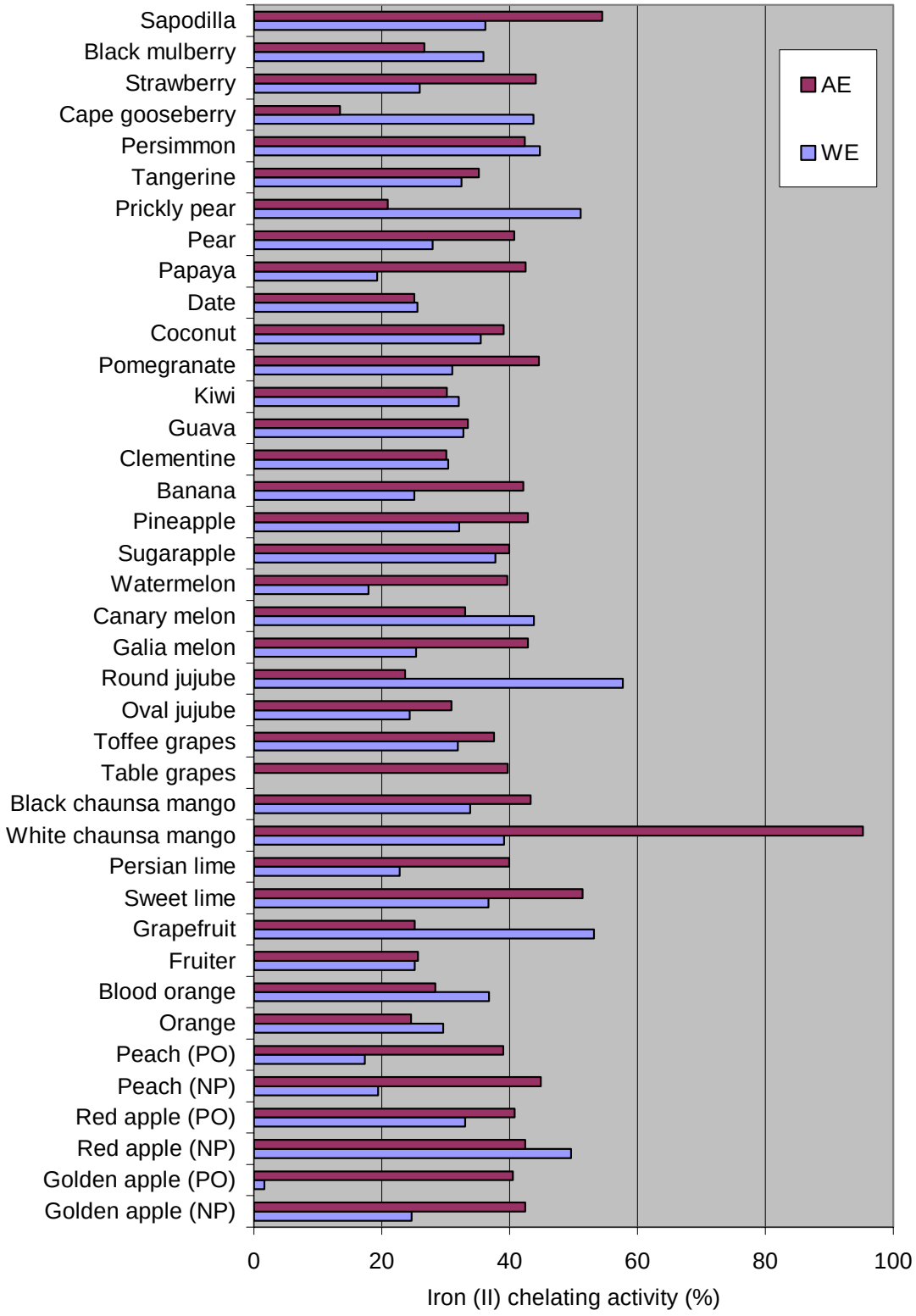

Figure 12. Comparison of Fe^{2+} chelating activity (%) in the fruits

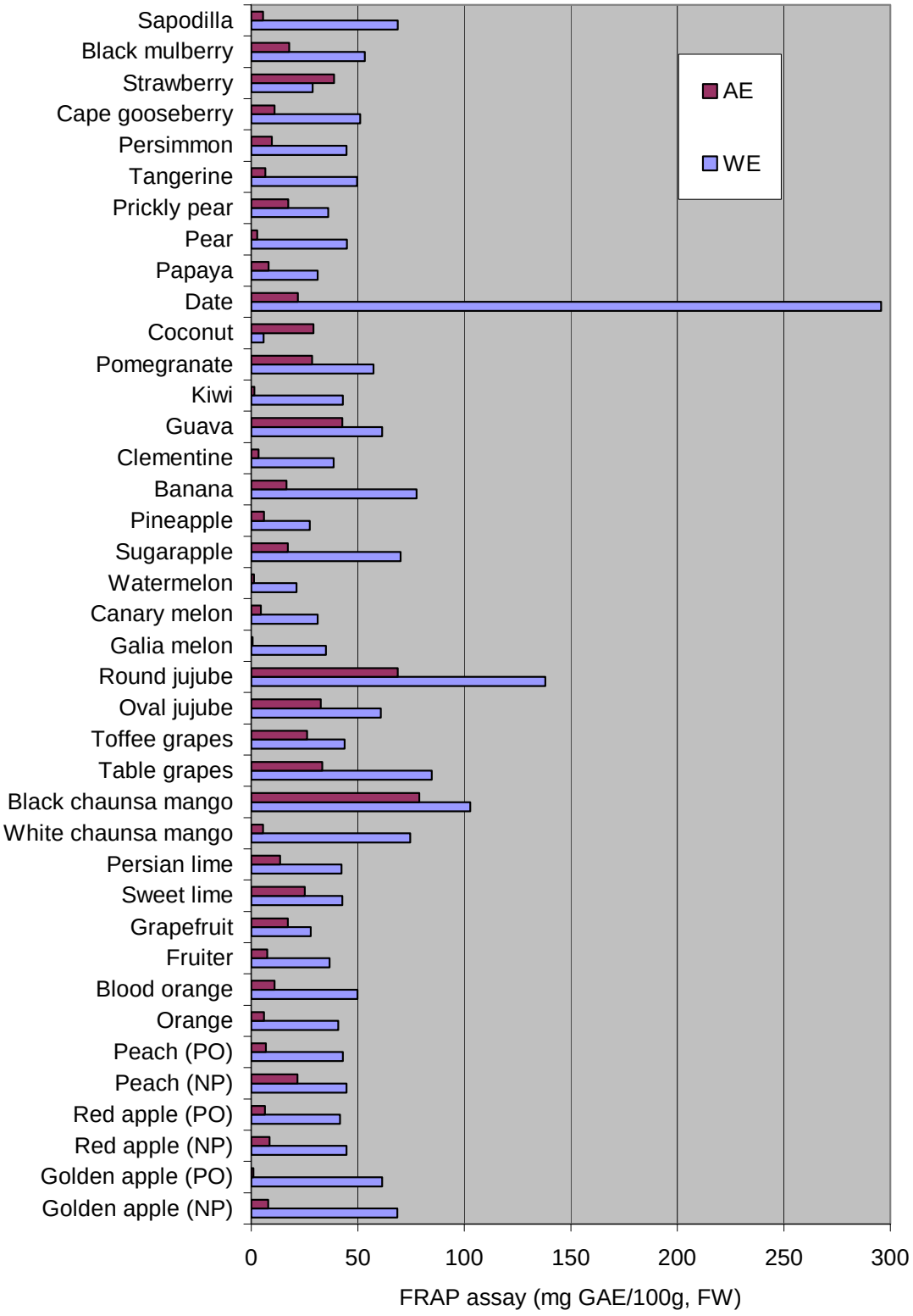

Figure 13. Comparison of ferric reducing antioxidant power (mg GAE/100 g, FW) in the fruits

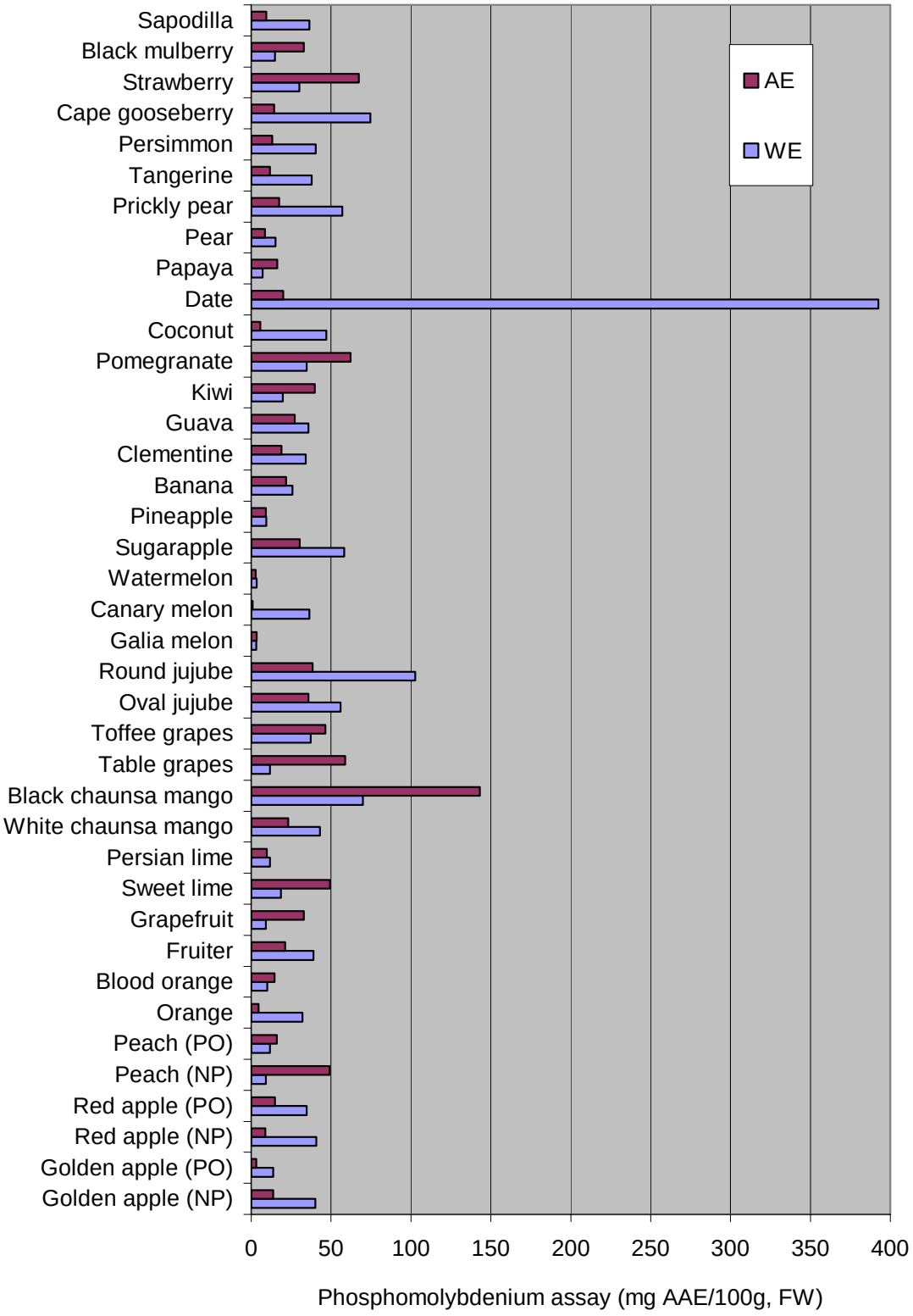

Figure 14. Comparison of total antioxidant activity by phosphomolybdenium assay (mg AAE/100 g, FW) in the fruits

3.7 Correlation Study of Antioxidant Activities in the Fruits

Correlation coefficients between various antioxidant activities were calculated to investigate their inter-relationships in the fruit samples as shown in the correlation coefficient matrix in Table 8, wherein the bold r-values are significant at $p < 0.05$. Some very strong positive correlations were noted among the antioxidant activities in both water and acetone extracts as indicated by following pairs; DPPH radical scavenging activity in WE with $OH^{\bullet}$ radical scavenging activity in WE (r = 0.546), DPPH radical scavenging activity in WE with Iron (II) chelating activity in WE (r = 0.585), DPPH radical scavenging activity in AE with FRAP assay in AE (r = 0.695), DPPH radical scavenging activity in AE with phosphomolybdenium assay in AE (r = 0.591), FRAP assay in WE with phosphomolybdenium assay in WE (r = 0.903) and FRAP in AE with phosphomolybdenium assay in AE (r = 0.781).

