ANTIOXIDANT CAPACITY AND LIPID CHARACTERIZATION OF GEORGIA-GROWN UNDERUTILIZED FRUIT CROPS

by

GARIMA PANDE

(Under the Direction of Casimir C. Akoh)

ABSTRACT

It is well known that certain bioactive compounds are involved in reducing the risk of diseases associated with oxidative stress. Five underutilized fruit crops of Georgia were investigated namely loquat (*Eriobotrya japonica*), mayhaw *Crataegus sp.),* fig (*Ficus carica*), pawpaw (*Asimina triloba*), and pomegranate (*Punica granatum*). Both hydrophilic and lipophilic antioxidant capacity were determined by FRAP and TEAC assays. Different fractions like seed, pulp, peel, whole fruit, and leaves were analyzed for phenolic compounds and organic acids. Lipid profile of seeds and fruits were also determined in terms of fatty acids, tocopherols, phytosterols, and phospholipids. The predominant organic acid in loquat, mayhaw, pawpaw, and fig was malic acid whereas in pomegranate it was citric acid. Among all the fruits investigated pomegranate had the highest content of phenolic compounds and antioxidant capacity. Leaves had the highest antioxidant capacity followed by peel in pomegranate and by seeds in other four fruits. The highest lipid content was found in pawpaw seed (21.5%). Pomegranate seed oil had high α and γ-tocopherols (167.3 and 84.6 mg/100 g, respectively) contents.

INDEX WORDS: antioxidant capacity; *Asimina triloba*; *Crataegus sp.; Eriobotrya japonica*; fatty acids; *Ficus carica*; organic acids; phospholipids; phytosterols; polyphenols; *Punica granatum;* tocopherols; underutilized fruits

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GARIMA PANDE

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by

GARIMA PANDE

Major Professor: Casimir C. Akoh

Committee: Ronald R. Eitenmiller
Robert L. Shewfelt

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2009

To my mother

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CHAPTER-1

Introduction

Phytochemicals, particularly antioxidants from natural sources like fruits and vegetables have gained popularity because many epidemiological studies have shown their protective properties against several chronic diseases like cancer and cardiovascular diseases (*1*). The most abundant antioxidants in fruits are polyphenols, vitamins, and carotenoids. A number of in vivo and in vitro assays have been developed to measure the antioxidant capacity of biological samples. By-products of food processing industries are a relatively untapped source of lipids and proteins (*2*). Furthermore, lipids from fruits and fruit seeds may be a rich source of bioactive compounds like essential fatty acids, tocopherols, and phytosterols.

The peels and seeds which are usually disposed as waste material in many food processing industries could be a rich source of beneficial phytochemicals. From economic and environment point of view disposing of such wastes should be avoided. There is an increasing public awareness of the need to recycle the wastes for conservation of the environment. Recovering of bioactive compounds helps towards making the recycling of wastes economically viable and also helps in value addition to these minor crops.

There is an increasing interest on research on different plant species for therapeutic, nutraceutical, pharmaceutical and other applications. With global functional food and beverage market expected to reach $109 billion by 2010 (*3*), diverse sources of phytochemicals are being

explored. As per 2008 Georgia data summary, only 1 in 5 high school children and 1 in 4 adults consume the minimum recommendation of 5 or more servings of fruits and vegetables per day. Cancer is the second leading cause of death in Georgia and accounts for almost a quarter of all deaths in the state. Cardiovascular diseases are the leading cause of death in Georgia and accounts for one third of deaths in the state (*4*). Georgia cultivates a wide range of fruits but some fruits with great economic and nutritional potential remain underexploited (*5*). Utilization of these crops may help in the diversification of the source of phytochemicals, conservation of these crops and economic advancement of the region.

The present thesis includes 6 chapters. The first chapter is an introduction which contains the overall objectives of this study. The second chapter is the literature review of topics related to oxidation, antioxidants, chemistry behind the antioxidant assays, phenolics, lipid profile, and underutilized fruits.

The third chapter presents the antioxidant capacity of four underutilized fruit crops loquat, mayhaw, pawpaw, and fig. Seed, peel, pulp, whole fruit and leaves were analyzed for carotenoids, organic acids, total polyphenols, phenolic compounds and antioxidant capacity.

The fourth chapter contains the lipid profile of the seeds and fruits of loquat, mayhaw, pawpaw, and fig. Total lipid was extracted by Folch method. Fatty acids and phytosterols were determined by GC. Normal phase HPLC was used for identification and quantification of tocopherols and phospholipids.

In the fifth chapter six cultivars of pomegranate, R19, R26, Cvg-Eve, North, Crab, and Cranberry were studied for their antioxidant capacity and lipid profile. Four fractions were prepared for antioxidant study – seed, pulp, peel, and leaves. For lipid profile only seed and fruit

fractions were used. The hydrolyzable tannins, punicalagins, were identified by MS-ESI and the presence of conjugated linolenic acid (punicic acid) was confirmed by GC-MS.

The last chapter is the conclusions of the whole study.

The objectives of the present study are:

1) To determine and compare the antioxidant content and capacity of peel, pulp, seeds, leaves and whole fruit fractions of underutilized fruits of Georgia.
2) To identify the phenolic compounds of these fruits.
3) To characterize the lipid profile of these fruits and seeds.

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CHAPTER -2

Literature Review

OXIDATION

Oxidation is the interaction between oxygen molecule and the different substances it comes in contact with, from metal to living tissue. Oxidation has both beneficial and detrimental effects on biological systems. Oxidative metabolism is an essential pathway for the survival of cells. Oxidation results in formation of free radicals. These free radicals are involved in a number of essential biological processes and their production is approximately balanced by the antioxidant defense system of the body. Most of the biomolecules of human body, unfortunately, are susceptible to oxidation. Oxidation of molecules like lipids, carbohydrates, proteins, and DNA has been associated with several diseases like atherosclerosis, diabetes, Alzheimer’s and cancer. Oxidation in a food system deteriorates its flavor, nutritional quality, functional attributes and poses several health risks.

Mechanism of oxidation

Free radical initiated oxidation occurs in three phases, initiation, propagation and termination as depicted in **Fig. 2.1**. The process may start by the action of heat, light, ionizing radiation, or by chemical reactions. Hydrogen is removed during initiation phase resulting in the formation of a free radical which reacts rapidly with molecular oxygen leading to a chain of

reactions. Termination reactions involve the combination of radicals to form non radical products (*1*).

Oxidative stress

Oxidative stress is a major cause of several chronic illnesses. Oxidative stress is the disturbance of the pro-oxidant – antioxidant balance in favor of the former, leading to potential damage (*2*). This damage, called oxidative damage, is defined as the biomolecular damage caused by attack of reactive species upon the constituents of living organisms (*2*). Oxidative stress can result from either reduced levels of antioxidant defenses or from increased production of free radicals. Free radicals are generated in the body (endogenous and exogenous) during normal metabolism (enzymes, heme proteins, mitochondrial electron transport chain, metabolic cycles, transition metals, autoradiation reaction). The main sources of free radicals are cellular metabolism, ischemia, ionizing radiations, air pollution, cigarette smoke, and tissue injury (*3*). These free radicals are involved in multiple normal regulatory pathways like ATP production (oxidative phosphorylation), apoptosis of defective cells, killing of microorganisms and cancer cells by microphages and lymphocytes, generation of prostaglandins and leukotrienes and also in cell signaling (*4*). In biological systems there are various levels of antioxidant defense namely, enzymes like superoxide dismutase, catalase, large molecules like transferrin, ferritin, albumin, small molecules like tocopherols, carotenoids, ascorbic acid, and hormones like melatonin and estrogen (*5*). These are able to balance the detrimental side effects of free radicals upto a certain limit. Upon exposure to environmental and pathophysiological stresses, if free radicals surpass the normal limit, they result in oxidative stress which poses a serious threat to the biological system.

Free radicals

A free radical is any species, atom or a group of atoms, capable of independent existence that contains one or more unpaired electrons. They can be formed by a loss or gain of an electron by a non-radical or during a hemolytic fission of a covalent bond. They are highly reactive, unstable and capable of causing a wide range of biological damage (*6, 7*). Once free radicals are formed they react with other non-radicals (most of metabolically significant biomolecules are non-radicals) to give rise to chain reactions generating more free radicals. The most common and important free radicals are oxygen centered (*8*). Reactive oxygen species (ROS) is a collective term for both oxygen radicals and non radical derivatives of oxygen which can easily convert to radicals. The most prominent sources of ROS in eukaryotic cells are the mitochondrial respiratory chain, microsomal cytochrome P450 enzymes, flavoprotein oxidases, and peroxisomal fatty acid metabolism. Nitric oxide is an important mediator of diverse physiologic process including neurotransmission, regulation of blood pressure, inhibition of platelet aggregation, and as an effector of immune responses. Some common reactive oxygen and nitrogen species are superoxide, hydroxyl radical, hydrogen peroxide, singlet oxygen, nitric oxide and peroxynitrite (*4*).

Cellular response to oxidation

There are diverse range of cellular responses to oxidative stress depending on the type of cell and severity of stress. The prominent ones are increased proliferation, adaptation, cell injury, senescence, and cell death. Reactive species are involved in the development of cancer, both by direct effects on DNA and by modulating signal transduction, cell proliferation, senescence and cell death. Free radicals attack the sugar moiety of DNA molecule by addition or removal of

hydrogen atom and results in products involved in carcinogenesis. Breakages in DNA strand may cause heritable mutations in germ cells or induce cancer in somatic cells. Membrane lipids are highly susceptible to oxidation and free radicals attack. Lipid oxidation includes three steps-initiation, propagation, and termination leading to formation of products like hydroperoxides, formaldehyde, malonaldehyde, alcohol and ketones which are detrimental to normal cell functioning . Free radicals also react with carbohydrates removing hydrogen atom and thus forming a carbon centered radical. With proteins, free radicals result in a number of stable as well as highly reactive undesirable products. Oxidation of amino acids in proteins may lead to formation of antibodies against modified protein and be a factor in auto immune diseases. Oxidized amino acids may catalyze further formation of oxygen radicals and are also involved in atherogenesis (*4*).

ANTIOXIDANTS

"Antioxidant is any substance that when present at low concentration compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate" (*9*). Antioxidants are important both for food and health industry.

Types of antioxidants

Antioxidants can be classified as primary or secondary, natural or synthetic, enzymatic or non enzymatic and lipophilic and hydrophilic (*10*). On the basis of their action on oxidation antioxidants are classified into two groups. The first group is primary (chain-breaking) antioxidants, which react directly with lipid radicals and convert them into stable products. The second group is secondary (preventive) antioxidants, which can lower the rate of oxidation by different mechanisms. Direct scavenging of free radicals is not involved (*11*). Most primary

antioxidants act by donating a hydrogen atom, and are consumed during the induction period (*12*). Secondary antioxidants may act by either binding metal ions capable of catalyzing oxidative processes, scavenging oxygen, absorbing UV radiation, inhibiting enzymes or decomposing hydroperoxides (*13*). It has been reported that some natural phenolic compounds function as both primary and secondary antioxidants (*12*).

The human body is capable of various antioxidant defenses both endogenous and diet derived like removal of reactive species by enzymes e.g., superoxide dismutase (SOD), agents that decrease reactive species formation, example mitochondrial uncoupling protein, agents that protect biomolecules against reactive species like chaperones. A number of synthetic antioxidants have also been formulated like BHA (butylated hydroxyanisol) and BHT (butylated hydroxytoluene). Increased intake of dietary antioxidants may help maintain an adequate antioxidant status, defined as the balance between antioxidants and oxidants in living organisms (*14*). Antioxidants are widely used in the manufacture, packaging, and storage of lipid-containing foods. Much interest has developed during the last few decades in naturally occurring antioxidants because of the adverse attention received by synthetic antioxidants, and also the worldwide trend to avoid or minimize the use of artificial food additives (*12*). Fruits, vegetables, spices, nuts, seeds, leaves, roots and barks have been considered as potential sources of natural antioxidants (*15*).

Mechanisms of antioxidants

Antioxidant can react in two methods, single electron transfer (SET) and hydrogen atom transfer (HAT) (*17*) as shown in **Fig. 2.2**. The end result of both mechanisms is same just the path and kinetics differ. These reactions may occur in parallel and usually occur together in all

samples (*5*). The factors that determine the mechanism of antioxidant activity in radical deactivation in a given system are antioxidant structure and properties, antioxidant solubility and partition coefficient, pH and the nature of the system (*17*).

The capability of an antioxidant to donate hydrogen atom to quench free radicals is measured by HAT-based methods whereas the ability of an antioxidant to transfer an electron to reduce any compound is measured by SET-based methods (*17*). HAT-based reactions are not dependent on the nature of the solvent or pH (*18*). They are usually relatively rapid, typically completed in seconds to minutes. Reducing agents, including metals, interfere with HAT-based reactions and may lead to erroneously high apparent capacities (*17*). SET-based reactions are pH dependent (*18*). Relative capacity in SET-based methods is based primarily on two factors, namely deprotonation (*19*) and ionization potential (*20*) of the reactive functional group. SET-based reactions are usually slow and can require long times to reach completion (*18*). The presence of trace components and contaminants (particularly metals) in samples could account for the high variability and poor reproducibility and consistency of results (*21*).

Examples of HAT-based methods are ORAC (Oxygen Radical Absorbance Capacity), TRAP (Total Radical Trapping Antioxidant Parameter), β-carotene bleaching method, PCL (Photochemiluminescence) and LDL (Low Density Lipoprotein) oxidation assays. FRAP (Ferric Reducing Antioxidant Power) is a SET-based method. Some methods utilizing both mechanisms are TEAC (Trolox Equivalent Antioxidant Capacity), DPPH (diphenyl-1-picrylhydrazyl) and FC (Folin Ciocalteau) method. All methods have their own advantages and disadvantages. With the presence of different types of free radicals, antioxidants, oxidation sources and mechanisms it is difficult to have a single validated method. Therefore, the use of atleast two different methods is always recommended.

BIOACTIVE COMPOUNDS

Phenolic compounds

Plants produce a variety of compounds in addition to other essential metabolites which are not directly involved in growth and development of the organism but play an important role in reproduction, appearance, protection, and defense against pathogens, pests, and environmental stress. These compounds are known as secondary metabolites and can be grouped into terpenoids, alkaloids, and phenolic compounds. Phenols, sometimes called phenolics, are a class of aromatic organic compounds consisting of one or more hydroxyl groups attached to an aromatic hydrocarbon group. Phenol is a benzene derivative and is the simplest member of the phenolic chemical. Phenolic compounds are produced in the plants via the shikimate or phenylpropanoid pathway. Most of the major classes of plant polyphenols are listed in **Table 2.1** according to the number of carbon atoms of the basic skeleton. Polyphenols are divided into various classes based on the number of phenol rings that they contain and on the structural elements that bind these rings to one another. The main groups of polyphenols are flavonoids, phenolic acids, phenolic polymers, stilbenes, and lignans (*22*). Among all these subclasses, flavonoids and phenolic acids account for 60 and 30%, respectively, of total dietary polyphenols (*23*).

Phenolic compounds play a significant role in the structure and protection of the plant, determining the texture, flavor, color, processing characteristics of the plant (*24*), and also in pollination and seed dispersal. Environmental stresses such as wounding, pathogen infection, and/or UV irradiation activate the biosynthesis of phenylpropanoid compounds. All plant

phenols are derived from the common intermediate phenylalanine or its precursor shikimic acid via the shikimic acid pathway.

Flavonoid represents the most common and widely distributed group of plant phenolics and consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle. They occur naturally in plants and are found in fruits, vegetables, grains, barks, roots, stems, flowers, tea, and wine (*25*). They can be subdivided into six classes: flavones, flavonones, isoflavones, flavonols, and anthocyanins (*26*). Flavonoids share a common carbon skeleton of diphenyl propanes, two benzene rings, joined by a linear three carbon chain. The central three carbon chain may form a closed pyran ring with one of the benzene rings (*22*). **Fig. 2.3** shows the basic structures of flavonoids. These flavonoids often occur as glycosides, rendering the molecule more water-soluble and less reactive toward free radicals. The flavonoid variants are all related by a common biosynthetic pathway, incorporating precursors from both the shikimate and the acetate-malonate pathways. Phenolic acids and their derivatives occur widely in the plant kingdom, *e.g.*, legumes, cereals, fruits and plant products such as tea, cider, oil, wine, beverages and medicinal plants (*27*). Phenolic acids are divided into two sub classes: (1) hydroxybenzoic acids, such as gallic acid and protocatechuic acids and (2) hydroxycinnamic acid, such as coumaric, caffeic, and ferulic acids. Hydroxycinnamic acids occur more frequently as simple esters with carboxylic acids or glucose whereas hydroxybenzoic acids are present as glucosides (*28*). Their structures are shown in **Fig. 2.4.**

In addition to their antioxidant properties, plant phenols also trap and scavenge free radicals, regulate nitric oxide, decrease leukocyte immobilization, induce apoptosis, inhibit cell proliferation and angiogenesis and exhibit phytoestrogenic activity (*29*).

Carotenoids

Carotenoids are a group of brightly colored compounds found in abundance in plant kingdom. Carotenoids have long chains of alternating double and single bonds which allows extensive electron delocalization. This causes carotenoids to absorb in the visible range and produce their bright colors. The basic skeleton of carotenoids has 40 carbon atoms and can be modified by cyclization at one or both ends, by reducing certain double bonds or by addition of oxygen containing functional groups (*2*). In humans and other animals carotenoids are the precursor of vitamin A which is essential for cell growth, differentiation, and vision. In plants, carotenoids play an important role in quenching singlet O_2 and preventing its formation during photosynthesis.

Organic acids

Organic acid are of great importance to plants, humans and commercial industries. As intermediates in the metabolic processes of the fruit, these acids are directly involved in growth, maturation, and senescence. Fruit juices have a low pH, because they contain high levels of organic acids. Organic acids also influence the growth of microorganisms in fruit juices and therefore have an effect on its shelf life. Another aspect of organic acids is their influence on the sensory properties and nutritional profile of juice products. Color is also related to the type and level of acids present. Organic acids accumulate during the early stages of fruit development and are used as respiratory substrate during ripening (*30*). Some of the major acids in fruits include citric, malic, and tartaric acids.

Fatty acid profile

The total lipid content is an important parameter used in several biochemical, physiological and nutritional studies. Lipids include those compounds that are soluble in organic solvents and sparingly soluble in water. The major lipid classes are phospholipids, triacylglcerols and free fatty acids. Dietary lipids play an important role in nutrition. Not only the amount but also the type of fat has a profound effect on health. The balance between saturated and unsaturated fatty acids, the amount and type of omega fatty acids are believed to have an influence on coronary heart disease. Determining the fatty acid profile of a substance helps in deciding its end use both in food and non food applications such as paints, coatings, oleochemicals and cosmetics.

Tocopherols

Tocopherols and tocotrienols are lipid soluble vitamins commonly grouped as vitamin E. Their structures are shown in **Fig. 2.5**. They play an important role in human growth and development. Tocopherols are the major lipid-soluble, membrane-localized antioxidants in humans. Their antioxidant properties have been extensively studied for more than forty years. Their structure consists of a polar chromanol head and a hydrophobic prenyl tail which results in amphipathic nature of these compounds. The only difference between tocopherols and tocotrienols is the degree of saturation of the hydrophobic tail. Tocopherols have a 4', 8' 12-trimethyltridecylphytol chain whereas tocotrienols have double bonds at 3', 7' and 11' positions. of the chain (*31*). Tocopherols and tocotrienols inhibit lipid peroxidation by scavenging lipid peroxyl radicals much faster than these radicals reaction with adjacent fatty acid side chains or membrane proteins (*32*). In plants tocopherols quench and react with singlet O_2, thereby

protecting chloroplasts which are one of the most important intracellular generators of ROS under stress.

Phytosterols

Phytosterols are 28 or 29 carbon alcohols resembling cholesterol structurally. They contain an extra methyl or ethyl group, or a double bond. Most phytosterols contain 9-10 carbon atom side chain as compared to 8 carbon atom side chain in cholesterol (*33*). The most common phytosterols found in nature are campesterol, β-sitosterol, stigmasterol, 5 α-hydrogenation of phytosterols form, saturated phytosterols such as campestanol and sitostanol (*34*). Phytosterols may decrease epithelial cell proliferation by suppressing bacterial metabolism of cholesterol and/or secondary bile acids in the colon and by increasing the excretion of cholesterol (*34*). Furthermore, phytosterols also show anticancer, anti sclerotic, anti-inflammatory, and antioxidative effects (*35*).

Phospholipids

Phospholipids are asymmetrical phosphoric acid diesters comprising of C-C bonds, ester bonds, and phosphoester bonds (*10*). Phospholipids are usually found in all biological membranes. In plants and animals they serve as structural components of membranes in addition to their role in enzyme activation. Phospholipids are amphiphilic in nature due to the presence of a polar head group and a nonpolar fatty acid region. This amphiphilic character of phospholipids is responsible for their emulsifying properties. They are widely used in food and cosmetic industries as well as manufacturing industries. The important classes of phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidylinositol (PI).

PROOXIDANT ACTIVITY

Most compounds with antioxidant activity may exhibit prooxidant behavior under certain conditions. Prooxidant activity can accelerate the damage to DNA, carbohydrates and proteins (*36, 37*). Ascorbic acid initiates lipid peroxidation in the presence of high concentration of ferric ion. Tocopherols which act as an antioxidant may accelerate lipid peroxidation at high concentrations. Some flavonoids have also shown mutagenic activities in vitro (*38*). The *O*-methylation of the hydroxyl substitutions in flavonoids inactivates their antioxidant and prooxidant activity whereas the conjugation between rings A and B does not affect its antioxidant activity but is very important for its prooxidant activity (*39*). This paradoxical role of antioxidants under certain conditions is related to 'redox signaling' of the antioxidants. When a cell is subjected to environmental stress, its defense level is lowered because of massive generation of ROS. The cell immediately responds to this stress by upregulating its antioxidant defense. The ROS function as signaling molecules during this process thereby aiding in the generation of antioxidants. Therefore, under such conditions even though endogeneous antioxidant level in the body is low, additional antioxidants can be harmful as they will prevent the function of ROS to perform signal transduction to induce intracellular antioxidants (*40*).

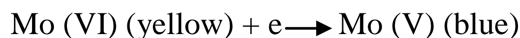
METHODS DESCRIPTION AND CHEMISTRY BEHIND THEM

The growing interest in the physiological benefits of antioxidants has been matched by acceleration in the development of analytical and biological methodologies for measurement of both the levels and quality of these compounds. A number of spectrophotometric methods have been developed for the quantification and identification of vitamins, carotenoids and polyphenols. Different assays are also available for determining the antioxidant capacity both in

vitro and in vivo. ORAC, TRAP, β-carotene bleaching method, PCL, and LDL oxidation assays, FRAP, TEAC, DPPH and FC method are a few of them. Each has its own advantages and disadvantages but the biggest problem is the lack of a single validated assay that can be used to measure the antioxidant capacity of foods and biological samples (*41*). Different researchers use different methods and therefore no consensus has been achieved. Most of the studies on antioxidant capacity emphasize the use of more than one method of analysis.

Total polyphenols assay using the Folin-Ciocalteau (FC) reagent

The total polyphenols assay originally was designed and used for the analysis of protein by reaction between the reagent and tyrosine (which contains a phenol group) residues in proteins. Singleton and others adopted this assay for the analysis of total polyphenols in wine. The total polyphenols assay actually measures the reducing capacity of a sample (*42*). Numerous publications reported excellent linear correlations between "total phenolic profiles" and "the antioxidant capacity" (*17*). Despite the undefined chemical nature of FC reagent, it is believed to contain heteropolyphosphotungstates-molybdates. Under basic conditions, FC reagent reacts with phenolic compounds and, consequently, a phenolate anion is formed, possibly (phenol MoW11O40)4-, by dissociation of a phenolic proton. This sequence of reversible one- or two-electron reduction reactions leads to blue-colored compounds being formed between phenolate and FC reagent. In essence, it is believed that the complex and electron-transfer reaction between Mo(VI) and reductants reduces the molybdenum (*5, 17*):



Mo (VI) (yellow) + e ⟶ Mo (V) (blue)

The total polyphenol assay has become a routine assay in studying phenolic antioxidants and it is simple, sensitive and precise (*17*). However, a number of substances, particularly sugars,

aromatic amines, sulfur dioxide, ascorbic acid and other enediols and reductones, organic acids, Fe (II) and Cu (I), interfere with the total phenolics method, so correction for interfering substances should be considered.

TEAC assay

Miller and Rice-Evans first described the TEAC assay, which is based on the ability of antioxidants to scavenge the long-life radical cation 2, 2'-azobis-(3- ethylbenzthiazoline-6-sulphonic acid) $ABTS^{\bullet+}$ (*43*). The TEAC assay was improved by Re and others (*44*). In this assay, peroxyl radicals or other oxidants oxidize ABTS to its radical cation, $ABTS^{\bullet+}$ (intense blue color). The antioxidant capacities of test compounds are determined by measuring decreases in the blue color as a result of reaction between the $ABTS^{\bullet+}$ radical and the antioxidant compounds in the sample. It has been shown that $ABTS^{\bullet+}$ has an absorption maximum at 415, 645, 734, and 815 nm out of which 415 and 734 nm are most widely accepted (*45*). In the most recent version of the TEAC method, the absorbance decrease of $ABTS^{\bullet+}$ in the presence of the test sample or Trolox at a fixed time point is measured, and the antioxidant capacity is expressed as Trolox equivalents (*46*). $ABTS^{\bullet+}$ is soluble in both aqueous and organic solvents and therefore can be used to determine both hydrophilic and lipophilic antioxidant capacities.

β-Carotene linoleic acid assay

Oxidation results in the bleaching of carotenoids. Oxidation is induced by light or heat (*47*) or by peroxyl radicals [*e.g.*, 2, 2'-azobis-(2-amidopropane)-dihydrochloride (AAPH) or oxidizing lipids] (*48*). Decolorization of β-carotene can be monitored spectrophotometrically at 470 nm (*47*). Heat-induced oxidation of an aqueous emulsion system of β-carotene and linoleic acid was reported as an antioxidant test reaction (*49*). An advantage of the β-carotene bleaching

method is that it is a simple method requiring no specialized instrumentation (*50*). β-Carotene bleaching can occur by multiple pathways, so interpretation of results may be complicated (*5*).

Reducing Power Assay- FRAP

FRAP assay was originally developed by Benzie and Strain (*51*) to measure the reducing power in plasma, but later the assay was modified and used for antioxidants. The reaction measures reduction of ferric 2, 4, 6-tripyridyl-s-triazine (TPTZ) to an intense blue colored product which can be read spectrophotometrically at 595 nm. Reducing power is related to the degree of hydroxylation and extent of conjugation in polyphenols. Similar compounds react to both FRAP and TEAC assays because the redox potential of FE (III)-TPTZ is comparable to $ABTS^{\bullet+}$ but the conditions in both assays differ. TEAC is carried out at neutral pH and FRAP at acidic pH (3.6) to maintain iron solubility. Often, FRAP values have a poor relationship to other antioxidant assays. FRAP assay is based fully on electron transfer mechanism. FRAP actually measures only the reducing ability of the compound based on ferric ion which has no correlation with the in vivo antioxidant activity. However, compared to other in vitro assays it is simple, speedy, inexpensive, and does not require specialized equipment (*5*).

UNDERUTILIZED/MINOR PLANTS

Interest in diversification of crops around the world has increased substantially. Various new crop species are being investigated for possible food and non food purposes. Determination of a crop as underutilized depends on the area e.g., pomegranate is a main fruit crop in India but it is considered as underutilized or minor crop in Georgia. Underutilized crops are found in several agroecosystems and often survive mainly in marginal lands. Underutilized crops are usually grouped into different categories based on their end uses as beverage, cereal, oil, spice and

flavoring, fruit and vegetables (*52*). Most of the underutilized crops are multipurpose. There is a need to create market for such crops to enable their conservation and enhance their industrial applications.

Loquat

Loquat (*Eriobotrya japonica*) or Japanese plum belongs to Rosaceae family. It originated in southeastern China and later became naturalized in Japan, India and many other areas. It is assumed that Chinese immigrants brought loquats to Hawaii. It was a common ornamental tree in California during 1870s. Loquat is adapted to sub tropical to mild temperate climate. The loquat is large evergreen shrub or a small tree with a rounded crown, short trunk and woolly twigs. Small, white, light fragrant flowers are borne in fall or early winter. Loquat fruit is usually oval or pear shaped and is borne in clusters. They are yellow or orange in color with the pulp varying from orange to yellow to white. The fruit bears three to five large brown seeds. The fruit should be allowed to ripen fully before harvesting during spring. The fruit is comparable to apple (of the same family) in high sugar, acid and pectin content. Both loquat fruit and leaves are used in Chinese traditional remedies for cough and asthma. In Japan loquat seed is called "good for health" (*53*).

In Georgia, the flowers and fruits are often destroyed if temperature falls below 27 °F. Loquat is more adaptable to south Georgia than northern parts. In south Georgia fruit production occurs about every three years in slightly protected locations. Some promising varieties for southeast area are Advance, Bartow, Fletcher Red, Hardee, Champagne, Thales, and Wolfe (*54*).

Mayhaw

Mayhaws (*Crataegus aestivalis, C.rufula* or *C.opaca*) belong to Rosaceae family and hawthorne genus. They are found usually in swampy area and may have their root systems flooded for several months in a year. They produce small red colored apple-like fruits that ripen during late April and May, hence the name. It is a relatively unexplored and underutilized indigenous fruit tree of southern states of USA. This is an attractive ornamental small tree growing abundant from North Carolina to Florida and west to Arkansas to Texas. The fruit has been used locally for making items like marmalades, jellies, deserts, syrups and wines.

Some promising cultivars suitable for south Georgia are T.O. Superberry, Mason's Superberry, Superspur, Saline, Big Red, Crimson, Big V, Turnage 57, Texas Star, and Turnage 88 (*54*).

Pawpaw

Pawpaw (*Asimina triloba*) is a native fruit of America and the only fruit of Annonaceae family adapted to temperate climate. It is a small tree with rounded canopy. Flowers vary from maroon to purple and bloom in May. Fruits are borne in clusters of one to six. The peel is green and turns yellowish black when fully ripe. Ripe pawpaws have a prominent fruity and floral flavor. The fruits are oblong to round with a number of large blackish brown seeds. Fruits have more than 430 calories per pound. Pawpaw has great potential as a commercial crop because of its adaptation to existing climatic and edaphic conditions, its nutritional and cosmetic value, its bioactive compounds, and its use as a component in residential edible landscapes (*55*). Pawpaw produces annonaceous acetogenins in leaf, bark, and twig that possess antitumor and pesticidal property (*56*).

Suitable varieties for Georgia are Davis, Mango, Mitchell, PA Golden, Sunflower, Taylor, Taytow, Wells, Wilson, and Overleese (*54*).

Fig

Fig (*Ficus carica*) of Moraceae family is considered one of the earliest fruits cultivated (*57*). It is a native crop of western Asia with wide distribution over the Mediterranean region. Fig is a flower inverted into itself with the seeds being the real fruit. Fig fruit is rich in mineral and vitamins, providing 30% iron, 15.8% calcium, 14% potassium, 7.1% thiamin, and 6.2% riboflavin per 100 g serving of dried figs (*58*). Fig color varies from green to dark purple. Figs can be consumed raw and whole or peeled. As quoted by Pliny (52-113 AD), "Figs are restorative. They increase the strength of young people, preserve the elderly in better health and make them look younger with fewer wrinkles" (*57*). In Ayurveda and Traditional Chinese Medicine (TCM) various parts of fig tree are used. All parts of fig trees have shown anti-inflammatory activity. Its role in the treatment of tumor, diarrhea, chronic cough, ulcers, dermatitis, and gout has also been reported in several studies (*59*).

Pomegranate

Pomegranate (*Punica granatum*) belonging to Punicaceae family has been used in several traditional medicine systems for centuries. The current total annual world production of pomegranate is estimated to be around 1.5 million tonnes and the four largest producers are Iran, India, China, and USA (*60*). It is a rich source of various bioactive compounds. The fruit contains numerous seeds surrounded by sweet pink, juicy, sub-acid pulp (together known as arils) covered with leathery brown to red, bitter skin (or peel or pericarp), which is easily peeled. Bell shaped orange red flowers appear in spring and summer (*61*). Pomegranates may be

damaged by low temperatures during their growth. Pomegranate seed oil comprising 12-20% of total seed weight consists of approximately 80% conjugated octadecatrienoic fatty acids mainly punicic acid (*61*). Studies show that conjugated linolenic acids significantly lower serum total cholesterol (*16*), apoB-100, and hepatic triacylglycerols (*62*). The peel (pericarp) is rich in both flavonoids and tannins especially punicalagin. Pomegranate juice is bright red in color owing to its high content of flavonoids and anthocyanins. Leaves, flowers, bark and roots also contain distinctive compounds having potent physiological effects. Pomegranate extracts have demonstrated protective and preventive properties in human skin fibroblast against UVA and UVB induced damage (*63*). Studies have provided evidence that pomegranate peel has much higher phenolic content than seed and juice and show high antimutagenic and antioxidant properties (*64, 65*).

Although pomegranates fruits grow poorly in Georgia, some suitable varieties are Belgal, Granada, early Foothill, Ruby Red, Sweet Spanish Papershell and Wonderful (*54*).