Table 8. Correlation coefficient matrix for antioxidant activities in the fruit samples

		DPPH		$OH^{\cdot}$		Iron (II)		FRAP		PMA	
		WE	AE	WE	AE	WE	AE	WE	AE	WE	AE
DPPH	WE	1.000									
	AE	0.150	1.000								
$OH^{\cdot}$	WE	**0.546**	-0.012	1.000							
	AE	-0.075	**-0.478**	0.198	1.000						
Iron (II)	WE	**0.585**	0.313	**0.446**	-0.123	1.000					
	AE	-0.194	-0.228	0.075	0.269	-0.162	1.000				
FRAP	WE	0.284	0.060	0.036	0.203	0.017	-0.086	1.000			
	AE	0.155	**0.695**	0.093	-0.216	0.272	-0.097	**0.340**	1.000		
PMA	WE	**0.365**	0.001	0.168	0.042	0.096	-0.216	**0.903**	0.233	1.000	
	AE	0.061	**0.591**	-0.160	-0.244	0.078	0.093	0.196	**0.781**	0.057	1.000

*r-values shown in bold are significant at $p < 0.05$

A few significantly positive correlations were also observed in some pairs, as; $OH^{\bullet}$ radical scavenging activity in WE with Iron (II) chelating activity in WE (r = 0.446), DPPH radical scavenging activity in WE with FRAP assay in WE (r = 0.365) and FRAP assay in WE with FRAP assay in AE (r = 0.340). However, strong inverse correlations

were not observed among the antioxidant activity pairs indicating similar distribution of these antioxidant activity assays in the fruit samples except for one significant inverse relationship of DPPH radical scavenging activity in AE with $OH^{\bullet}$ radical scavenging activity in AE (r = -0.478).

3.8 Correlation Study of Selected Metals, Phytochemical Constituents and Antioxidant Activities in the Fruits

Correlation study was also carried out to find out the relationships between the selected metal levels, phytochemical constituents and antioxidant assays in the fruits as shown in Tables 9-11. The correlation coefficient matrix for selected metals and phytochemical constituents in the fruit samples is shown in Table 9, wherein significant r-values are shown in bold ($p<0.05$). In this case, ascorbic acid in WE showed the strongest positive correlation with Cr (r = 0.796). Similarly; phenolics showed very strong positive correlation with Zn (r = 0.604) and considerably strong correlation with Cd (r = 0.594). flavonoids in WE showed very strong positive correlation with Cd (r = 0.576), while flavonols in AE showed strong positive correlation with Zn (r = 0.504). Ascorbic acid in WE showed significantly strong positive correlation with Mg (r = 0.575), Cr (r = 0.796), Co (r = 0.574) and Cd (r = 0.692) while; ascorbic acid in AE showed very strong positive correlations with Mg (r = 0.527), Cr (r = 0.792), Co (r = 0.736) and Cd (r = 0.729). Overall, among the phytochemicals, ascorbic acid depicted strong positive correlations with many metals in both the water and acetone extracts and among the metals, Zn, Cr, Mg, Co and Cd manifested strong positive correlations with most of the phytochemicals especially in their water extracts. Other significant correlations were also recorded as; phenolics in WE with Mg (r = 0.346), Cr (r = 0.491), Ni (r = 0.398) and Co (r = 0.378); phenolics in AE with Cu (r = 0.482) and Cd (r = 0.334); flavonoids in WE with Cr (r = 0.478) and Co (r = 0.337); flavonoids in AE with Zn (r = 0.335) and Co (r = 0.353); flavonols in WE with Li (r = 0.427) and Cr (r = 0.487); flavonols in AE with Ni (r = 0.469) and Co (r = 0.394); ascorbic acid in WE with K (r = 0.415), Fe (r = 0.440) and Cu (r = 0.440); ascorbic acid in AE with K (r = 0.445), Ca (r = 0.384), Fe (r = 0.435), Li (r = 0.358) and Cu (r = 0.432). No significant inverse correlation was observed between metals and phytochemical constituents. Among the selected metals, Na, Mn and Pb depicted very weak correlation with almost all the phytochemical constituents.

Table 9. Correlation coefficient (r)* matrix for selected metals and phytochemical constituents in the fruit samples

	Phenolics		Flavonoids		Flavonols		Ascorbic Acid	
	WE	AE	WE	AE	WE	AE	WE	AE
Na	-0.180	-0.173	-0.147	-0.133	-0.133	-0.171	-0.041	0.017
K	0.151	0.105	0.192	0.005	0.006	0.195	**0.415**	**0.445**
Ca	0.250	-0.161	0.172	-0.060	-0.029	0.052	0.233	**0.384**
Mg	**0.346**	0.269	0.298	0.218	0.140	0.304	**0.575**	**0.527**
Fe	0.239	0.229	0.284	-0.021	0.063	0.081	**0.440**	**0.435**
Zn	0.262	**0.604**	0.202	**0.335**	0.131	**0.504**	0.285	0.187
Li	0.046	-0.025	0.044	-0.108	**0.427**	-0.017	0.045	**0.358**
Sr	-0.224	-0.005	-0.162	-0.074	-0.278	0.046	0.021	0.120
Cr	**0.491**	-0.079	**0.478**	0.213	**0.487**	0.214	**0.796**	**0.729**
Mn	-0.180	-0.018	-0.096	0.066	-0.138	-0.109	-0.014	0.060
Cu	0.128	**0.482**	0.119	0.231	0.259	0.243	**0.440**	**0.432**
Ni	**0.398**	0.015	0.186	0.069	0.076	**0.469**	0.211	0.147
Co	**0.378**	0.288	**0.337**	**0.353**	0.200	**0.394**	**0.574**	**0.736**
Cd	**0.594**	**0.334**	0.576	0.171	0.274	0.309	**0.692**	**0.729**
Pb	-0.090	0.012	-0.134	-0.088	-0.015	-0.025	0.174	**0.376**