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Table 2.1. The Major Classes of Phenols (*66*)

number of carbon atom	basic skeleton	class
6	C_6	simple phenols, benzoquinones
7	C_6- C_1	phenolic acids
8	C_6- C_2	acetophenone, phenylacetic acid, hydroxycinnamic acid
9	C_6- C_3	polypropene, coumarin, isocoumarin
10	C_6- C_4	naphtoquinone
13	C_6- C_1- C_6	xanthone
14	C_6- C_2- C_6	stilbene, anthrachinone
15	C_6- C_3- C_6	flavonoids, isoflavonoids
18	$(C_6$- $C_3)_2$	lignans, neolignans
30	$(C_6$- C_3- $C_6)_2$	biflavonoids
n	$(C_6$- $C_3)_n$	lignins
n	$(C_6)_n$	catecholmelanine
n	$(C_6$- C_3- $C_6)_n$	(condensed tannins)

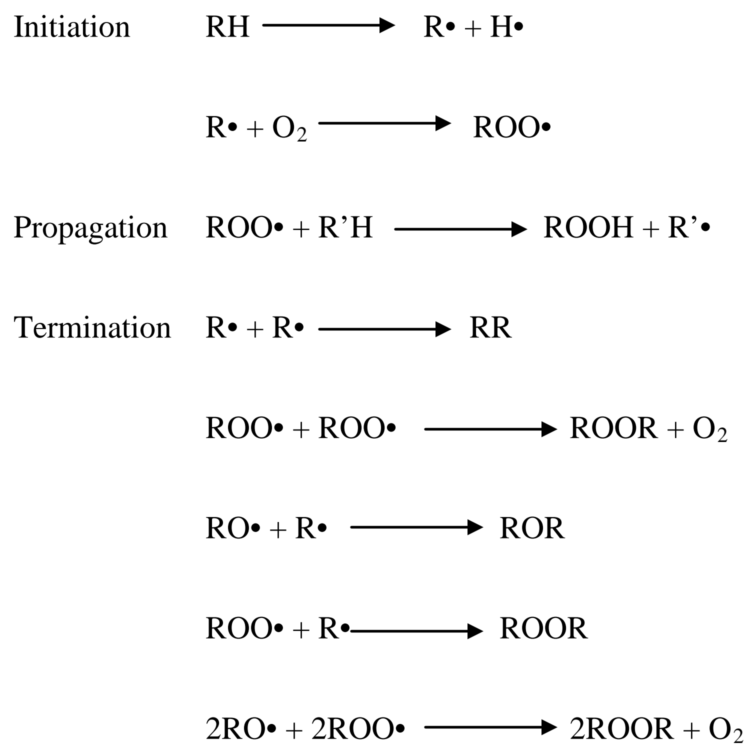
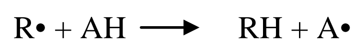
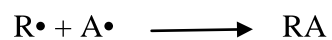


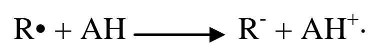
Fig 2.1. Oxidation reactions

HAT – hydrogen atom transfer





SET- single electron transfer



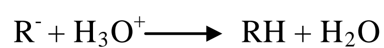


Fig. 2.2. Antioxidant reactions by two mechanisms, hydrogen atom transfer (HAT) and single electron transfer (SET) (*17*)

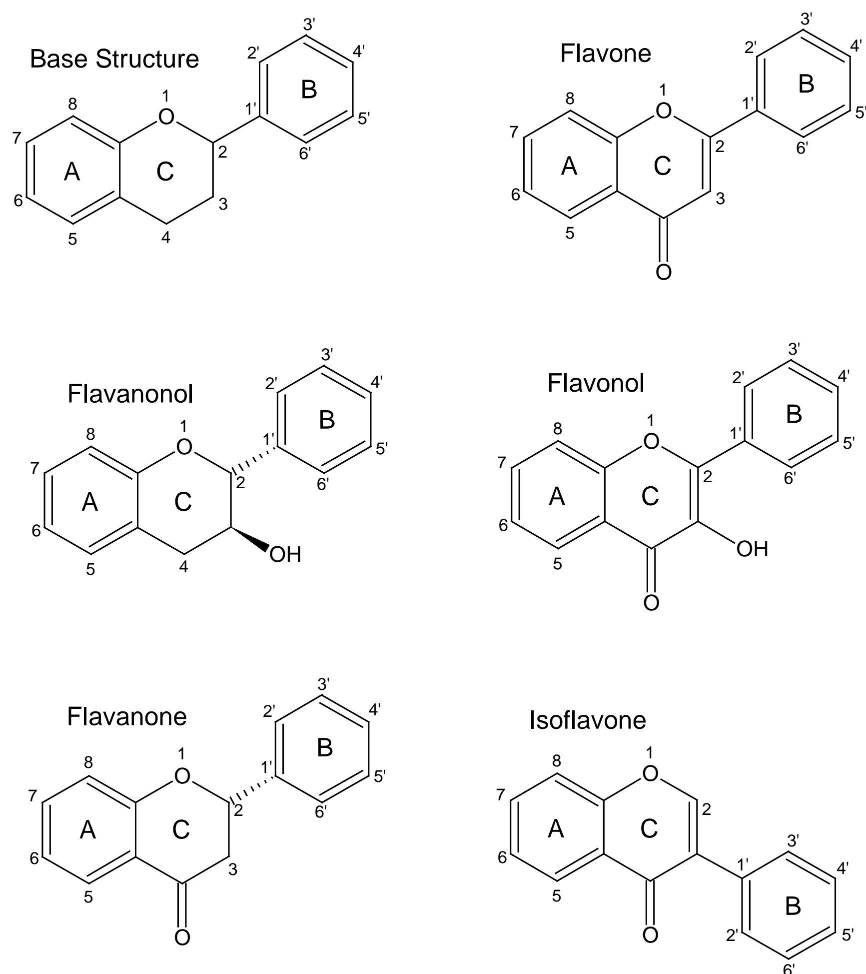
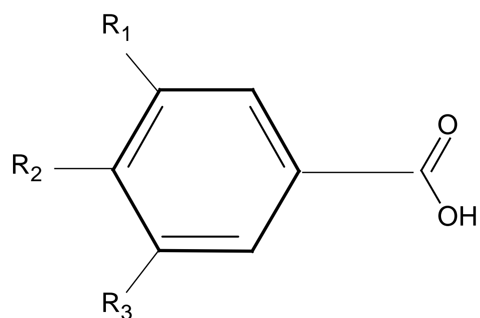
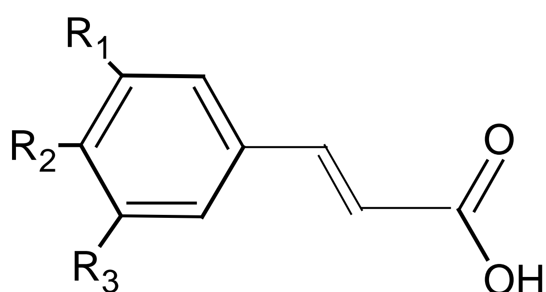


Fig 2.3. Basic structures of flavonoids (*67*)



(a) Hydroxybenzoic acid derivatives

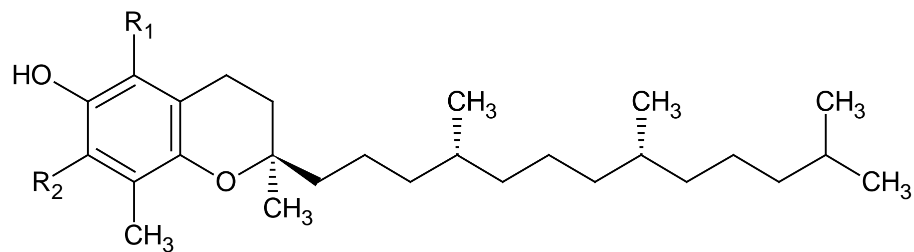
hydroxybenzoic acids	R_1	R_2	R_3
3-hydroxybenzoic acid	H	H	OH
gallic acid	OH	OH	OH
protocatechuic acid	H	OH	OH
vanillic acid	H	OH	OCH_3



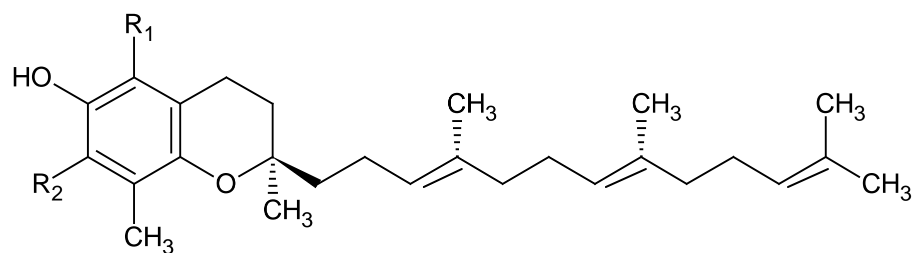
(b) Hydroxycinnamic acid derivatives

hydroxycinnamic acid	R_1	R_2	R_3
caffeic acid	OH	OH	H
p-coumaric acid	H	OH	H
ferulic acid	OCH_3	OH	H

Fig 2.4. Structures of phenolic acids (*68*)



(a) Tocopherol



(b) Tocotrienol

homologues	R1	R2
α	CH3	CH3
β	CH3	H
γ	H	CH3
δ	H	H

Fig. 2.5. Structures of tocopherols and tocotrienols (*31*)

CHAPTER-3

Organic Acids, Phenolic Content, and Antioxidant Capacity of Georgia-Grown Underutilized Fruit Crops

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Garima Pande and Casimir C. Akoh
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ABSTRACT

Four underutilized Georgia–grown fruit crops namely loquat (*Eriobotrya japonica*), mayhaw *Crataegus sp.),* fig (*Ficus carica*), and pawpaw (*Asimina triloba*) were separated into seed, pulp, and peel. Both hydrophilic and lipophilic fractions were prepared and each fraction was analyzed for total polyphenols by Folin Ciocalteau method and antioxidant capacity by trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) assays. Organic acids and phenolic compounds were identified by RP-HPLC. The leaves and the whole fruits were also studied. The major organic acids identified in loquat and mayhaw was malic acid, in pawpaw malic, citric, and ascorbic acids, and in fig malic, citric, and oxalic acids. In general, the hydrophilic fractions had higher total polyphenols and antioxidant capacity than the lipophilic fractions with the highest concentration being in the seeds and leaves. High correlation was observed between total polyphenols and TEAC in hydrophilic fractions (R^2=0.82) and lipophilic fractions (R^2=0.79). The predominant phenolic acids in all the fruits were gallic and ellagic acids and the most abundant flavonoid was catechin.

KEYWORDS: antioxidant capacity; *Asimina triloba*; *Crataegus sp.; Eriobotrya japonica*; *Ficus carica*; organic acids; polyphenols; underutilized fruits

INTRODUCTION

Oxidative stress caused by either reduced level of antioxidant defense or increased free radical production is a major factor in pathogenesis of degenerative diseases such as cardiovascular diseases and cancer (*1*). Both endogenous and diet-derived antioxidants prevent biomolecules from damage caused by free radicals which are generated as a result of oxidation. Epidemiological studies over the last decade have shown positive correlation between intake of antioxidant rich diet and treatment and prevention of chronic diseases (*2*). Vitamins, carotenoids and polyphenols are believed to contribute the most to the protective nature of fruits and vegetables (*3*). In addition to the metabolic processes in plants, organic acids and phenolic compounds play a major role in determining the nutritional profile, sensory qualities and shelf life of the fruit and its derived products (*4*).

With the global functional food and beverage market expected to reach $109 billion by 2010 (*5*), diverse sources of phytochemicals are being explored. Georgia cultivates a wide range of fruits but some fruits with great economic and nutritional potential remain underexploited. The rationale for studying these fruit crops is their conservation, diversification of sources of phytochemicals, agronomic, and economical advancement.

Loquat or Japanese plum (*Eriobotrya japonica*) belongs to Rosaceae family. It originated in southeastern China and later became naturalized in Japan, India, and many other areas. In China, both fruit and leaves of loquat are used for treating cough and asthma (*6*). Mayhaws (*Crataegus aestivalis, C. rufula or C. opaca*) also belong to Rosaceae family and hawthorne genus. They produce small red colored apple like fruits that ripen during late April and May, hence the name. The fruit has been used locally for making marmalades, jellies, deserts, syrups and wines. It is a relatively unexplored and underutilized indigenous fruit tree of southern states

of USA. Pawpaw (*Asimina triloba*) is a native fruit of America and the only fruit of Annonaceae family adapted to temperate climate (*7*). Pawpaw plant contains annonaceous acetogenins that exhibits high antitumor and pesticidal properties (*8*). Fig (*Ficus carica*) of Moraceae family is considered to be one of the earliest fruits cultivated (*9*). In Ayurveda and Traditional Chinese Medicine various parts of fig tree are used. Its role in the treatment of tumor, diarrhea, chronic cough, ulcers, dermatitis, and gout has been reported in several studies (*10*).

The peels and seeds which are usually disposed as waste material in many food processing industries could be used as a rich source of beneficial phytochemicals. Furthermore, this could prevent environmental pollution and economic losses. Higher antioxidant capacity has been identified in citrus peel and seeds (*11*), mango seed kernels (*12*), and muscadine skin and seeds (*13*). Very few studies have been documented on the antioxidant capacity of loquat, mayhaw, pawpaw, and fig. Thus, the objective of this study was to identify and quantify the major organic acids, phenolic compounds and determine the antioxidant capacity of different fruit parts and leaves of these underutilized fruit crops.

MATERIALS AND METHODS

Plant material

All fruits were harvested at their optimum ripe stage along with the leaves and transported in ice coolers to the University of Georgia. Loquat (*Eriobotrya japonica*, Rosaceae) was collected from seedling trees on Lincoln Street, Savannah, GA. Mayhaw *(Crataegus sp.,* Rosaceae*)* (Turnage 57) was collected from Tifton, GA. Pawpaws (*Asimina triloba,* Annonaceae) were collected randomly from different cultivars at university experimental farm, Tifton, GA, and figs (*Ficus carica,* Moraceae) (Brown Turkey) were collected from Briar Patch farm, Dacula, GA. The fractions were prepared immediately to prevent degradation of the fruits.

Chemicals

Pure standards of succinic acid (99%), DL-malic acid (98%), oxalic acid (99%), BHT (99%), β – carotene (95%), gallic acid (90%), quercetin (85%), ellagic acid (95%), (+)-catechin (98%), ferulic acid (99%), *p*-coumaric acid (≥98.0%), caffeic acid (≥98.0%), (-)- epicatechin (≥98%), Folin Ciocalteau reagent, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (98%), citric acid (99.5%), and potassium persulfate (≥99.0%) were purchased from Sigma Chemical Co. (St. Louis, MO). 2, 4, 6-Tripyridyl-s-triazine (TPTZ) (99%) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (97%) were purchased from Acros Organics (Morris Plains, NJ), L-Ascorbic acid from Mallinckrodt Baker Inc.(Phillipsburg, NJ), and $FeCl_3.6H_2O$ (98%) from Fluka (Milwaukee, WI). Other solvents and chemicals were purchased from Sigma Chemical Co., J.T. Baker Chemical Co. (Phillipsburg, NJ), and/or Fischer Scientific (Norcross, GA)

Sample preparation

The samples were washed with water and dried properly. 500 g of loquat fruits were weighed and divided into peel, pulp, edible portion (pulp and peel), and seeds. For mayhaw, 500 g of fruit was used. It was not possible to separate the peel from the pulp. Therefore, for mayhaws three fractions were prepared, pulp and seed, peel, and edible portion (whole fruit). 500 g of pawpaw fruits were divided into pulp (edible portion), peel, and seeds. 500 g of fig fruits were divided into peel, pulp containing minute inseparable seeds, and edible portion (whole fruit). The leaves of each crop were also analyzed. All sample preparation was performed under cool and dark conditions. All fractions were packaged in amber bottles, labeled, and stored at -80 ºC after flushing with nitrogen until analyzed.

Dry weight determination

Dry weight (DW) was determined following the guidelines of the official AOAC method 967.03 (*14*). A small portion of each fraction was placed on a pre-weighed aluminum pan and weighed. The pans were kept in oven at 105 ºC for 16 h. After the drying time, the pans were removed from the oven, allowed to cool in a dessicator and weighed again. Sample dry weight (g/g FW) was calculated as shown in equation 1.

$$DW = (c-a) / (b-a) \quad (1)$$

where, a is the weight of empty pan (g), b is the weight of pan and fresh sample (g), and c is the weight of pan and dried sample (g). All samples were analyzed in triplicates and average values were reported.

Preparation of hydrophilic and lipophilic fractions

Hydrophilic and lipophilic fractions were prepared using the method of Jimenez-Alvarez et al. (*15*). 2 g of each fruit fraction was weighed and 10 mL hexane was added to each. The samples were centrifuged for 10 min at 2500 rpm and the supernatants were collected. This procedure was repeated once more; supernatants were pooled in, dried under nitrogen and reconstituted in 10 mL of 95% ethanol. These lipophilic fractions were stored at -20 ºC until analyzed. For the hydrophilic fraction, the remaining residue was dried under nitrogen to remove all hexane and 5 mL acetone/water/acetic acid (70:28:2, v/v/v) was added. The samples were centrifuged for 10 min at 2500 rpm and the supernatants were collected. This procedure was repeated once again and the supernatants were pooled. The volume was made up to 10 mL with the acetone/water/acetic acid (70:28:2, v/v/v) and samples were stored at -20 ºC until analyzed. These lipophilic and hydrophilic fractions (0.2 g/mL) were used for total polyphenols and antioxidant assays.

Total carotenoids

Total carotenoids were determined using the method by Duvivier et al. (*16*) with few modifications. Briefly, 0.2 g sodium carbonate, 0.1 g diatomaceous earth, 5 mL methanol and 10 mL hexane/acetone (1:1, v/v) with 0.001% BHT was added to 2 g of each sample. The samples were stirred for 2 h and then filtered through Whatman No.1 filter paper. The residue was washed twice with 5 mL methanol and once with 10 mL hexane/acetone mixture. The volume was made up to 50 mL with deionized water. The lipophilic layer was transferred to a volumetric flask and the volume was made up to 10 mL with hexane. The absorption was measured at 450 nm using Shimadzu 300 UV-Vis spectrophotometer (Shimadzu UV-1601, Norcross, GA). Quantification was based on the standard curve generated with 5-10 μg/mL of β-carotene and the total carotenoids were expressed as mg β-carotene equivalents/100 g FW. All samples were analyzed in triplicates and average values reported.