*r-values shown in bold are significant at $p < 0.05$

The correlation coefficient matrix for selected metals and antioxidant activities in the fruit samples is shown in Table 10, wherein the bold r-values are significant at $p<0.05$. Only few strong positive correlations were recorded among the selected metals and total antioxidant activities (FRAP assay & PMA). Strongest positive correlation was found for phosphomolybdenium assay in WE with Cd (r = 0.873), followed by phosphomolybdenium assay in WE with Mg (r = 0.584), Cr (r = 0.590) and Co (r = 0.768) while only one strong positive correlation was recorded for PMA in AE with Zn (r = 0.677). Similarly, considerably strong positive correlation was recorded for the FRAP assay in WE with Cr (r = 0.534), Co (r = 0.651) and Cd (r = 0.776), while only one strong positive correlation was recorded among FRAP assay in AE and Zn (r = 0.687). Other significant positive correlations were also recorded for; DPPH radical scavenging activity in AE with K (r = 0.373) and Fe (r = 0.408); $OH^{\bullet}$ radical scavenging activity in WE with Co (r = 0.404) and Pb (r = 0.427); Iron (II) chelating activity in WE with K (r = 0.342), Li (r = 0.401) and Sr (r = 0.407); FRAP assay in WE with K (r = 0.486), Mg (r = 0.414), Fe

(r = 0.338) and Ni (r = 0479); FRAP assay in AE with K (r = 0.495), Fe (r = 0.378), Cu (r = 0.424) and Co (r = 0439) and PMA in WE with K (r = 0.389), Fe (r = 0.361) and Ni (r = 0.398). In case of DPPH radical scavenging activity in WE and Iron (II) chelating activity in AE no strong or significant positive or inverse correlation was found with any of the selected metals.

Table 10. Correlation coefficient (r)* matrix for selected metals and antioxidant activities in the fruit samples

	DPPH		$OH^{\cdot}$		Iron (II)		FRAP		PMA	
	WE	AE	WE	AE	WE	WE	WE	AE	WE	AE
Na	-0.021	-0.276	0.201	-0.082	0.139	-0.045	-0.113	-0.155	0.013	-0.231
K	0.112	**0.373**	0.251	0.006	**0.342**	-0.260	**0.486**	**0.495**	**0.389**	0.106
Ca	0.151	0.014	0.153	-0.034	-0.018	-0.177	0.221	-0.141	0.180	-0.024
Mg	0.078	-0.184	0.196	-0.100	0.098	-0.078	**0.414**	0.233	**0.584**	0.125
Fe	-0.076	**0.408**	0.153	**-0.348**	0.042	-0.080	**0.338**	**0.378**	**0.361**	0.321
Zn	-0.039	0.279	0.092	-0.057	0.101	-0.066	0.268	**0.687**	0.243	**0.677**
Li	0.256	-0.252	0.188	0.217	**0.401**	0.012	0.113	0.012	0.202	-0.078
Sr	0.245	0.237	0.087	**-0.360**	**0.407**	-0.311	0.111	0.245	0.186	-0.010
Cr	0.102	-0.210	0.305	0.143	-0.001	-0.032	**0.534**	0.239	**0.590**	-0.097
Mn	0.142	0.117	-0.050	**-0.333**	0.286	-0.224	-0.052	0.015	0.071	-0.052
Cu	-0.022	0.099	0.304	-0.305	0.074	0.067	0.138	**0.424**	0.268	0.273
Ni	0.246	0.066	-0.133	-0.161	-0.033	-0.068	**0.479**	0.118	**0.398**	0.252
Co	0.293	0.037	**0.404**	-0.014	0.283	-0.094	**0.651**	**0.439**	**0.768**	0.159
Cd	0.256	-0.146	0.257	0.119	-0.021	-0.007	**0.776**	0.220	**0.873**	0.114
Pb	0.151	-0.136	**0.427**	0.061	0.125	-0.163	-0.095	0.151	0.076	-0.121

*r-values shown in bold are significant at $p < 0.05$

A few significant inverse correlations were also found among the selected metals and antioxidant activity in the fruits, such as; OH radical scavenging activity in AE with Fe (r = -0.348), Sr (r = -0.360) and Mn (r = -0.333) indicating dissimilar distribution of the antioxidant activity and respective metals in the fruit samples. Similar to the correlation coefficient matrix for selected metals and phytochemical constituents, Na indicated very

weak correlations for almost all the antioxidant activities. On the other hand, Iron (II) chelating activity in AE also showed weak inverse correlation for all the metals except Li and Cu levels in the fruits.

The correlation coefficient matrix for phytochemical constituents and antioxidant activities in the fruit samples is shown in Table 11, wherein significant r-values are shown in bold ($p<0.05$). In this case, phenolics in AE showed the strongest positive correlation with phosphomolybdenium assay in AE (r = 0.883). Similarly, significantly strong positive correlations were recorded for phenolics in WE with FRAP assay in WE (r = 0.762) and with PMA in WE (r = 0.561). Phenolics in AE also showed significantly strong positive correlations with DPPH radical scavenging activity in AE (r = 0.630), FRAP assay in AE (r = 0.739) and PMA in AE (r = 0.833). Flavonoids in WE depicted strong positive correlations with FRAP assay in WE (r = 0.744) and PMA in WE (r = 0.558) while flavonoids in AE showed strong positive correlations with FRAP assay in AE (r = 0.513) and PMA in AE (r = 0.588). Flavonols in WE did not show strong positive correlation with any antioxidant activity but on the other hand, flavonols in AE manifested considerably strong positive correlations with FRAP assay in AE (r = 0.625) and PMA in AE (r = 0.759). Ascorbic acid in WE depicted strong positive correlation with FRAP assay in WE (r = 0.742) and PMA in WE (r = 0.673), while, ascorbic acid in AE, showed strong positive correlation with FRAP assay in WE (r = 0.673) and PMA in WE (r = 0.778). Generally, phosphomolybdenium assay showed significantly strong positive correlation with phenolics, flavonoids, flavonols and ascorbic acid either in water or acetone extract. Other significant positive correlations were also recorded among; phenolics in WE and $OH^{\bullet}$ scavenging activity in AE (r = 0.350); phenolics in AE and FRAP assay in WE (r = 0.340); flavonoids in AE and DPPH radical scavenging activity in AE (r = 0.474); flavonols in WE and OH radical scavenging activity in AE (r = 0.345); flavonols in WE and FRAP assay in WE (r = 0.344); flavonols in AE and DPPH radical scavenging activity in AE (r = 0.358); flavonols in AE and FRAP assay in WE (r = 0.394) and ascorbic acid in AE and OH radical scavenging activity in WE (r = 0.339). A few significant inverse correlations were also noted among the phytochemical constituents and antioxidant activities, such as; flavonoids in WE and iron (II) chelating activity in WE (r = -0.424); flavonols in WE and $OH^{\bullet}$ radical scavenging activity in WE (r = -0.406) and flavonols in WE and iron (II) chelating activity in WE (r = -0.372).