Major organic acids

2 g sample was mashed with 10 mL of 1 M HCl. The volume was made up to 20 mL with 1 M HCl. The samples were flushed with nitrogen and centrifuged at 2000 rpm for 15 min. The samples were placed in water bath at 90 ℃ for 30 min, allowed to cool to room temperature and the supernatant was collected and filtered through 0.45 μm membrane filter. Organic acids were analyzed and identified using Hewlett-Packard (Avondale, PA) HP 1100 HPLC system with diode array and fluorescence detectors based on Chen et al. (*17*) method. The injection volume was 20 μL and column temperature was maintained at 40 ℃. Agilent Zorbax Eclipse® XDB-C18, 3.5 μm, 4.6 x 150 mm column and an isocratic mobile phase of 0.5% ammonium phosphate, pH adjusted to 2.8 with phosphoric acid was used at a flow rate of 0.5 mL/min.

Detection was done at 214 nm. Triplicate determinations were made and averaged based on the external standards (10-1600 μg/mL).

Major phenolic compounds

Major phenolic compounds were determined following the method described by Pastrana-Bonilla et al. (*13*). 1 g sample was mashed and diluted with 10 mL 80% methanol in 6 N HCl. The samples were vortexed for 1 min and placed in a water bath shaker at 60 °C and 200 rpm for 2 h. Once again the samples were vortexed and then cooled to room temperature. The supernatants were filtered through 0.45 μm membrane filter and 20 μL was injected into a Hewlett-Packard (Avondale, PA) HP 1100 HPLC system with diode array detector. The Beckman Ultrasphere® C18, 5 μm, 4.6 x 250 mm column with temperature set at 40 °C was used as the stationary phase. The mobile phase consisted of solvent A, methanol/acetic acid/water (10:2:88, v/v/v); solvent B, acetonitrile; and, solvent C, water. A linear gradient was used as follows: at 0 min, 100% solvent A; at 5 min, 90% solvent A and 10% solvent B; and at 25 min, 30% solvent A and 70% solvent B, with 5 min postrun of 100% solvent C. The flow rate was 1 mL/min. Detection was done at 260, 280, 320, and 360 nm. Identification was based on the retention times and characteristic UV spectra and quantification was done by the external standard curves. All analysis was performed in triplicates.

Total polyphenols

Total polyphenols were determined according to the Folin-Ciocalteau reagent method (*18*). 200 μL of extracted sample, in triplicate, was added to 1 mL of 0.2 N Folin-Ciocalteau reagent and 0.8 mL of 7.5% sodium carbonate solution, mixed well and allowed to stand for 30 min at room temperature. Absorption at 765 nm was read using a Shimadzu 300 UV-Vis spectrophotometer

(Shimadzu UV-1601, Norcross, GA). Quantification was based on the standard curve generated with 100 - 400 mg/L of gallic acid.

Antioxidant capacity

Ferric Reducing Antioxidant power (FRAP) assay

FRAP assay was carried out as described by Benzie and Strain (*19*) with slight modifications. Stock solutions of 300 mM acetate buffer, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine solution in 40 mM HCl), and 20 mM $FeCl_3.6H_2O$ were prepared. Working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3.6H_2O$. The solution was heated to 37 °C and maintained at pH 3.6. 50 µL of sample was mixed with 2 mL of the working solution and absorbance was read at 593 nm against appropriate blank for 4 min. The change in absorbance was calculated and related to the standard curve generated with trolox. Results were expressed as µM TE/g FW. All assays were in triplicates and averages reported.

Trolox Equivalent Antioxidant Capacity (TEAC) assay

The antioxidant capacity was measured by the radical cation decolorization assay based on the methods of Re et al. (*20)* and van den Berg et al. (*20, 21*). Trolox solution of 0-15 µM final concentration was used as standard. Briefly, 7 mM ABTS solution and 2.45 mM potassium persulfate solution were mixed and kept in the dark at room temperature for 12-16 h. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. 50 µL of trolox standard or sample was added to 2 mL of diluted $ABTS^{\bullet+}$ solution and the absorbance was read for 6 min at 734 nm. Appropriate solvent blanks were also ran in each assay. The percentage inhibition of absorbance was calculated and plotted as a function of concentration of trolox. TEAC values of samples were calculated based on the standard curve and reported as µM TE/g FW from average of triplicate determinations.

Statistics

All samples were analyzed in triplicates and the results are expressed as average ± standard deviation. All statistical analysis was carried out using the Microsoft Excel software package (Microsoft Corp. Redmond, WA).

RESULTS AND DISCUSSION

All results were expressed on fresh weight basis. The dry matter content of the samples is reported in **Table 3.1**. The seeds and leaves have the highest dry matter. Total carotenoids of the fruits are given in **Table 3.2**. Carotenoids were determined only in pulp, peel, and edible portions of the fruits. The highest carotenoids were found in loquat whole fruit (2.8 mg/100 g FW) and the least in pawpaw pulp (0.6 mg/100 g FW). These results are in accordance with the visual appearance (color) of the fruits. The loquats had bright yellowish pulp with orange peel whereas pawpaw had pale yellowish pulp with green peel.

Organic acid content of a fruit is an important factor for the development of its flavor. **Table 3.3** shows the organic acid composition of the fruit parts, whole fruits, and the leaves. As a characteristic of rosaceae family, high malic acid content was found in loquat (462.1-1917.7 mg/100 g FW) and mayhaw (402.0-1645.8 mg/100 g FW) fruits with the highest being in loquat peel. The major organic acids in loquat and mayhaw were malic, citric, and ascorbic acids with traces of oxalic acid. Succinic acid (4.4-12.8 mg/100 g FW) was found in all samples except loquat fruit and leaves. Highest ascorbic acid was found in mayhaw pulp and peel fraction (14.5 mg/100 g FW) followed by fig whole fruit (14.2 mg/100 g FW). Edible portion of loquat contained the lowest ascorbic acid (6.0 mg/100 g FW) when compared with the edible portions of the other three fruits. Fig fruit contained high citric (20.2 mg/100 g FW) and oxalic (17.9

mg/100 g FW) acids. Oxalic acid, usually found in green leaves, was found in leaves of all the fruits in considerable amount (13.4-18.6 mg/100 g FW).

Major phenolic compounds of the fruit fractions and leaves are reported in **Table 3.4**. Phenolic compounds can be grouped into several sub-classes with flavonoids and phenolic acids constituting 60 and 30%, respectively, of total dietary polyphenols (*22*). In terms of phenolic acids, the highest gallic acid content was found in loquat seed (6.4 mg/100 g FW) whereas all other samples showed comparable gallic acid content. The highest ellagic acid content was found in fig leaves (33.8 mg/100 g FW). Caffeic acid was detected only in the leaves ranging from 0.8 mg/100 g FW in pawpaw leaves to 7.8 mg/100 g FW in fig leaves. Ferulic acid content varied from not detected in fig fruit and pawpaw seed to 22.1 mg/100 g FW in mayhaw pulp and peel fraction. The predominant flavonoid detected was catechin with the highest content in loquat seed (37.8 mg/100 g FW). In contrast to previous results, catechin was found in fig fruit (16.7 - 21.2 mg/100 g FW) (*23*). This difference may be due to cultivar and agroecological variations between the studies. Epicatechin was found in all the samples except pawpaw seed, peel and pulp. Quercetin was found only in the leaves with the exception of loquat seed. Although there were differences in the phenolic compounds content both among and within the fruits, in general the seeds and leaves of all the fruits showed much higher phenolic compounds than the pulp or peel.

Total polyphenols of the hydrophilic and lipophilic fractions of samples are shown in **Tables 3.5** and **3.6**, respectively. Total polyphenols were calculated using the Folin Ciocalteau reagent assay which is a convenient, simple, and precise method. The hydrophilic fraction showed higher total phenols as compared to the lipophilic fraction. The highest total polyphenols content among hydrophilic fractions (**Table 3.5**) was found in loquat leaves (178.1 mg GAE/100

g FW) whereas among lipophilic fractions it was the pawpaw seed (64.1 mg GAE/100 g FW) (**Table 3.6**).

Antioxidant capacity was measured by two methods namely, FRAP and TEAC assays. FRAP measures the reducing ability of the compound and TEAC measures its scavenging ability. The results for hydrophilic and lipophilic fractions are shown in **Tables 3.5** and **3.6,** respectively. FRAP assay resulted in higher values than TEAC assay in both fractions. Similar to the major phenolic compounds content and total polyphenols, the results of both assays were much higher in hydrophilic fractions than lipophilic fractions. In the hydrophilic fractions, the highest FRAP and TEAC values were found in loquat leaves (17.2 µM TE/g FW and 7.5 µM TE/g FW, respectively) which correlates with its high total polyphenols content. Among the lipophilic fractions, the highest FRAP value was found in loquat leaf (2.8 µM TE/g FW) although the total polyphenols content of pawpaw seed was the highest. The highest TEAC value in the lipophilic fraction was found in pawpaw seed (1.8 µM TE/g FW) which is in accordance with its total polyphenols content.

The correlation between total polyphenols, TEAC, and FRAP were calculated together for all four fruits rather than individual fruits. High correlation was found between total polyphenols content and TEAC ($R^2_{hydrophilic}$=0.82 and $R^2_{lipophilic}$=0.79) (**Figures 3.1a** and **3.2a**). This indicates that TEAC is strongly related to total polyphenols content of the samples which is in agreement to previous studies (*24*). The correlation coefficient between total polyphenols and FRAP was 0.74 for hydrophilic fraction (**Figure 3.1a**) and only 0.35 (**Figure 3.2a**) for lipophilic fractions indicating that FRAP assay is better suited for hydrophilic fraction than lipophilic fraction. Correlation between TEAC and FRAP was much higher in the hydrophilic fraction (R^2=0.86) (**Figure 3.1b**) than lipophilic fractions (R^2=0.46) (**Figure 3.2b**).

Table 3.7 shows a comparison between the edible portions of loquat, mayhaw, pawpaw, and fig with other Georgia-grown crops and also with some other common fruits. The total polyphenols and antioxidant capacity as measured by TEAC assay were compared. TEAC was used for comparison because it is the most common in vitro antioxidant assay and also our results showed that it is better suited for both hydrophilic and lipophilic fractions. Therefore, TEAC assay was used as a comparative index for antioxidant capacity. These four fruits had much lower total polyphenols and TEAC when compared to rabbiteye blueberries (556.1 mg GAE/100 g FW, 27.6 µM TE/g FW) *(24)*, southern highbush blueberries (399.3 mg GAE/100 g FW, 14.8 µM TE/g FW) *(24)*, blackberries (486.5 mg GAE/100 g FW, 20.4 µM TE/g FW) *(24)*, and muscadines (247.7 mg GAE/100 g FW, 17.6 µM TE/g FW) *(13)* but higher than Vidalia onion (var. Nirvana) (73.3 mg GAE/100 g FW, 1.1 µM TE/g FW) *(25)* among Georgia-grown crops. With respect to other common fruits, these underutilized fruits show greater antioxidant capacity than apple (1.6 µM TE/g FW) *(26)*, watermelon (0.7 µM TE/g FW) *(26)*, and banana (0.6 µM TE/g FW) *(26)* but much lower antioxidant capacity than pineapple (9.9 µM TE/g FW) *(26),* and strawberry (11.0 µM TE/g FW) *(26)*.

The results of our study indicate that these underutilized fruits and leaves contain considerable amount of bioactive compounds and may find use in nutraceutical, pharmaceutical, and other applications although more extensive studies are required to screen for toxic compounds, if any, before any commercial applications of these fruits.

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Figure captions:

Figure 3.1a. Correlation between total polyphenols and antioxidant assays for hydrophilic fractions

Figure 3.1b. Correlation between FRAP and TEAC for hydrophilic fractions

Figure 3.2a. Correlation between total polyphenols and antioxidant assays for lipophilic fractions

Figure 3.2b. Correlation between FRAP and TEAC for lipophilic fractions

Table 3.1. Dry Matter (DM) Content of Fruit Parts and Leaves (g/g FW).

fruits	parts	DM[a]
loquat	seed	0.5±0.1
	pulp	0.1±0.0
	peel	0.1±0.0
	whole fruit	0.1±0.0
	leaf	0.6±0.1
mayhaw	seed	0.4±0.0
	pulp + peel	0.1±0.0
	whole fruit	0.2±0.0
	leaf	0.6±0.2
pawpaw	seed	0.7±0.1
	pulp	0.3±0.0
	peel	0.3±0.0
	leaf	0.4±0.1
fig	pulp + seed	0.1±0.0
	peel	0.1±0.0
	whole fruit	0.2±0.0
	leaf	0.5±0.2

[a] Values are the average of triplicates ± standard deviation

Table 3.2. Total Carotenoids Content of Fruit Parts (mg/100 g FW)

fruit	parts	carotenoids[a]
loquat	pulp	1.2±0.2
	peel	1.8±0.5
	whole fruit	2.8±0.4
mayhaw	pulp + peel	2.6±0.2
	whole fruit	1.6±0.4
pawpaw	pulp	0.6±0.1
	peel	1.5±0.1
fig	pulp + seed	1.1±0.1
	peel	1.9±0.3
	whole fruit	1.6±0.2

[a] Values are the average of triplicates ± standard deviation

Table 3.3. Major Organic Acids in Fruit Parts, Whole Fruit, and Leaves (mg/100 g FW)[a]

fruit	parts	malic acid	citric acid	ascorbic acid	succinic acid	oxalic acid
loquat	seed	462.1±8.1	10.9±5.7	3.2±0.1	nd[b]	nd
	pulp	1644.2±12.6	14.2±8.1	5.3±1.1	nd	nd
	peel	1917.7±25.3	16.6±2.4	6.4±0.6	nd	10.6±1.2
	whole fruit	1078.1±32.2	16.1±3.8	6.0±2.8	nd	4.8±0.6
	leaf	602.0±16.3	4.6±1.3	2.3±1.2	nd	14.8±0.6
mayhaw	seed	402.0±14.5	11.6±5.6	5.8±1.1	5.1±1.4	nd
	pulp + peel	1645.8±33.1	13.9±7.2	14.5±2.4	4.4±1.9	nd
	whole fruit	1459.2±45.3	17.1±3.8	13.6±4.5	6.2±0.6	nd
	leaf	658.0±22.4	8.5±3.0	3.9±2.8	8.6±2.6	18.6±3.9
pawpaw	seed	202.6±17.1	10.6±4.6	7.4±2.3	12.8±4.7	13.5±1.8
	pulp	322.1±26.1	16.2±7.3	9.8±1.1	4.8±2.1	nd
	peel	493.1±18.4	18.7±10.5	11.5±2.4	5.5±1.	nd
	leaf	177.2±9.7	9.8±4.9	5.6±2.0	5.4±2.7	14.5±5.6
fig	pulp + seed	404.9±32.0	24.7±2.9	11.7±1.8	8.7±3.9	17.2±2.8
	peel	450.0±11.0	18.8±6.3	10.6±5.2	6.4±1.9	19.4±3.5
	whole fruit	430.6±19.8	20.2±8.0	14.2±2.6	10.2±3.4	17.9±1.3
	leaf	177.5±22.5	9.7±2.1	8.8±2.2	6.1±4.5	13.4±8.4

[a] Each value is the mean of triplicates ± standard deviation. [b] nd, not detected

Table 3.4. Individual Phenolic Compounds in Fruit Parts, Whole Fruit and Leaves (mg/100 g FW)[a]

fruit	parts	phenolic acids					flavonoids		
		gallic acid	ellagic acid	caffeic acid	*p*-coumaric acid	ferulic acid	catechin	epicatechin	quercetin
loquat	seed	6.4±0.2	0.5±0.1	nd[b]	nd	0.5±0.1	37.8±3.8	12.2±2.8	5.6±2.1
	pulp	2.6±1.7	0.2±0.1	nd	nd	1.9±0.8	12.2±2.7	16.2±0.8	nd
	peel	2.4±0.8	0.8±0.3	nd	0.6±0.1	1.5±0.3	14.5±3.3	8.1±1.0	nd
	whole fruit	2.3±1.8	0.4±0.1	nd	0.5±0.1	0.7±0.1	12.7±0.8	13.6±0.7	nd
	leaf	5.7±1.6	1.3±0.1	2.1±0.6	1.8±0.6	2.3±1.0	14.5±4.6	26.3±4.2	9.8±3.1
mayhaw	seed	4.7±0.5	1.1±0.1	nd	nd	10.2±3.3	28.9±2.9	16.8±0.4	nd
	pulp+peel	2.8±0.2	3.6±0.2	nd	3.1±0.2	22.1±4.2	18.7±0.4	12.6±0.2	nd
	whole fruit	2.6±0.3	3.9±0.2	nd	1.4±0.4	8.9±1.8	15.9±2.0	13.1±2.8	nd
	leaf	3.8±0.2	11.9±1.4	1.4±0.8	2.4±1.1	1.6±0.4	26.6±5.1	24.8±3.9	9.2±2.9
pawpaw	seed	3.9±1.6	1.1±0.1	nd	nd	nd	20.7±3.9	nd	nd
	pulp	1.5±0.1	1.0±0.1	nd	nd	3.0±0.4	12.2±0.5	nd	nd
	peel	1.9±0.8	1.4±0.2	nd	1.5±0.7	1.6±0.1	14.2±0.7	nd	nd
	leaf	3.7±1.0	2.9±0.3	0.8±0.1	6.2±1.1	3.6±0.5	20.2±1.3	15.6±0.4	7.6±1.3
fig	pulp+seed	2.6±0.9	0.5±0.1	nd	nd	nd	21.2±0.4	28.6±3.9	nd
	peel	2.8±1.1	0.4±0.1	nd	nd	nd	17.1±1.8	31.8±2.5	nd
	whole fruit	2.8±0.3	0.2±0.1	nd	nd	nd	16.7±3.2	32.1±3.3	nd
	leaf	3.8±0.2	33.8±9.6	7.8±2.3	5.9±2.0	20.6±5.3	20.8±4.9	17.8±.4	8.4±2.0

[a] Each value is the mean of triplicates ± standard deviation. [b] nd, not detected

Table 3.5. Total Polyphenols and Antioxidant Capacity of Hydrophilic Fractions[a]

fruit	part	total polyphenols[b]	FRAP[c]	TEAC [c]
loquat	seed	152.8±3.6	15.8±1.9	7.2±0.5
	pulp	43.5±0.2	6.9±0.1	1.1±0.1
	peel	89.3±1.1	10.1±0.4	4.2±0.7
	whole fruit	61.1±1.2	8.4±0.4	3.5±0.1
	leaf	178.1±4.4	17.2±2.1	7.5±0.2
mayhaw	seed	82.9±8.8	9.2±0.7	3.1±0.3
	pulp+peel	120.2±3.1	10.2±1.3	5.5±0.5
	whole fruit	124.5±8.5	12.6±2.3	5.8±0.1
	leaf	148.3±4.8	15.1±0.8	6.1±0.1
pawpaw	seed	72.1±1.2	12.8±1.1	4.8±0.2
	pulp	41.3±2.5	4.8±0.2	1.6±0.4
	peel	59.8±1.3	8.1±0.5	2.8±0.3
	leaf	78.5±2.1	11.4±1.2	5.6±0.3
fig	pulp+seed	41.9±2.6	3.5±0.5	0.8±0.1
	peel	105.0±7.1	7.7±0.1	3.8±0.8
	whole fruit	54.3±0.9	6.2±0.3	1.3±0.6
	leaf	69.3±3.1	11.6±0.3	3.2±0.2

[a] Values are averages of triplicate analyses ±standard deviation. [b] Total polyphenols measured as mg GAE/ 100 g FW). [c] Antioxidant capacity measured as FRAP and TEAC (μM TE/g FW).

Table 3.6. Total Polyphenols and Antioxidant Capacity of Lipophilic Fractions[a]

fruit	part	total polyphenols [b]	FRAP[c]	TEAC [c]
loquat	seed	29.0±2.5	1.6±0.1	0.7±0.1
	pulp	13.4±3.5	1.2±0.0	0.4±0.0
	peel	18.1±1.3	1.7±0.1	0.7±0.0
	whole fruit	15.4±0.5	2.2±0.1	0.8±0.1
	leaf	26.3±2.5	2.8±0.1	0.9±0.1
mayhaw	seed	32.0±1.2	2.1±0.1	0.8±0.0
	pulp+peel	15.8±1.3	1.4±0.1	0.6±0.0
	whole fruit	17.1±2.7	1.9±0.0	0.7±0.1
	leaf	27.6±2.0	2.1±0.5	0.7±0.0
pawpaw	seed	64.1±2.8	2.6±0.2	1.8±0.5
	pulp	16.5±1.9	1.2±0.1	0.8±0.0
	peel	19.4±0.6	1.8±0.0	0.9±0.1
	leaf	34.3±0.8	2.1±0.3	1.1±0.3
fig	pulp+seed	19.1±1.2	1.8±0.4	0.8±0.0
	peel	20.9±0.8	1.3±0.0	0.5±0.1
	whole fruit	18.4±0.2	1.0±0.0	0.4±0.0
	leaf	45.4±1.9	2.2±0.3	1.3±0.2

[a] Values are averages of triplicate analyses ±standard deviation. [b] Total polyphenols measured as mg GAE/ 100 g FW). [c] Antioxidant capacity measured as FRAP and TEAC (μM TE/g FW).

Table 3.7. Comparison of Loquat, Mayhaw, Pawpaw, and Fig with other Georgia-grown Crops and Other Common Fruits

fruits	total polyphenols (mg GAE/100 g FW)	TEAC (μM TE/g FW)	ref
loquat[b]	76.5±1.7[a]	4.3±0.2[a]	
mayhaw[c]	141.6±11.2[a]	6.5±0.2[a]	
pawpaw[d]	57.8±4.4[a]	2.4±0.4[a]	
fig[e]	72.7±1.1[a]	1.7±0.6[a]	
Georgia-grown other main crops			
rabbiteye blueberries	556.1±216.9	27.6±5.3	*(24)*
southern highbush blueberries	399.3±149.1	14.8±8.2	*(24)*
blackberries	486.5±97.1	20.4±3.3	*(24)*
muscadine-purple (whole fruit)	247.7±100.5	17.6±7.1	*(13)*
Vidalia onion (var Nirvana)	73.3±1.1	1.1 ±0.0	*(25)*
other fruits			
apple (red delicious)	-	1.6	*(26)*
banana	-	0.6	*(26)*
pineapple	-	9.9	*(26)*
cherry	-	2.7	*(26)*
watermelon	-	0.7	*(26)*
strawberry (cultivated)	-	11.0	*(26)*

[a]Sum of hydrophilic and lipophilic fractions. [b]Edible portion (whole fruit). [c]Edible portion (whole fruit). [d]Edible portion (pulp). [e]Edible portion (whole fruit).

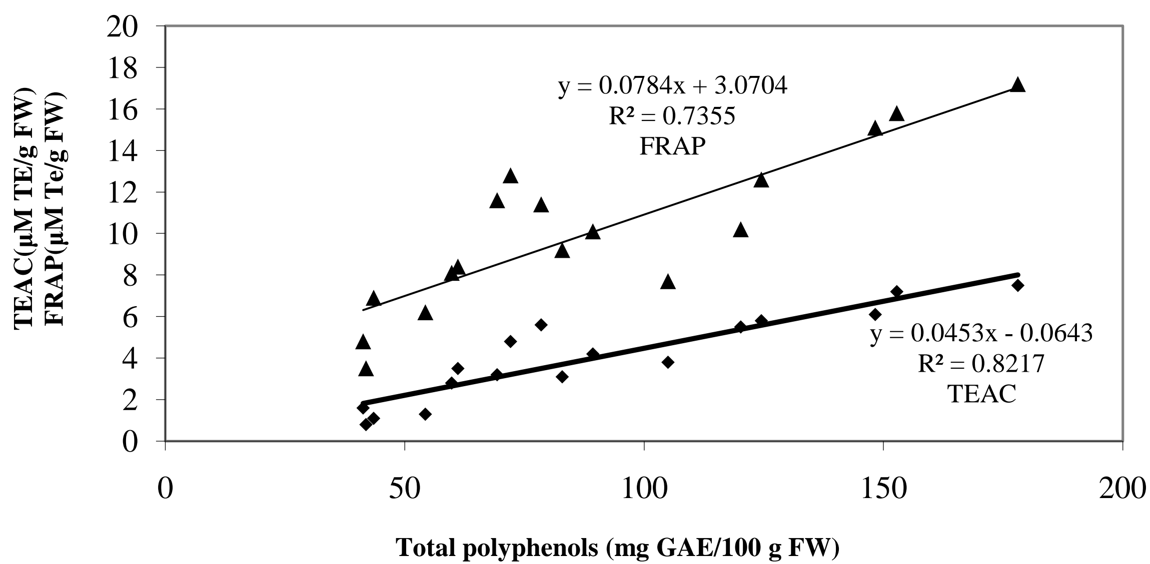

Figure 3.1a

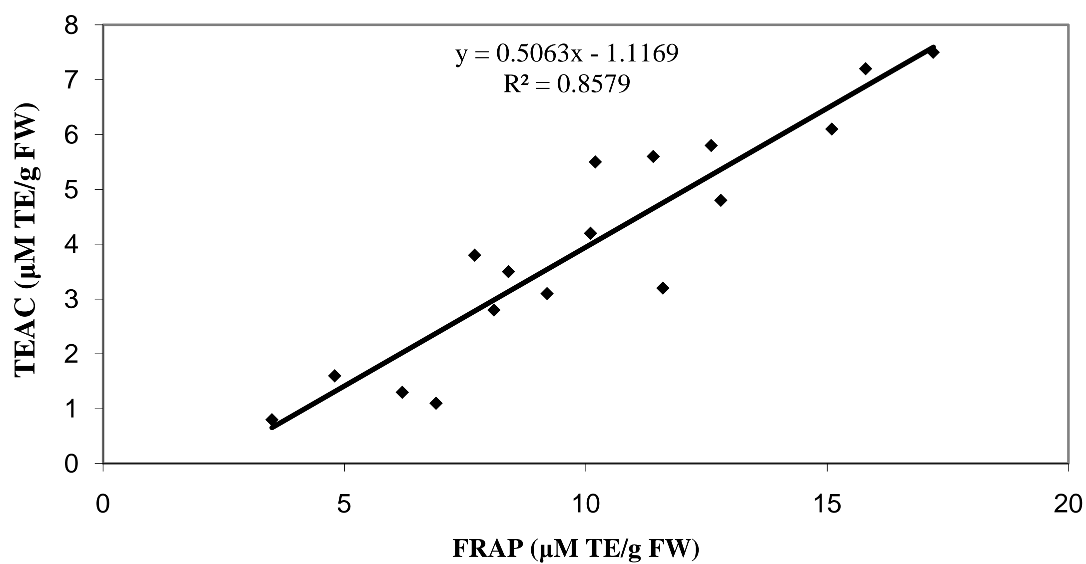

Figure 3.1b

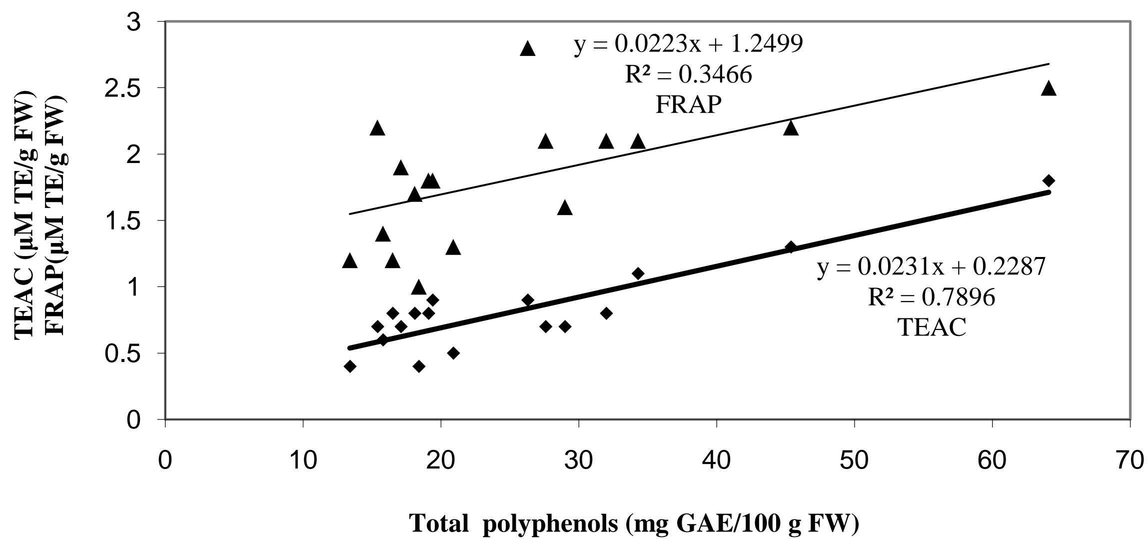

Figure 3.2a

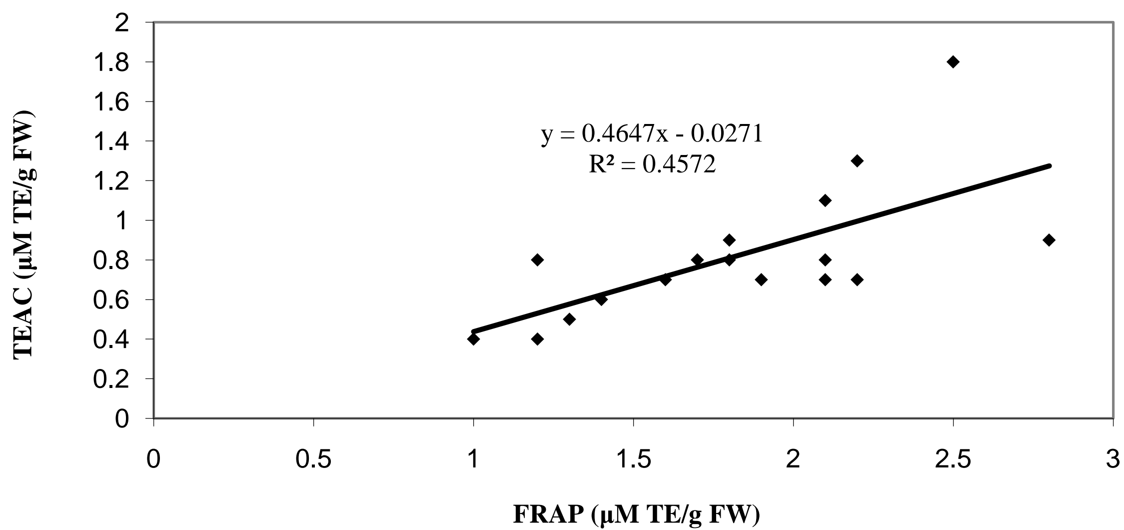

Figure 3.2b

CHAPTER-4

Determination of Fatty Acids, Tocopherols, Phytosterols, and Phospholipids in Underutilized Fruits and Seeds of Georgia

Garima Pande and Casimir C. Akoh
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ABSTRACT

Lipid profile of four Georgia-grown underutilized fruits namely, loquat (*Eriobotrya japonica*), mayhaw (*Crataegus sp.),* fig (*Ficus carica*), and pawpaw (*Asimina triloba*) were characterized. Fruits were separated into two fractions – seed and fruit (i.e., without seed) and each fraction was analyzed. Lipid was extracted using the Folch method. Fatty acid compositions and phytosterols were determined by gas chromatography. Tocopherols were analyzed using NP-HPLC and phospholipids by HPLC-ELSD (Evaporative Light Scattering Detector). Total lipid content varied from 0.1% in mayhaw fruit to 21.5% in pawpaw seed. Linoleic acid was the predominant fatty acid in all the samples. β-Sitosterol was the most abundant phytosterol in all the fractions. Seeds had higher total phytosterols content compared to the fruits. Loquat fruit had the highest total tocopherol content of 4.8 mg/100 g.

KEYWORDS: *Asimina triloba*; *Crataegus sp.; Eriobotrya japonica*; fatty acids*; Ficus carica*; phospholipids; phytosterols; tocopherols; total lipids; underutilized fruits

INTRODUCTION

Recently, interest in diversification of crops around the world has increased substantially. Various new crop species are being investigated for possible food and non food purposes. Georgia cultivates a diverse range of fruits with less attention to some minor fruit crops which may have great economic and nutritional potential. Loquat or Japanese plum (*Eriobotrya japonica*) belonging to Rosaceae family originated in southeastern China and later spread to Japan, India, and many other areas. In China, both fruits and leaves of loquat are used for treating cough and asthma (*1*). Loquat seed is called "good for health" in Japan (*1*). Mayhaws (*Crataegus aestivalis, C. rufula or C. opaca*) also belong to Rosaceae family. Small red colored apple-like fruits are borne that ripen during late April and May. It is a relatively unexplored and underutilized indigenous fruit tree of southern states of USA. Pawpaw (*Asimina triloba*) is a native fruit of America and the only fruit of Annonaceae family adapted to temperate climate (*2*). Ripe pawpaws have a prominent fruity and floral flavor. The fruits are oblong to round in shape with a number of large blackish brown seeds. Pawpaw plant produces annonaceous acetogenins that exhibit high antitumor and pesticidal properties (*3*). Fig (*Ficus carica*) which is believed to be one of the earliest cultivated fruit crops belongs to Moraceae family (*4*). It is a native crop of western Asia with wide distribution over the Mediterranean region. Its role in the treatment of tumor, diarrhea, chronic cough, ulcers, dermatitis, and gout has been extensively investigated (*5*).

Previous studies have shown that lipids from fruits possess bioactive compounds such as tocopherols and phytosterols. Seed oils from fruits like raspberry (*6*), Bulgarian chokeberry, black currant (*7*), apricot, cherry, nectarine, peach, and plum (*8*) have been isolated and characterized. The balance between saturated and unsaturated fatty acids, the amount and type of

omega fatty acids are believed to have an influence on coronary heart disease. Determining the fatty acid profile of a substance helps in deciding its end use both in food and non food applications such as paints, coatings, oleochemicals, and cosmetics. Phytosterols are bioactive compounds, structurally similar to cholesterol and possessing cholesterol lowering activity (*9*). Tocopherols play an important role in human growth and development. Their antioxidant properties have been extensively studied for several years. Phospholipids are usually found in all biological membranes of plants and animals. They are widely used in food and cosmetic industries as well as manufacturing industries.

The seeds are usually disposed as waste material in many food processing. Disposing of such wastes is highly undesirable both economically and environmentally. It would be beneficial to utilize these by products as a source of bioactive compounds and add value to these minor fruit crops. Very little work has been done on these crops especially their lipid characterization. Thus, the main objective of this research was to identify and quantify the major lipid classes namely, fatty acids, tocopherols, phytosterols, and phospholipids in the fruits and seeds of these underutilized crops.

MATERIALS AND METHODS

Plant material

All fruits were harvested at their optimum ripe stage and transported in ice coolers to the University of Georgia. Loquat (*Eriobotrya japonica*, Rosaceae) was collected from seedling trees on Lincoln Street, Savannah, GA. Mayhaw *(Crataegus sp.,* Rosaceae*)* (Turnage 57) was collected from Tifton, GA. Pawpaws (*Asimina triloba,* Annonaceae) were collected randomly from different cultivars at university experimental farm, Tifton, GA, and figs (*Ficus carica,*

Moraceae) (Brown Turkey) were collected from Briar Patch farm, Dacula, GA . The fractions were prepared immediately to prevent degradation of the fruits.