Table 11. Correlation coefficient (r)* matrix for phytochemical constituents and antioxidant activities in the fruit samples

		Phenolics		Flavonoids		Flavonols		Ascorbic Acid	
		WE	AE	WE	AE	WE	AE	WE	AE
DPPH	WE	-0.058	0.089	-0.148	-0.070	-0.331	0.165	-0.105	0.279
	AE	-0.124	**0.630**	-0.150	**0.474**	-0.296	**0.358**	-0.106	-0.106
$OH^{\cdot}$	WE	-0.257	-0.035	-0.312	0.007	**-0.406**	-0.052	-0.077	**0.339**
	AE	**0.350**	-0.252	0.320	-0.071	**0.345**	-0.068	0.126	0.098
Iron (II)	WE	-0.307	0.124	**-0.424**	0.020	**-0.372**	0.116	-0.240	0.000
	AE	0.061	0.040	0.001	0.082	0.122	0.057	-0.135	-0.240
FRAP	WE	**0.762**	**0.340**	**0.744**	0.257	**0.344**	**0.394**	**0.742**	**0.673**
	AE	0.224	**0.739**	0.134	**0.513**	0.039	**0.625**	0.217	0.072
PMA	WE	**0.561**	0.249	**0.558**	0.126	0.147	0.283	**0.673**	**0.778**
	AE	0.303	**0.883**	0.183	**0.588**	0.132	**0.759**	0.071	-0.206

*r-values shown in bold are significant at $p < 0.05$

3.9 Multivariate Cluster Analysis of Selected Metals in the Fruits

Source apportionment of selected metals refers to the assessment of the contribution from different source categories to the concentrations measured in the fruits. A multivariate statistical method, namely; Cluster Analysis (CA), was applied for source identification and apportionment during the present study. Cluster analysis refers to a set of techniques designed to classify observations so members of the resulting groups are similar to each other but distinct from other groups. Hierarchical clustering, which successively joins the most similar observations, is the most common approach. Cluster analysis, may be thought of as a useful way of objectively organizing a large data-set into groups on the basis of matching characteristics. This can ultimately assist in the recognition of potentially meaningful patterns and sources of the pollutants (Yongming et al., 2006). The CA using Ward's method, for the selected metals in the available commercial fruits samples is reported as dendrogram in Figure 15, showing different metal clusters, corresponding to the sources of these metals in the fruits. Two major clusters were found corresponding to the natural and anthropogenic sources of the metals in the fruit samples. Predominantly natural contributions were recorded for Na-Sr-Ca-Ni as

manifested by their strong and distinct cluster in Figure 15. The strongest cluster was observed among Mn-Cu-Pb-K-Cr which was mostly contributed by the excessive use of fertilizers. Other mutual clusters were noted among Co-Cd-Mg and Fe-Li-Zn which were mostly emanating from the combustion processes (particularly agricultural products) and industrial emissions (particularly iron-based industries), respectively. Consequently, significant anthropogenic contributions were noted for most of the selected metals in the fruits.

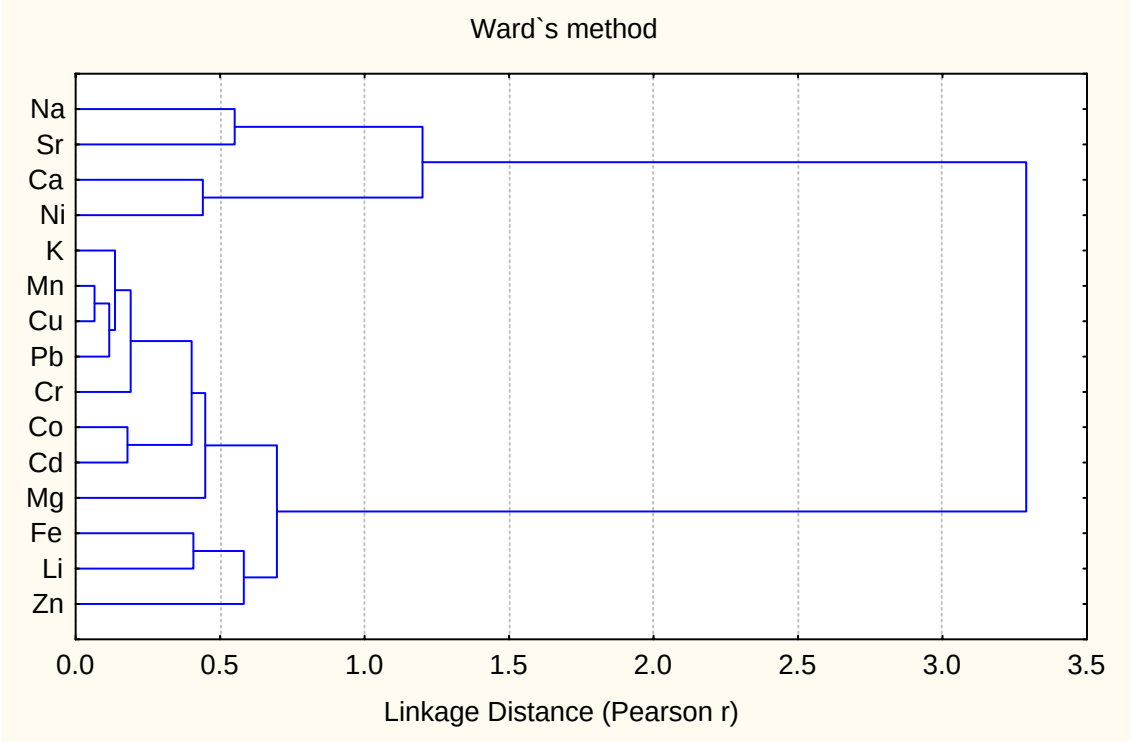

Figure 15. Cluster analysis of selected metals in the fruit samples

3.10 Health Risk Assessment of Selected Metals in Fruits

Health risk indices of selected metals for each fruit were calculated on individual basis as shown in Table 12. In the present study, HRI values of the metals for most of the fruits were less than unity, which is considered as safe for human consumption. However, in few cases HRI values were exceeding the safe limit unity (> 1); for instance, Pb contents in cape-gooseberry (1.120) and coconut (2.986) and Cd contents in date (1.729). Nevertheless, mean HRI values for rest of the metals in all fruit samples were less than unity; consequently, the consumption of these fruits was considered to be safe and their consumption on regular basis was recommended.