Chemicals

Supelco 37 FAME mix, heptadecanoic acid, 14% boron trifluoride in methanol, 5β-cholestan-3β-ol, 5% DMDS in toluene (Sylon-CT™), α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, and L-α- phosphatidylethanolamine were purchased from Sigma Chemical Co. (St. Louis, MO). Plant sterol mixture was purchased from Matreya LLC (Pleasant Gap, CA). BSTFA with 1% TMCS was purchased from Thermo Scientific (Rockford, IL) and L-α- phosphatidylcholine from Avanti Polar Lipids, Inc. (Alabaster, AL). All other reagents and chemicals were purchased from Sigma Chemical Co., J.T. Baker Chemical Co. (Phillipsburg, NJ), and/or Fischer Scientific (Norcross, GA)

Sample preparation

The samples were washed with water and dried properly. 500 g of each fruit was weighed and divided into two fractions, seeds and fruits (i.e., without the seed). In case of figs the seeds were too minute to be separated. Therefore, whole fruit was taken as one fraction. All sample preparation was performed under cool and dark conditions. All fractions were packaged in amber bottles, labeled, and stored at -80 ºC after flushing with nitrogen until analyzed.

Lipid extraction

Total lipids were extracted using the method of Folch et al. (*10*). Briefly, the lipids were extracted with chloroform/methanol (2:1, v/v) in a ratio of 1:20, sample to solvent, for 2 h at room temperature. 0.37 M KCl (0.2% of its volume) was added, mixed well, and centrifuged at 3000 rpm for 10 min. The lower lipophilic layer was recovered after washing with chloroform/methanol/water (3:48:47, v/v/v). All the solvents were removed in rotary evaporator

at ~40 ºC. The dried samples were reconstituted in hexane and filtered through anhydrous sodium sulfate column. Hexane was removed under nitrogen and the samples were kept in a dessicator for 10-12 h. The constant weight was recorded and the samples were stored at -80 ºC in hexane with 0.005% BHT to prevent oxidation.

Fatty acid profile

Lipid samples were converted to fatty acid methyl ester following the AOAC official method 996.01 (*11*). 50 mg oil was placed in a screw capped, teflon lined test tube and 20 µL internal standard (C17:0, 20 mg/mL) was added. The samples were dried completely under nitrogen. 1 mL 0.5 N NaOH in methanol solution was added and samples were kept at 100 ºC for 5 min. 1 mL BF_3-methanol solution was added, vortexed for 1 min and again kept at 100 ºC for 5 min. 1 mL hexane and 1 mL saturated NaCl solution was added, vortexed for 2 min, and centrifuged at 1000 rpm for 5 min. The upper hexane layer was recovered and analyzed in a Hewlett-Packard 6890 series II gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) using Supelco SP-2560, 100 m x 0.25 mm x 0.2 µm column. Helium was used as the carrier gas at a flow rate of 1.1 mL/min at constant pressure. Injection volume was 1 µL and a spilt ratio of 5:1 was used. Detection was done with flame ionization detector at 260 ºC. The oven program was as follows: setpoint 150 ºC was held for 3 min and then increased to 215 ºC at 10 ºC/min and held at 215 ºC for 40 min. All samples were analyzed in duplicates and average values reported.

Phytosterols

Samples were prepared based on Kim et al. method (*12*). 50 mg was weighed into a screw-capped tube and 20 µL of internal standard solution (2 mg/mL of 5β-cholestan-3β-ol) was added. After flushing with nitrogen, the samples were saponified with 250 µL of saturated KOH and 2 mL of 3% pyrogallol (in ethanol) at 80 °C for 30 min. After cooling to room temperature, 5 mL

of hexane and 4 mL of water was added to the samples. The samples were vortexed for 5 min to extract the unsaponifiables. The top layer was recovered and dried completely under nitrogen. Derivatization was done by adding 50 µL TMS/pyridine (1:1, v/v) and placing in a water bath at 70 ºC for 1 h. The samples were dried under nitrogen and reconstituted in 0.5 mL hexane and 1 µL of this solution was injected into the GC. A Hewlett-Packard 6890 series II gas chromatograph equipped with a FID was used. The column was an HP-5, 5% phenylmethyl siloxane from Agilent, 0.25 µm, 30 m x 0.32 mm. The carrier gas was helium at a flow rate of 1.5 mL/min at constant pressure. The injector and detector temperatures were maintained at 300 and 320 °C, respectively. The column was initially held at 260 °C and programmed to increase to 300 °C at the rate of 3 °C/min and held at 300 °C for 6.7 min. The sterols in the samples were identified by comparison with chromatogram of plant sterol mixture, and calculated as milligrams per 100 g as shown in equation 1(*13*)

$$\text{Phytosterol amount (mg/100 g)} = 100\,(PA_s)\,(m_{is}) / (PA_{is})\,(m_s) \qquad (1)$$

where, PA_s is the sterol peak area, m_{is} is weight of the internal standard (mg), PA_{is} is the internal standard peak area, and m_s is the weight of the sample (g). All samples were analyzed in duplicates and average values were reported.

Tocopherols

Tocopherols were analyzed following the method described by Chun et al. (*14*). 50-100 mg sample was weighed and 6% ethanolic pyrogallol solution was added. The samples were vortexed for 1 min and 0.5 mL 60% KOH solution was added and again vortexed for 1 min. After flushing with nitrogen, the samples were kept in shaking water bath for 30 min at 80 °C. The samples were cooled to room temperature and 2 mL of 2% NaCl solution was added. 1 mL hexane/ethyl acetate (85:15, v/v) containing 0.005% BHT was added and vortexed. The upper

layer was recovered. This extraction was carried out two to three times and the upper hexane layer fractions were pooled. The samples were dried under nitrogen, reconstituted in 2 mL hexane, and filtered through 0.45 µm membrane filter. 50 µL aliquot was injected into Hewlett-Packard (Avondale, PA) HP 1090 HPLC system with fluorescence detector. Agilent Zorbax RX-SIL, 5 µm, 4.6 x 250 mm silica column was used. Fluorescence detector was set with excitation at 290 nm and emission at 330 nm. The mobile phase consisted of hexane/isopropanol (99.3:0.7, v/v) with a flow rate of 1 mL/min. The tocopherols were identified by comparing their retention times with those of authentic standards. Tocopherol quantity was calculated based on the standard calibration curves and reported as mg/100 g from average of duplicate determinations.

Phospholipids

To analyze phospholipids (PL) by HPLC-ELSD, solid phase extraction method was used as described by Descalzo et al. (*15*). The sample was fractionated on a normal phase silica cartridge (Supelclean LC-Si, 500 mg, 3 mL) previously conditioned with 10 mL chloroform. Sequential elution was done with 20 mL chloroform, 5 mL acetone, and 20 mL methanol. The methanol extract was dried under nitrogen, reconstituted in 1 mL chloroform, and filtered through 0.2 µm membrane filter. PL analysis was performed with a Hewlett-Packard (Avondale, PA) HP 1100 HPLC system with Sedex 55 ELSD (Sedere Inc., NJ) based on Zhang et al. (*16*) method with few modifications. Agilent Zorbax RX-SIL, 5µm, 4.6 x 250 mm silica column was used as stationary phase and the mobile phase consisted of a gradient elution of isopropanol, water, and hexane as shown in **Table 4.1**. Injection volume was 10 µL. ELSD parameters were 60 °C drift tube temperature, 2 bar pressure and a gain of 5. Duplicate determinations were made and averaged based on the individual PL standards (0.5- 4 mg/mL).

Statistics

All samples were analyzed in duplicates and the results are expressed as average ± standard deviation. All statistical analysis was carried out using the Microsoft Excel software package (Microsoft Corp. Redmond, WA).

RESULTS AND DISCUSSION

Among the seeds, the highest lipid content was found in pawpaw (21.5%) closely followed by loquat (18.5%). Mayhaw seed contained 16.0% total lipids. Pawpaw fruit had the highest lipid content among the fruit fractions, 6.2%. Total lipid content in loquat fruit, mayhaw fruit and fig was 0.3, 0.1, and 2.5%, respectively.

Fatty acids in these fruits and seeds were determined as their methyl esters by gas chromatography. The fatty acid profiles are shown in **Table 4.2**. A typical gas chromatogram of fatty acid profile of fig is shown in **Fig.4.1**. In all the samples linoleic acid was the predominant fatty acid with the highest content being in mayhaw seed (55.7%) and the lowest in fig (28.2%). The highest content of linolenic acid (20.7%) was found in fig fruit lipids. Previous studies have reported 53.1% linolenic acid in dried fig fruit (*17*). Pawpaw fruit contained the highest amount of saturated fatty acids (35.8%). The lowest saturated/unsaturated fatty acid was found in loquat seed (0.1) and the highest in pawpaw fruit (0.6).

Phytosterol, tocopherol, and phospholipid content of the samples are presented in **Table 4.3.** For phytosterols and tocopherols, the crude lipid samples were saponified before analysis. TMS derivatization of phytosterols was done in order to obtain better resolution and peak shape. The major phytosterols analyzed were brassicasterol, campesterol, stigmasterol, and β-sitosterol as these are the most common phytosterols in nature (*18*). Identification of sterols was based on plant sterol mixture while quantification was based on an internal standard method. 5β-

Cholestan-3β-ol was used as an internal standard because it is structurally similar to sterols, commercially available, and absent from plant samples (*19*). Retention time of all four phytosterols was within 15 min. β-Sitosterol, which is usually more abundantly present and frequent in plants than other sterols, was found in all the samples with the highest content being in loquat seed (303.1 mg/100 g) and the lowest in loquat fruit (2.4 mg/100 g). Seeds had higher phytosterols content compared to the fruits. Brassicasterol and campesterol were found only in mayhaw seeds and fig fruit. Overall, mayhaw seed contained the highest content of total phytosterols (sum of those analyzed), 320.8 mg/100 g.

Tocopherols were identified and quantified using normal phase liquid chromatography. Linearity was observed for all four standards within the range 10 to 200 ppm with high correlation coefficient $R^2 > 0.99$ (**Fig. 4.2**). α-Tocopherol was found in all the samples with the highest content being in mayhaw seed (1.0 mg/100 g). The highest γ-tocopherol content was found in loquat fruit (3.2 mg/100 g). β-Tocopherol content varied from not being detected in mayhaw fruit and pawpaw fruit to 0.5 mg/100 g in mayhaw seed. Loquat fruit had the highest total tocopherol content (4.8 mg/100 g). The tocopherol content of fig fruit was similar to previously reported values (*14*).

Phospholipids were analyzed using HPLC-ELSD. Various mobile phases have previously been used for PL analysis of several samples, the basic two being isopropanol-based or acetonitrile-based. A gradient elution mode using hexane, isopropanol, and water was used to separate PL owing to the difference in their polarities. Calibration curves of the principal PL classes are shown in **Fig. 4.3**. Linear curves were obtained for standards within 5 to 40 μg range with correlation coefficient $R^2 > 0.99$. This linearity range is in accordance with other literature values (*15*). The highest content of phosphatidylethanolamine (PE) was found in loquat seed

(44.1 mg/100 g). Phosphatidylcholine (PC) content ranged from 0.1 mg/100 g in pawpaw fruit to 33.9 mg/100 g in mayhaw fruit.

The results of our study indicate that these underutilized fruits and their seeds contain considerable amount of compounds with possible potent physiological and industrial benefits. To the best of our knowledge, this is the first report on the lipid profile of most of these underutilized fruits. Determining the lipid profile of these samples would aid in assessment of their physical properties, oxidative stability, and physiological activity. Economical extraction of these compounds may lead to their eventual use in both food and non food applications. However, further studies are warranted to screen for toxic or anti-nutritional compounds, if any, before commercial food applications.

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Figure captions:

Figure 4.1. Gas chromatogram of fig fatty acid profile. Supelco SP-2560, 100 m x 0.25 mm x 0.2 µm column was used. Carrier gas was helium at a flow rate of 1.1 mL/min and constant pressure. Flame ionization detector was at 260 ºC. The oven was initially held at 150 ºC for 3 min, and then increased to 215 ºC at 10 ºC/min and held at 215 ºC for 40 min.

Figure 4.2. Calibration curves for tocopherol standards

Figure 4.3. Calibration curves for phospholipid standards

Table 4.1. Gradient Elution for Phospholipid Determination by HPLC-ELSD

time (min)	flow rate (mL/min)	isopropanol (vol%)	water (vol%)	hexane (vol%)
0.0	0.8	42.0	5.0	53.0
8.0	0.8	42.0	5.0	53.0
8.1	1.2	42.0	5.0	53.0
25.0	1.0	54.0	10.0	36.0
25.1	0.8	66.0	17.0	17.0
35.0	0.8	66.0	17.0	17.0
38.0	0.8	42.0	5.0	53.0
45.0	0.8	42.0	5.0	53.0

Table 4.2. Fatty Acid Content of Seeds and Fruits (%)[a].

fatty acids	loquat seed	loquat fruit	mayhaw seed	mayhaw fruit	pawpaw seed	pawpaw fruit	fig fruit
10:0	0.7±0.1	1.6±0.2	0.8±0.0	1.9±0.3	nd[b]	3.0±0.3	nd
11:0	1.8±0.1	0.9±0.1	nd	nd	nd	nd	nd
12:0	2.3±0.1	nd	3.1±0.0	nd	nd	nd	nd
14:0	0.5±0.3	0.7±0.1	0.6±0.7	0.8±0.8	3.7±0.3	1.1±0.0	nd
14:1	2.3±0.1	3.8±0.3	1.6±0.1	2.9±0.3	nd	nd	nd
16:0	2.7±0.2	25.6±0.1	7.8±0.3	27.2±0.3	5.3±0.4	30.7±0.0	27.5±0.8
16:1	nd	nd	0.8±0.0	0.8±0.1	0.8±0.1	nd	nd
18:0	2.6±0.1	1.7±0.1	2.4±0.3	1.8±0.1	2.5±0.2	1.0±0.1	1.7±0.1
18:1	31.2±0.3	6.4±0.1	27.7±0.3	4.6±0.3	33.0±0.9	6.7±0.3	20.2±0.4
18:2	52.5±0.3	48.8±0.4	55.7±0.7	50.0±0.5	51.8±0.3	45.5±0.3	28.2±0.3
18:3	1.9±0.1	8.8±1.1	1.4±0.1	9.2±0.9	0.5±0.1	12.1±0.2	20.7±0.1
20:0	2.2±0.0	nd	2.6±0.1	nd	0.3±0.1	nd	1.8±0.1
20:1	nd	1.8±0.1	0.2±0.0	1.3±0.1	0.1±0.0	nd	nd
21:0	nd	nd	nd	nd	1.4±0.3	nd	nd
22:0	0.3±0.0	nd	0.2±0.0	nd	nd	nd	nd
∑saturated	12.1	30.7	17.5	31.7	13.2	35.8	31.0
∑ unsaturated	87.9	69.4	82.5	68.4	86.7	64.3	69.1
saturated/unsaturated	0.1	0.4	0.2	0.5	0.2	0.6	0.4

[a] Each value is the mean of duplicates ± standard deviation. [b] nd, not detected

Table 4.3. Tocopherol, Phytosterol, and Phospholipid Content of Seeds and Fruits (mg/100 g)[a].

samples	tocopherols				phytosterols				phospholipids	
	α	β	γ	δ	brassicasterol	campesterol	stigmasterol	β-sitosterol	PC	PE
loquat seed	0.1±0.0	tr[b]	0.1±0.0	0.1±0.0	nd[c]	nd	nd	303.1±7.4	16.0±2.1	44.1±6.0
loquat fruit	0.9±0.1	0.3±0.1	3.2±0.6	0.4±0.0	nd	nd	1.8±0.1	2.4±0.3	0.4±0.1	nd
mayhaw seed	1.0±0.2	0.5±0.1	1.5±0.1	tr	15.7±2.1	8.6±1.1	48.7±3.3	247.8±4.8	3.5±0.3	9.8±021
mayhaw fruit	0.3±0.0	nd	1.0±0.3	0.2±0.0	nd	nd	18.3±1.6	4.8±0.6	33.9±3.1	nd
pawpaw seed	0.1±0.0	0.1±0.0	0.2±0.0	tr	nd	nd	10.1±2.0	28.4±1.1	0.7±0.0	1.5±0.1
pawpaw fruit	0.7±0.1	nd	nd	0.1±0.0	nd	nd	nd	2.8±0.1	0.1±0.0	nd
fig fruit	0.2±0.0	tr	0.3±0.0	nd	11.8±1.9	5.2±0.9	54.3±3.8	58.5±2.5	3.2±0.2	1.0±0.0

[a] Each value is the mean of duplicates ± standard deviation. [b] tr, trace. [c] nd, not detected. PC, phosphatidylcholine ; PE, phosphatidylethanolamine