Table 12. Health risk index (HRI) of selected metals in each fruit sample

S #	Fruit Samples	Na	K	Ca	Mg	Fe	Zn	Li	Sr	Cr	Mn	Cu	Ni	Co	Cd	Pb
1	Banana	0.00	0.02	0.15	0.06	0.01	0.01	0.06	0.00	0.35	0.02	0.03	0.31	0.06	0.15	0.37
2	Blood orange	0.00	0.01	0.39	0.03	0.02	0.00	0.00	0.00	0.26	0.01	0.00	0.00	0.05	0.35	0.70
3	Galia melon	0.01	0.01	0.09	0.04	0.01	0.00	0.08	0.00	0.07	0.00	0.01	0.06	0.02	0.07	0.08
4	White chaunsa mango	0.00	0.02	0.15	0.03	0.01	0.00	0.11	0.00	0.25	0.01	0.04	0.10	0.30	0.53	0.06
5	Coconut	0.01	0.03	0.05	0.12	0.02	0.03	0.16	0.00	0.65	0.06	0.16	0.05	0.71	0.62	2.99
6	Peach (NP)	0.00	0.01	0.03	0.02	0.01	0.03	0.04	0.00	0.04	0.01	0.01	0.04	0.02	0.02	0.18
7	Peach (P)	0.00	0.01	0.12	0.01	0.01	0.00	0.00	0.00	0.03	0.00	0.01	0.02	0.04	0.20	0.64
8	Date	0.00	0.05	0.34	0.12	0.03	0.02	0.15	0.00	0.66	0.08	0.06	0.00	0.98	1.73	0.50
9	Fruiter	0.00	0.01	0.21	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.12	0.12	0.23	0.52
10	Golden apple (NP)	0.00	0.01	0.02	0.01	0.01	0.00	0.00	0.00	0.03	0.00	0.01	0.00	0.00	0.24	0.65
11	Golden apple (P)	0.00	0.01	0.14	0.01	0.00	0.00	0.08	0.00	0.17	0.00	0.01	0.14	0.06	0.19	0.25
12	Grapefruit	0.00	0.01	0.07	0.01	0.00	0.00	0.03	0.00	0.06	0.00	0.00	0.01	0.03	0.01	0.22
13	Table grapes	0.00	0.02	0.03	0.03	0.01	0.01	0.09	0.00	0.30	0.01	0.05	0.03	0.02	0.19	0.93
14	Guava	0.00	0.01	0.14	0.02	0.01	0.01	0.03	0.00	0.18	0.01	0.03	0.10	0.23	0.27	0.92
15	Oval jujube	0.00	0.01	0.22	0.02	0.02	0.00	0.05	0.00	0.07	0.01	0.01	0.29	0.07	0.16	0.54
16	Round jujube	0.00	0.12	0.08	0.01	0.02	0.02	0.00	0.02	0.19	0.02	0.03	0.00	0.54	0.16	0.41
17	Clementine	0.00	0.01	0.18	0.02	0.01	0.00	0.07	0.00	0.22	0.00	0.01	0.04	0.05	0.11	0.42
18	Kiwi	0.00	0.01	0.39	0.03	0.00	0.00	0.06	0.00	0.05	0.00	0.03	0.34	0.00	0.15	0.50
19	Canary melon	0.02	0.01	0.13	0.06	0.01	0.01	0.16	0.00	0.06	0.00	0.01	0.01	0.08	0.19	0.38
20	Sweet lime	0.00	0.01	0.14	0.03	0.02	0.00	0.05	0.01	0.08	0.00	0.01	0.08	0.07	0.00	0.56
21	Black chaunsa mango	0.00	0.01	0.13	0.08	0.01	0.07	0.13	0.00	0.00	0.01	0.06	0.00	0.48	0.60	0.41
22	Orange	0.00	0.01	0.14	0.03	0.00	0.00	0.07	0.00	0.08	0.00	0.01	0.00	0.01	0.04	0.55
23	Pineapple	0.00	0.01	0.14	0.04	0.01	0.01	0.01	0.00	0.02	0.01	0.01	0.05	0.06	0.02	0.26
24	Pomegranate	0.00	0.01	0.21	0.04	0.01	0.01	0.03	0.00	0.00	0.01	0.02	0.00	0.00	0.13	0.21
25	Papaya	0.01	0.01	0.10	0.03	0.01	0.00	0.04	0.01	0.01	0.00	0.00	0.00	0.07	0.12	0.21
26	Pear	0.00	0.01	0.41	0.01	0.01	0.00	0.02	0.00	0.16	0.00	0.02	0.00	0.24	0.21	0.52
27	Persimmon	0.00	0.01	0.11	0.02	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.14	0.16	0.34	0.86
28	Red apple (NP)	0.00	0.01	0.06	0.01	0.00	0.00	0.58	0.00	0.00	0.00	0.01	0.06	0.36	0.18	0.43
29	Red apple (P)	0.00	0.01	0.07	0.01	0.00	0.00	0.06	0.00	0.14	0.00	0.01	0.00	0.10	0.00	0.43
30	Cape gooseberry	0.00	0.02	0.07	0.05	0.01	0.01	0.12	0.00	0.12	0.01	0.03	0.14	0.13	0.28	1.12
31	Sugarapple	0.01	0.01	0.30	0.09	0.01	0.01	0.03	0.01	0.15	0.01	0.02	0.28	0.23	0.02	0.45
32	Tangerine	0.00	0.01	0.21	0.02	0.00	0.00	0.03	0.00	0.09	0.00	0.01	0.00	0.14	0.15	0.51
33	Strawberry	0.00	0.01	0.11	0.04	0.05	0.01	0.01	0.00	0.07	0.04	0.09	0.00	0.16	0.15	0.18
34	Black mulberry	0.00	0.07	0.57	0.01	0.05	0.02	0.03	0.00	0.08	0.03	0.04	0.00	0.24	0.09	0.25
35	Prickly pear	0.01	0.01	0.17	0.06	0.01	0.01	0.13	0.03	0.14	2.42	0.03	0.00	0.39	0.20	0.21
36	Persian lime	0.00	0.01	0.22	0.03	0.00	0.01	0.02	0.00	0.21	0.01	0.01	0.08	0.13	0.19	0.46
37	Sapodilla	0.00	0.01	0.21	0.02	0.00	0.00	0.02	0.00	0.07	0.01	0.01	0.12	0.01	0.02	0.55
38	Toffee grapes	0.00	0.01	0.03	0.02	0.00	0.00	0.00	0.00	0.02	0.01	0.01	0.12	0.05	0.34	0.67
39	Watermelon	0.00	0.01	0.01	0.02	0.00	0.00	0.03	0.00	0.12	0.00	0.01	0.00	0.01	0.08	0.12
	Mean	0.00	0.02	0.16	0.03	0.01	0.01	0.06	0.00	0.13	0.07	0.02	0.07	0.16	0.22	0.52