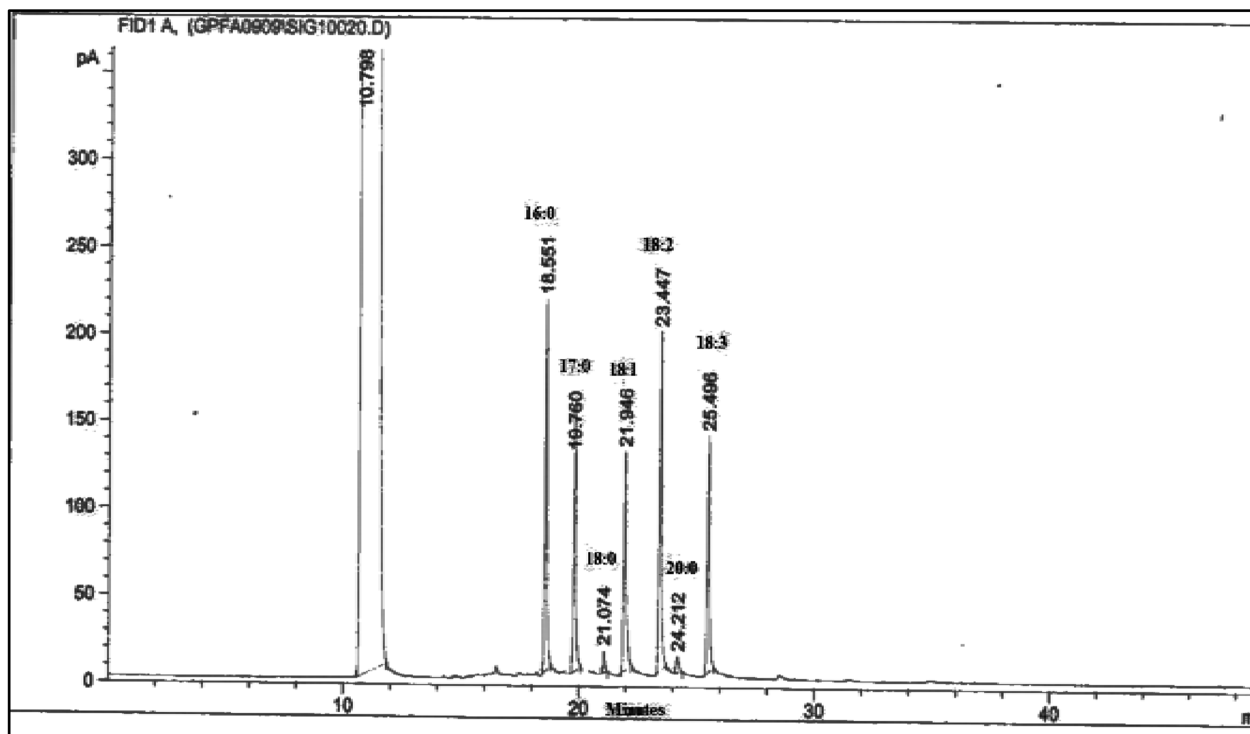

Figure 4.1

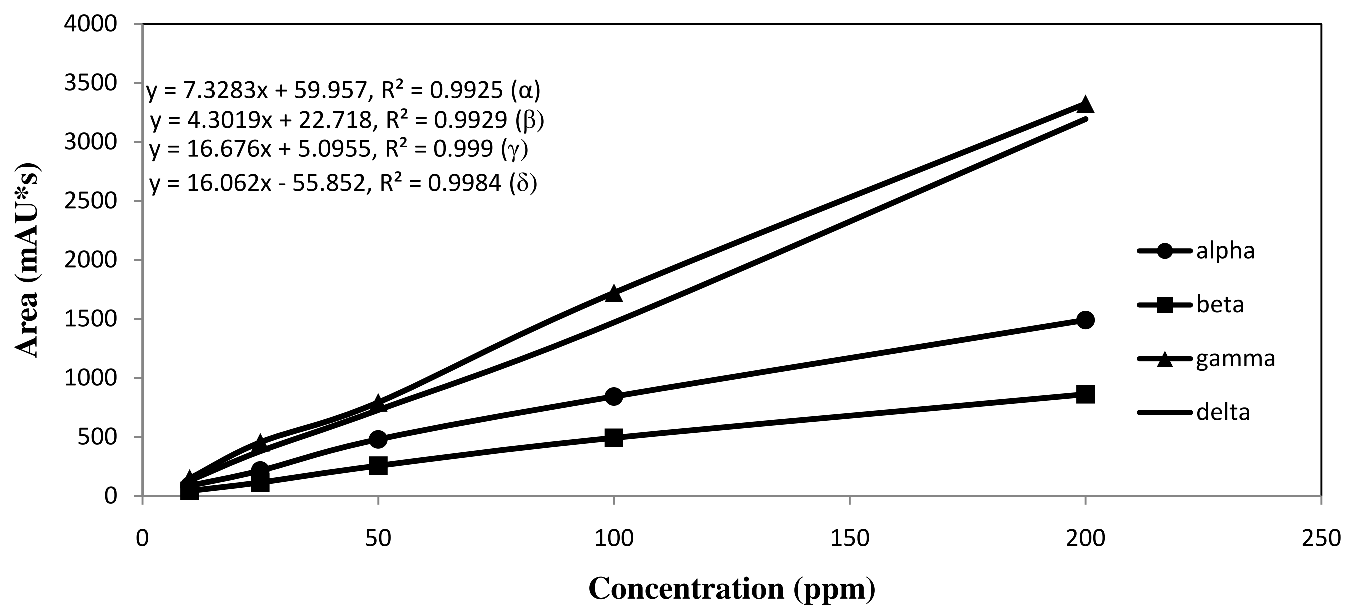

Figure 4.2

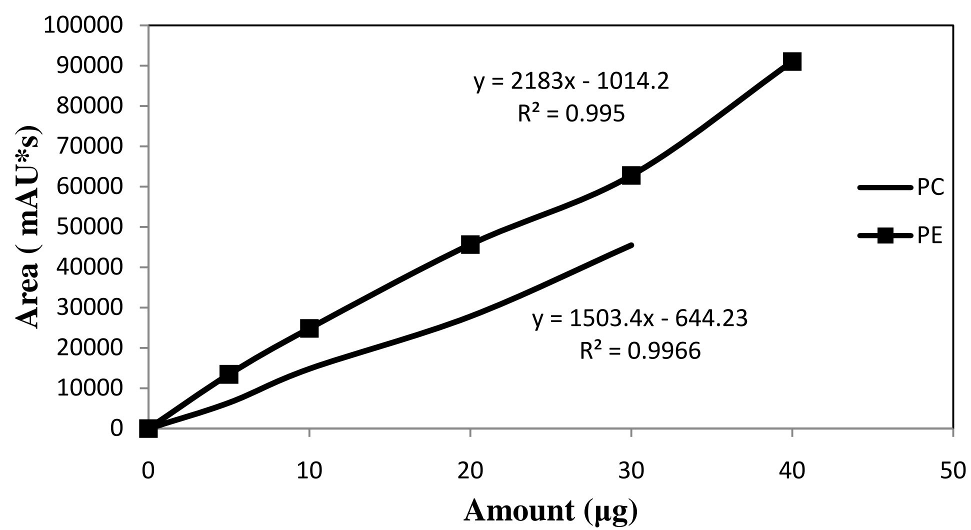

Figure 4.3

CHAPTER-5

Antioxidant Capacity and Lipid Characterization of Six Georgia-Grown Pomegranate Cultivars

Garima Pande and Casimir C. Akoh
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ABSTRACT

Six pomegranate (*Punica granatum*) cultivars were investigated for their antioxidant capacity and lipid profile. Total polyphenols were determined by Folin Ciocalteau method. Major organic acids and phenolic compounds were analyzed by RP-HPLC. Two in vitro antioxidant assays were used, ferric reducing antioxidant power (FRAP) and trolox equivalent antioxidant capacity (TEAC). Total lipid was extracted by Folch method and fatty acid methyl esters were determined by GC. Tocopherols and phospholipids were identified and quantified by NP-HPLC using fluorescence detector for tocopherols and evaporative light scattering detector (ELSD) for phospholipid analysis, and phytosterols were analyzed by GC. The predominant organic acid was citric acid followed by malic acid. Peel fraction had the highest total hydrolyzable tannins content. Overall, the highest antioxidant capacity was found in leaves followed by peel, pulp, and seed. Pomegranate seed had an average lipid content of 19.2% with punicic acid as the predominant fatty acid. Pomegranate seed had high content of α- and γ-tocopherols.

KEYWORDS: antioxidant capacity; fatty acids; organic acids; phospholipids; phytosterols polyphenols; *Punica granatum;* tocopherols

INTRODUCTION

Phytochemicals, particularly antioxidants from natural sources like fruits and vegetables have gained popularity because many epidemiological studies have shown their protective properties against several chronic diseases like cancer and cardiovascular diseases (*1*). Products and intermediates of oxidative stress pathways are associated with several chronic diseases. Oxidative stress is the disturbance of the pro-oxidant – antioxidant balance in favor of the former, leading to potential damage (*2*). Interest in natural antioxidants has increased during the last few decades because of the adverse effects shown by synthetic antioxidants, and also due to the worldwide trend to avoid or minimize the use of artificial food additives (*3*). The most abundant antioxidants in fruits are polyphenols, vitamins and carotenoids.

The lipid profile of several fruits and their seeds have been characterized and various bioactive compounds isolated (*4, 5*). Determination of the lipid classes of the sample may aid in deciding its application in food, health, and other manufacturing industries. This is in turn will help in identifying the potential of the crop as a mainstream agricultural product.

Pomegranate (*Punica granatum*) belonging to family Punicaceae has been used in several traditional medicine systems. It is a rich source of various bioactive compounds demonstrating antioxidant and anti-inflammatory activities (*6*). Pomegranate seed oil comprising 12-20% of total seed weight consists of approximately 80% conjugated octadecatrienoic fatty acids, mainly punicic acid (*6*). Highest antioxidant activity has been shown in pomegranate peel compared to pulp and seed fractions (*7*) which can be attributed to its high content of tannins, especially punicalagin isomers. Pomegranate juice is bright red in color due to high content of flavonoids and anthocyanins. Pomegranate juice exhibits anti-atherogenic activities (*8*). Leaves, flowers, bark, and roots also contain distinctive compounds having potent physiological effects.

Pomegranate is a minor fruit crop of Georgia but with the current commercial and nutritional potentials a detailed study may help in its cultivar selection and application. The main objective of this research was to compare six Georgia-grown pomegranate cultivars in terms of their organic acid content, antioxidant capacity, and lipid profile.

MATERIALS AND METHODS

Plant material

Six pomegranate (*Punica granatum*, Punicaceae) cultivars R19, R26, Cvg-Eve, North, Crab, and Cranberry along with their leaves were obtained from Ponder farm, a University of Georgia operated farm near Tifton, GA. The samples were transported in ice coolers from the farm to the University of Georgia. All fruits were at ripe stage ready for fresh consumption.

Chemicals

Pure standards of succinic acid, DL-malic acid, oxalic acid, BHT, gallic acid, quercetin, ellagic acid, (+)-catechin, ferulic acid, *p*-coumaric acid, caffeic acid, (-)- epicatechin, Supelco 37 FAME mix, heptadecanoic acid, 14% boron trifluoride in methanol, 5β-cholestan-3β-ol, 5% DMDS in toluene (Sylon-CT™), α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, and L-α-phosphatidylethanolamine, Folin Ciocalteau reagent, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), citric acid, and potassium persulfate were purchased from Sigma Chemical Co. (St. Louis, MO). 2, 4, 6-Tripyridyl-s-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Acros Organics (Morris Plains, NJ), L-Ascorbic acid from Mallinckrodt Baker Inc.(Phillipsburg, NJ), and $FeCl_3.6H_2O$ from Fluka (Milwaukee, WI). Plant sterol mixture was purchased from Matreya LLC (Pleasant Gap, CA). BSTFA with 1% TMCS was purchased from Thermo Scientific (Rockford, IL) and L-α- phosphatidylcholine from Avanti Polar Lipids, Inc. (Alabaster,

AL).Other solvents and chemicals were purchased from Sigma Chemical Co., J.T. Baker Chemical Co. (Phillipsburg, NJ), and/or Fischer Scientific (Norcross, GA).

Sample preparation

The samples were washed with water and dried properly. 500 g of the fruits were divided into three fractions as peel, pulp (juice), and seeds for antioxidant capacity. The leaves of each crop were also analyzed. For lipid profile 500 g fruits were divided into two fractions, seeds and fruits (without the seed i.e., peel and juice together). All sample preparation was done under cool dry conditions. All fractions were packaged in amber bottles, labeled, and stored at -80 ºC after flushing with nitrogen until analyzed.

Dry weight determination

Dry weight (DW) was determined following the guidelines of the official AOAC method 967.03 (*9*). Sample dry weight (g/g FW) was calculated as shown in equation 1.

$$DW = (c-a) / (b-a) \quad (1)$$

where a is the weight of empty pan (g), b is the weight of pan and fresh sample (g), and c is the weight of pan and dried sample (g). All samples were analyzed in triplicates and average values were reported.

Preparation of hydrophilic and lipophilic fractions

Hydrophilic and lipophilic fractions were prepared using the method of Jimenez-Alvarez et al. (*10*). 2 g of each fruit fraction was weighed and 10 mL hexane was added to each. After centrifuging for 10 min at 2500 rpm the supernatants were collected. This procedure was repeated once again and the supernatant was dried under nitrogen and reconstituted in 10 mL of 95% ethanol. These lipophilic fractions were stored at -20 ºC until analyzed. For the hydrophilic fraction, after removing all the hexane from the residues 5 mL acetone/water/acetic acid

(70:28:2, v/v/v) was added. Similar centrifugation step was followed as described above and supernatants were collected. The volume was made up to 10 mL with the acetone/water/acetic acid (70:28:2, v/v/v) and samples were stored at -20 ºC until analyzed. These lipophilic and hydrophilic fractions (0.2 g/mL) were used for total polyphenols and antioxidant assays.

Major organic acids

1 g sample was mashed with 10 mL of 1 M HCl. After flushing with nitrogen the samples were centrifuged at 2000 rpm for 15 min and placed in water bath at 90 ºC for 30 min. The samples were then allowed to cool to room temperature and the supernatant was filtered through 0.45 µm membrane filter. Organic acids were analyzed and identified using Hewlett-Packard (Avondale, PA) HP 1100 HPLC system with diode array detector based on Chen et al. (*11*) method. Agilent Zorbax Eclipse® XDB-C18, 3.5 µm, 4.6 x 150 mm column and an isocratic mobile phase of 0.5% ammonium phosphate, pH adjusted to 2.8 with phosphoric acid was used at a flow rate of 0.5 mL/min. The injection volume was 20 µL and column temperature was maintained at 40 ºC. Detection was done at 214 nm. Triplicate determinations were made and averaged and quantification was based on external standards (10-1600 µg/mL).

Major phenolic compounds

Major phenolic compounds were determined following the method described by Pastrana-Bonilla et al. (*12*). 1 g sample was mashed and diluted with 10 mL 80% methanol in 6 N HCl. The samples were vortexed and placed in a water bath shaker at 60 °C and 200 rpm for 2 h. After cooling to room temperature the supernatants were filtered through 0.45 µm membrane filter and injected into a Hewlett-Packard (Avondale, PA) HP 1100 HPLC system with diode array detector. The column used was Beckman Ultrasphere® C18, 5 µm, 4.6 x 250 mm with temperature set at 40 °C. The injection volume was 1 µL. The mobile phase at a flow rate of 1

mL/min consisted of solvent A, methanol/acetic acid/water (10:2:88, v/v/v); solvent B, acetonitrile; and, solvent C, water. A linear gradient was used as follows: at 0 min, 100% solvent A; at 5 min, 90% solvent A and 10% solvent B; and at 25 min, 30% solvent A and 70% solvent B, with 5 min postrun of 100% solvent C. Detection was done at 260, 280, 320, and 360 nm. Identification was based on the retention times and characteristic UV spectra and quantification was done by the external standard curves. All analysis was performed in triplicates and average reported.

For identification of punicalgin isomers MS-ESI (Electro-spray ionization) was performed on PE Sciex API 1 (Perken Elmer, Waltham, MA) quadrupole mass spectrometer operated in the negative ESI mode. 20 μL sample was injected with methanol as carrier. The carrier flow rate was 0.2 mL/h. continuous mass spectra were recorded over the range 295-1150 m/z with 0.2 m/z step.

Total polyphenols

Total polyphenols were determined according to the Folin-Ciocalteau reagent method (*13*). To each 200 μL of sample 1 mL of 0.2 N Folin-Ciocalteau reagent and 0.8 mL of 7.5% sodium carbonate solution were added, mixed well and allowed to stand for 30 min at room temperature. Absorption at 765 nm was read using a Shimadzu 300 UV-Vis spectrophotometer (Shimadzu UV-1601, Norcross, GA). Quantification was based on the standard curve generated with 100 - 400 mg/L of gallic acid and average results from triplicate determination were reported as mg GAE/100 g FW.

Antioxidant capacity

Ferric Reducing Antioxidant Capacity (FRAP) assay

FRAP assay was carried out as described by Benzie and Strain (*14*) with slight modifications. Stock solutions of 300 mM acetate buffer, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine solution in 40 mM HCl), and 20 mM $FeCl_3.6H_2O$ were prepared. Working solution was prepared by mixing the stock solutions in 10:1:1 ratio. The solution was maintained at 37 °C and pH 3.6. Then, 50 µL of sample was mixed with 2 mL of the working solution and absorbance was read at 593 nm against appropriate blank for 4 min. The change in absorbance was calculated and related to the standard curve generated with trolox. Results were expressed as µM TE/g FW. All assays were in triplicates and averages reported.