Average HRI values for the selected metals in the fruits are shown in Figure 16. for a comparative evaluation. Among the metals, highest HRI value was noted for Pb, followed by Cd, nonetheless, the index values were safe for all the metal contents in the fruits included in the present study. Although on individual basis, consumption of coconut, date and cape-gooseberries may be associated with some adverse health effects but overall no significant health effects were recorded.

Non-carcinogenic health effects associated with the metals levels in the fruits were also calculated in terms of target hazard quotient (THQ). The health protection standard of life time risks for THQ is 1.0 (USEPA, 2006). As shown in Figure 17, the THQ values for all the metals were lower than the safe standard unity (1.0); however, Pb showed relatively higher THQ value than rest of the metals. It demonstrated that ingestion of the fruits pertaining to the metals will not cause any non carcinogenic risks in the consumers.

The carcinogenic risk to human associated with Cd, Cr and Pb contents through the fruits consumption was also assessed in terms of target cancer risk (TCR) as depicted in Figure 18. The health protection standard of life time risks for TCR is 10^{-4} to 10^{-6}. The TCR values for Cd, Cr and Pb were lower than the acceptable risk limit; however, relatively higher contribution was noted for Cd among these metals. Hence, no significant carcinogenic risk for life time consumption of the fruits was noted.

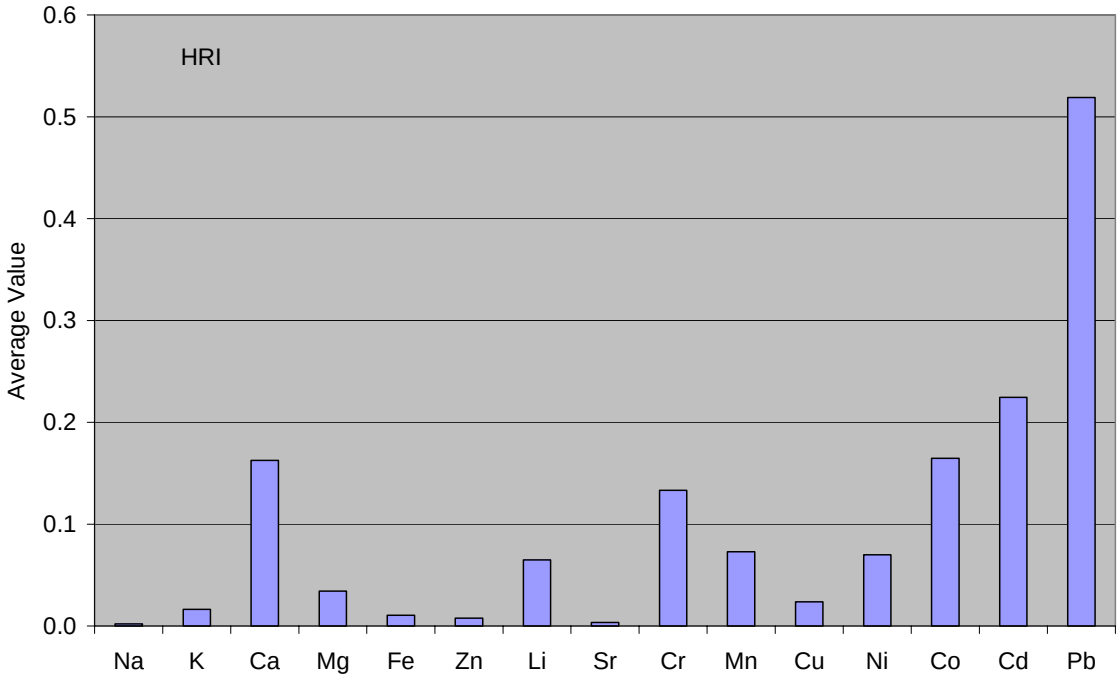

Figure 16. Health risk index (HRI) of selected metals in the fruit samples

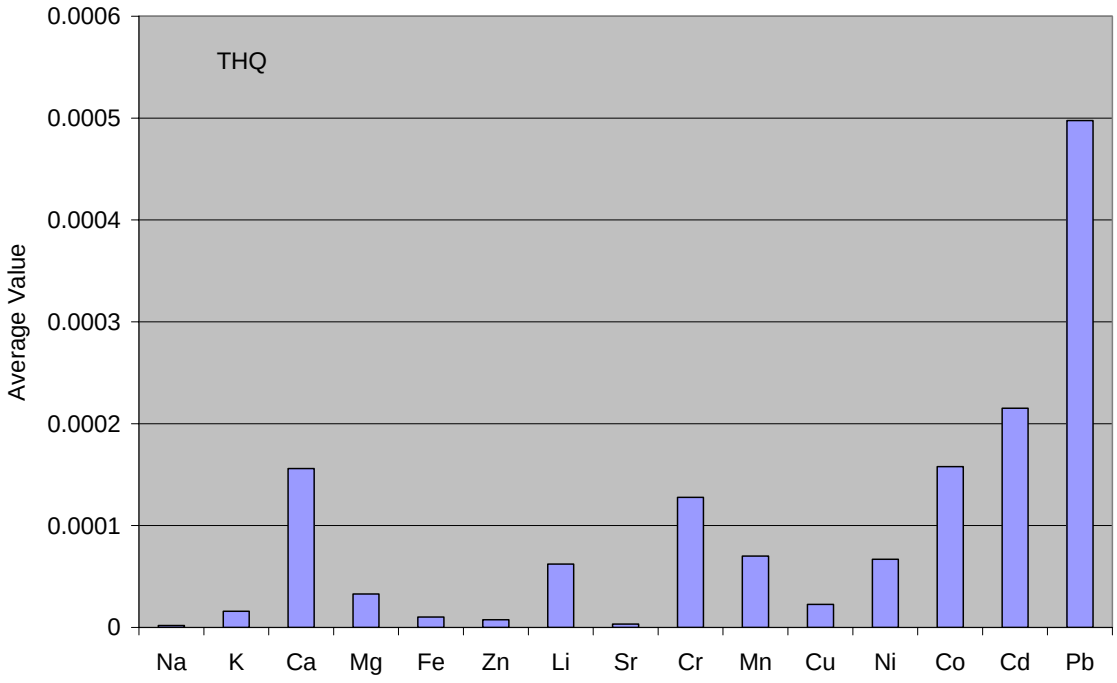

Figure 17. Target hazard quotient (THQ) of selected metals in the fruit samples

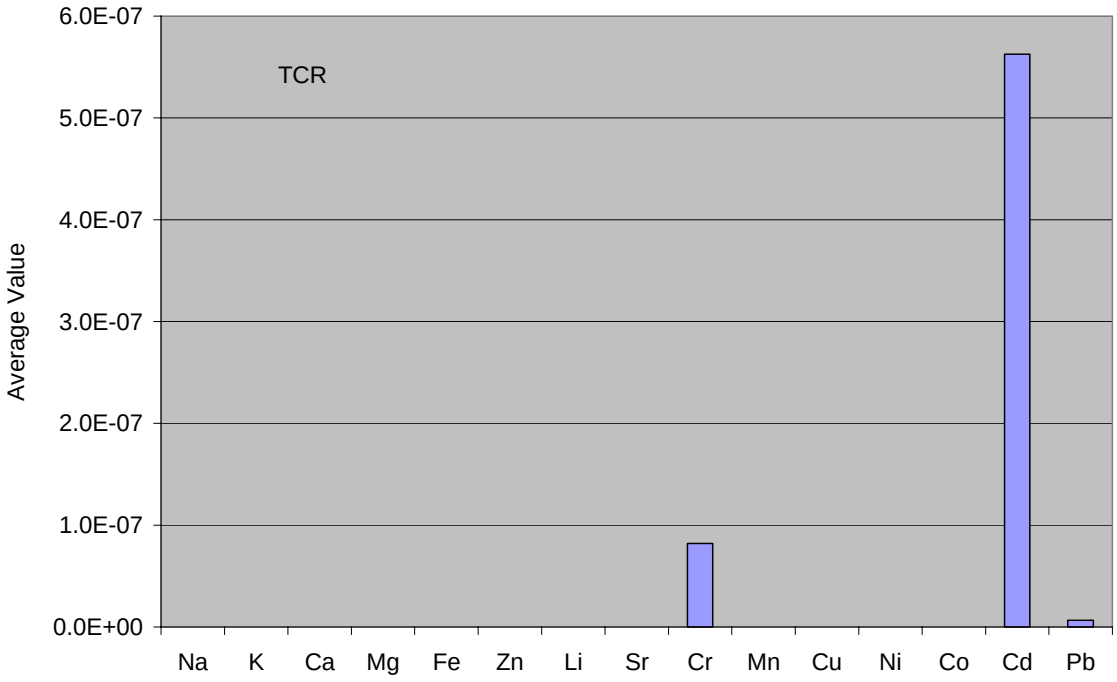

Figure 18. Target cancer risk (TCR) of selected metals in the fruit samples

3.11 Salient Findings

Based on the discussion and deliberations in foregoing sections, following salient findings emerged from the present study.

1. Relatively broad and asymmetric distribution was observed for almost all the selected metals in the available commercial fruits.
2. Average levels of Ca, K, Mg and Na were appreciably higher in the fruits while lowest contribution was noted for Li, Cr and Cd.
3. In the fruits very strong positive correlations were noted for; Cr-Mg, Cr-Li, Mn-Sr, Cu-Mg, Cu-Fe, Cu-Zn, Cu-Cr, Ni-Ca, Co-K, Co-Mg, Co-Zn, Co-Cr, Co-Cu, Cd-Mg, Cd-Cr, Cd-Co, Pb-Cr and Pb-Cu.
4. Distribution of phytochemical constituents/antioxidant activities in the fruits was relatively random and asymmetric with comparatively higher mean values in the water extracts.
5. Among the phytochemicals, total flavonoids showed the highest levels in the fruits.
6. Highest phenolic contents were recorded in water extracts of date, table grapes, banana and black chaunsa mango.
7. Elevated flavonoid contents were found in water extracts of date, table grapes, peeled-off golden apple and banana.
8. Highest flavonol contents were shown by the water extracts of table grapes, non-peeled red apple, peeled-off golden apple, date and banana.
9. Relatively higher ascorbic acid was found in the water extract of date, banana and table grapes.
10. Strong mutual positive correlations were noted among the phytochemical constituents of the fruits.
11. Most of the fruits showed significant DPPH and OH radical scavenging activity; relatively higher in their acetone extracts.
12. Highest DPPH radical scavenging activity was shown by the sapodilla, persimmon, canary melon, date, jujube berries, strawberry and guava.
13. Elevated OH radical scavenging activity was shown by coconut, peeled-off and non-peeled golden apple, non-peeled red apple, pear, peeled-off peach, and sugar apple.
14. Divergent variations were noted in Fe^{2+} chelating activity in the fruits with best chelating power shown by the white chaunsa mango.

15. Considerably higher ferric reducing antioxidant power was shown by date, round jujube berries and black chaunsa mango
16. Markedly higher phosphomolybdenum assay value was noted in date, round jujube berries and black chaunsa mango.
17. Overall, appreciably elevated phytochemical constituents and antioxidant activities were shown by date among the fruits.
18. Strong correlations were found for DPPH radical scavenging activity with OH radical scavenging activity, Iron (II) chelating activity, FRAP assay and phosphomolybdenium assay.
19. Among the phytochemical constituents, ascorbic acid showed strong correlations with various metals (Mg, Cr, Co and Cd).
20. Among the antioxidant activities, phosphomolybdenium assay showed strong correlations with some metals (Mg, Zn, Cr, Co and Cd).
21. FRAP and phosphomolybdenium assay depicted significant relationships with all phytochamicals in both water and acetone extracts.
22. Cluster analysis revealed significant anthropogenic contributions of most of the metals in the fruits.
23. Health risk assessment in terms of HRI and THQ showed no significant adverse heath effect related to the metal contents in the fruits.
24. In terms of TCR, consumption of the fruits was found to be safe and lifetime carcinogenic effects related to Cd, Cr and Pb were non-significant.

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