Trolox Equivalent Antioxidant Capacity (TEAC) assay

The antioxidant capacity was measured by the radical cation decolorization assay based on the methods of Re et al. (*20)* and van den Berg et al. (*15, 16*). Briefly, 7 mM ABTS solution and 2.45 mM potassium persulfate solution were mixed and kept in the dark at room temperature for 12-16 h. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. To each 50 µL aliquot of trolox standard or sample, 2 mL of diluted $ABTS^{\bullet+}$ was added and the absorbance was read for 6 min at 734 nm. Appropriate solvent blanks were also ran in each assay. The percentage inhibition of absorbance was calculated and plotted as a function of trolox concentration. TEAC values of samples were calculated based on the standard curve and reported as µM TE/g FW from average of triplicate determinations.

Lipid extraction

Total lipids were extracted using the Folch method (*17*). The constant weight was recorded and the samples were stored at -80 ºC in hexane with 0.005% BHT to prevent oxidation.

Fatty acid profile

Lipid samples were converted to fatty acid methyl ester following the AOAC official method 996.01 (*18*) and analyzed with Hewlett-Packard 6890 series II gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) using Supelco SP-2560, 100 m x 25 mm x 0.2 µm column. Helium was the carrier gas at a flow rate of 1.1 mL/min at constant pressure. Injection volume was 1 µL and a spilt ratio of 5:1 was used. Detection was with flame ionization detector at 260 ºC. The column was initially held at 150 ºC for 3 min and then increased to 215 ºC at 10 ºC/min and held at 215 ºC for 40 min. All samples were analyzed in triplicates and average values reported.

The position of *cis*-*trans*-*cis* isomer was identified with Shimadzu 300 UV-Vis spectrophotometer (Shimadzu UV-1601, Norcross, GA) based on Sita Devi method (*19*). The fatty acid methyl esters (FAME) were purified by TLC on silica gel plates using hexane/diethyl ether (94:6, v/v) as developing solvent. Fatty acids isolated by TLC were dissolved in cyclohexane (10 µg/mL) and absorbance was read at wavelength range of 200-300 nm.

Identification of punicic acid was done with Hewlett-Packard 5890 series gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) coupled with MS. Supelco SP-2560, 100 m x 25 mm x 0.2 µm column was used. The injector and detector were maintained at 250 and 230 °C, respectively. The column was initially held at 150 ºC for 3 min, then increased to 215 ºC at 10 ºC/min, held at 215 ºC for 20 min and further increased to 230 °C at 1.5 °C/min and held at 230 °C for 30 min. The pressure was maintained at 24 psi. 50-550 m/z scans were done. The mass spectrum of the sample was identified by computer comparison against a mass spectral library.

Phytosterols

For phytosterols analysis, the samples were saponified based on Kim et al. (*20*) method. 50 mg of sample was weighed and 20 µL of internal standard solution (2 mg/mL of 5β-cholestan-3β-ol) was added. The samples were flushed with nitrogen and 250 µL of saturated KOH and 2 mL of 3% pyrogallol in ethanol were added. The samples were placed in a water bath at 80 °C for 30 min. After cooling to room temperature, 5 mL of hexane and 4 mL of water were added and the samples were vortexed. The upper hexane layer was recovered and dried completely under nitrogen. Derivatization was done by adding 50 µL TMS/pyridine (1:1, v/v) and placing in a water bath at 70 ºC for 1 h in order to obtain better resolution and peak shape. The samples were dried under nitrogen and reconstituted in 0.5 mL hexane and 1 µL of this solution was injected into the GC. A Hewlett-Packard 6890 series II gas chromatograph equipped with a FID was used. The column was HP-5, 5% phenylmethyl siloxane from Agilent (30 m x 0.32 mm x 0.25 µm) and the carrier gas was helium with a flow rate of 1.5 mL/min at constant pressure. The injector and detector temperatures were maintained at 300 and 320 °C, respectively. The column was initially held at 260 °C and programmed to increase to 300 °C at the rate of 3 °C/min and held at 300 °C for 6.7 min. Identification of sterols was based on plant sterol mixture while quantification was based on an internal standard method as shown in equation 2(*21*)

$$\text{Phytosterol amount (mg/100 g)} = 100\,(PA_s)\,(m_{is})\,/\,(PA_{is})\,(m_s) \quad (2)$$

where, PA_s is the sterol peak area, m_{is} is weight of the internal standard (mg), PA_i is the internal standard peak area, and m_s is the weight of the sample (g). All samples were analyzed in triplicates and average values were reported.

Tocopherols

Tocopherols were analyzed based on the method described by Chun et al. (*22*). 50-100 mg sample was weighed and 6% ethanolic pyrogallol solution was added. The samples were vortexed and 0.5 mL 60% KOH solution was added. After flushing with nitrogen, the samples were kept in a shaking water bath for 30 min at 80 °C. The samples were allowed to cool to room temperature and 2 mL of 2% NaCl solution was added. 1 mL hexane/ethyl acetate (85:15, v/v) containing 0.005% BHT was added and vortexed. The upper hexane layer was recovered and this extraction was repeated two to three times. The hexane fractions were pooled, dried under nitrogen, reconstituted in 2 mL hexane, and filtered through 0.45 µm membrane filter. 50 µL aliquot was injected into Hewlett-Packard (Avondale, PA) HP 1090 HPLC system with fluorescence detector with excitation set at 290 nm and emission at 330 nm. Agilent Zorbax RX-SIL, 5 µm, 4.6 x 250 mm silica column was used. The mobile phase consisted of hexane/isopropanol (99.3:0.7, v/v) with a flow rate of 1 mL/min. The tocopherols were identified by comparing their retention times with those of authentic standards. Tocopherol quantity was calculated based on the standard calibration curves and reported as mg/100 g from average of triplicate determinations.

Phospholipids

To analyze phospholipids by HPLC-ELSD, solid phase extraction was used to separate neutral lipids, glycolipids, and polar lipids as described by Descalzo et al. (*23*). The sample was fractionated on a normal phase silica cartridge (Supelclean LC-Si, 500 mg, 3 mL) previously conditioned with 10 mL chloroform. Sequential elution was carried out with 20 mL chloroform, 5 mL acetone, and 20 mL methanol. The methanol extract containing the sample was dried under nitrogen and reconstituted in 1 mL chloroform. The samples were filtered through 0.2 µm

membrane filter and 10 µL was injected into the HPLC system. Phospholipid analysis was performed with a Hewlett-Packard (Avondale, PA) HP 1100 HPLC system with ELSD (Sedex 55) based on Zhang et al. (*24*) method with few modifications. The stationary phase was Agilent Zorbax RX-SIL, 5µm, 4.6 x 250 mm silica column and the mobile phase consisted of a gradient elution of isopropanol, water, and hexane as shown in **Table 5.1**. Drift tube temperature was set at 60 °C, pressure at 2 bar and gain at 5. Triplicate determinations were made and averaged. Quantification was based on the individual phospholipid standards (0.5-4 mg/mL).

Statistics

All samples were analyzed in triplicates and the results are expressed as average ± standard deviation. All statistical analysis was carried out using the Microsoft Excel software package (Microsoft Corp. Redmond, WA). Single factor ANOVA and multiple range test for variables was used to determine significant differences. Significance was determined at $p \leq 0.05$.

RESULTS AND DISCUSSION

The dry matter content of different parts of the cultivars is shown in **Table 5.2**. Dry matter content of different fractions was comparable within the cultivars. The highest dry matter content was found in seeds (0.4-0.5 g/g FW).

Table 5.3 shows the major organic acids in different pomegranate cultivars. The predominant organic acid was citric acid followed by malic acid. This trend is similar to previously reported results (*25*). In all the cultivars, pulp fraction had the highest organic acids content followed by peel, leaf, and seeds. Highest ascorbic acid was found in Crab pulp. Citric acid ranged from 113.6 mg/100 g FW in North leaf fraction to 1926.6 mg/100 g FW in R26 pulp.

Several phenolic compounds were identified in the samples. These were mainly phenolic acids like caffeic, *p*-coumaric, and ferulic acids, flavonoids like catechin, epicatechin, and quercetin with the highest being hydrolyzable tannins as shown in **Table 5.4**. Hydrolyzable

tannins include gallotannins, ellagic acid derivatives, and gallagyl tannins which comprises mainly of punicalagin isomers and punicalin. Two major peaks were obtained at 280 nm along with gallic and ellagic acids which were further purified. MS-ESI analyses of the peaks indicated the presence of a quasimolecular parent ion at 1084 m/z (M-H) and a daughter ion at 601 m/z (gallagic acid) characteristic of punicalagin. Both the peaks had the same UV spectra with maxima at 258 and 380 nm. These observations are in accordance with previous studies on pomegranate (*26*). Gallic acid and its derivative, ellagic acid and its derivatives, and punicalgin isomers were quantified as total hydrolyzable tannins using HPLC-UV detection. Where applicable external standards were used for quantification. The highest hydrolysable tannins were found in leaves followed by peels and ranged from 22.8 mg/100 g FW in North seed to 6054.3 mg/100 g FW in Crab leaf as shown in **Table 5.4.** Leaves contained the highest amount of caffeic and *p*-coumaric acids. Among the flavonoids, epicatechin and quercetin were found in all the samples while catechin was absent in the seeds of all the cultivars. In pulp and peel fractions, the predominant flavonoid was catechin with the highest concentration in Crab peel (126.7 mg/100 g FW). The average concentration of epicatechin ranged from 6.1 mg/100 g FW in seeds to 61.2 mg/100 g FW in leaves (**Table 5.4**).

The total polyphenol content, FRAP and TEAC values are shown in **Figs. 5.1 and 5.2**. Considerable difference was found in lipophilic and hydrophilic fractions of the samples with the hydrophilic fraction showing higher total polyphenols and antioxidant capacity. Among the lipophilic fractions, the highest concentration of total polyphenols was found in Crab leaves (48.1 mg GAE /100 g FW). The lipophilic pulp fraction had the lowest total polyphenol content (**Fig. 5.1**). The highest FRAP value was found in Cranberry leaves 17.4 µM TE/g FW and the lowest in North seed (7.8 µM TE/g FW). TEAC values were lower than the FRAP values with

the highest being in R19 leaf (13.7 μM TE/g FW) and the lowest, 5.2 μM TE/g FW, in R26 seed. Among the hydrophilic fractions, the highest total polyphenol content, FRAP, and TEAC values were found in Cranberry leaf (332. 6 mg GAE/100 g FW, 32.8 and 27.7 μM TE/g FW, respectively).

Total lipids and fatty acid compositions are given in **Tables 5.5** and **5.6**. Seeds had an average of 19.2% and fruit fraction had 0.3% total lipids content. The total lipid content of Cvg-Eve and North seeds was not significantly different from each other but significantly different from the other four cultivars **(Table 5.5)**. Comparing the fruit lipid content no significant difference was observed between North and R19 cultivars but they were significantly different from the other cultivars **(Table 5.6)**. The mass spectrum of FAME was characterized by an abundant molecular ion at 292 m/z, which was identified as a methyl ester of octadecatrienoic acid isomer (**Fig. 5.3**). Furthermore, its UV spectra showed the maxima at 265, 275, and 287 nm which corresponds to cis-trans-cis configuration (*19*). With the help of these results the compound was identified as punicic acid (9cis, 11trans, 13cis octadecatrienoic acid) which was the predominant fatty acid in the seed lipids. This is in accordance with previously reported studies (*27*). The highest punicic acid content was in R19 cultivar (83.4%). Linoleic acid was the major fatty acid in fruit fractions with the highest content in R26 (47.2%) and the lowest in Cranberry (45.0%). Fruit fractions also had higher palmitic and oleic acid contents than the seeds. The saturated/unsaturated ratio was the same in all the seeds (0.1) and in the fruits (0.5).

Table 5.7 shows the tocopherol, phytosterol and phospholipid contents. Pomegranate seed has high content of α- tocopherol (average, 167.3 mg/100 g) and γ-tocopherol (average, 84.6 mg/100 g). β-Tocopherol was not detected in any of the samples. Among the phytosterols, β-sitosterol was the most abundant in all the samples ranging from 345.8 mg/100 g in Crab seed

to 32.7 mg/100 g in R19 fruit. The phospholipids were analyzed by HPLC-ELSD. The highest content of phosphatidylcholine (PC) was found in Crab seed (26.1 mg/100 g) and the lowest in Cranberry fruit (3.7 mg/100 g). Crab seed had the highest content of phosphatidylethanolamine (PE) (74.2 mg/100 g).

Table 5.8 shows the comparison of seed, pulp, peel, and leaves of different cultivars in terms of their total organic acids, total hydrolyzable tannins, and total polyphenols. For total organic acid content the order of the cultivars was R26>Crab>Cranberry>Cvg-Eve>R19>North in terms of pulp fractions. Comparing the peel fractions of different cultivars the following trend was observed for total hydrolyzable tannins: Crab>Cranberry>Cvg-Eve>R19>R26>North. Among the pulp fractions, the trend was R19>Cranberry>R26>Crab>Cvg-Eve>North. Comparing the pulp and peel fractions among the cultivars in terms of total polyphenols, a similar trend was obtained as above. This pattern bears strong correlation to the visual appearance of the pulp and peel of the cultivars. North which has the lowest total polyphenols, total phenolic and antioxidant capacity is light pink in color.

A comparison of pomegranate with other Georgia-grown crops and other fruits and fruit juices is shown in **Table 5.9**. Pomegranate pulp had higher antioxidant capacity (26.5 µM TE/g FW) than Georgia-grown blackberries (20.4 µM TE/g FW) *(28)*, muscadines (17.6 µM TE/g FW) *(12)*, southern highbush blueberries (14.8 µM TE/g FW) *(28),* and other fruits and juices in terms of TEAC. TEAC was used for comparison because it is the most common antioxidant assay and also our results showed that it is better suited for both hydrophilic and lipophilic fractions. Pomegranate peel (34.3 µM TE/g FW) had the highest antioxidant capacity among all Georgia-grown crops and other fruits. The commercial pomegranate juice shows higher antioxidant capacity (41.6 µM TE/g FW) *(31)* because during processing extracts of peel and

seeds are also incorporated into the juice. Thus, pomegranate has a much higher antioxidant capacity than most of the commonly consumed fruits and fruit juices.

The results of our research also indicate that the by-products of pomegranate i.e., peel and seeds are a rich source of several high value compounds with potential beneficial physiological activities. The rich bioactive profile of pomegranate makes it a highly nutritious and desirable fruit crop. Comparing these cultivars in terms of their antioxidant capacity and lipid profile will also aid in proper cultivar selection, propagation, and commercialization.

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Figure captions:

Figure 5.1. Total polyphenols, FRAP, and TEAC of lipophilic fractions

Figure 5.2.Total polyphenols, FRAP, and TEAC of hydrophilic fractions

Figure 5.3. GC-MS spectrum of pomegranate seed fatty acids. Injection was done at 250 °C and detection at 235 °C. Pressure was maintained at 24 psi. Scan was done over the range 50-550 m/z.

Table 5.1. Gradient Elution for Phospholipid Determination by HPLC-ELSD

time (min)	flow rate (mL/min)	isopropanol (vol%)	water (vol%)	hexane (vol%)
0.0	0.8	42.0	5.0	53.0
8.0	0.8	42.0	5.0	53.0
8.1	1.2	42.0	5.0	53.0
25.0	1.0	54.0	10.0	36.0
25.1	0.8	66.0	17.0	17.0
35.0	0.8	66.0	17.0	17.0
38.0	0.8	42.0	5.0	53.0
45.0	0.8	42.0	5.0	53.0

Table 5.2. Dry Matter (DM) Content of Cultivars (g/g FW)

cultivar	part	DM[a]
R19	seed	0.4±0.1
	pulp	0.1±0.0
	peel	0.2±0.0
	leaf	0.3±0.0
R26	seed	0.5±0.1
	pulp	0.1±0.0
	peel	0.3±0.0
	leaf	0.3±0.0
Cvg-Eve	seed	0.4±0.1
	pulp	0.1±0.0
	peel	0.2±0.0
	leaf	0.4±0.1
North	seed	0.4±0.1
	pulp	0.1±0.0
	peel	0.3±0.0
	leaf	0.3±0.0
Crab	seed	0.4±0.1
	pulp	0.1±0.0
	peel	0.2±0.0
	leaf	0.4±0.1
Cranberry	seed	0.3±0.0
	pulp	0.1±0.0
	peel	0.2±0.0
	leaf	0.3±0.0

[a] Values are the average of triplicates ± standard deviation

Table 5.3. Major Organic Acids in Fruit Parts and Leaves (mg/100 g FW)[a]

cultivar	part	malic acid	citric acid	oxalic acid	ascorbic acid	succinic acid
R19	seed	93.5±0.7	315.7±1.0	8.6±0.2	1.4±0.4	nd[b]
	pulp	156.7±4.5	826.7±5.4	23.8±0.3	48.2±0.3	17.8±0.6
	peel	99.0±1.0	766.9±3.2	7.4±0.4	24.5±0.3	13.0±0.9
	leaf	71.2±3.0	130.2±1.6	28.6±0.5	12.5±0.4	12.8±0.3
R26	seed	102.2±2.6	482.6±5.7	11.2±0.4	4.2±0.2	5.4±0.4
	pulp	183.4±9.3	1926.6±33.7	34.1±0.5	65.9±1.3	25.3±0.4
	peel	116.5±2.4	1678.2±8.4	9.3±0.4	37.3±5.7	16.5±0.7
	leaf	78.1±1.5	177.3±1.6	38.8±3.5	16.0±2.1	14.2±0.9
Cvg-Eve	seed	92.7±2.3	305.1±32.8	8.7±0.7	nd	nd
	pulp	149.4±2.5	857.9±12.7	22.5±0.7	43.3±1.3	16.4±0.5
	peel	98.2±4.1	606.0±5.1	7.7±0.6	23.6±1.8	13.5±0.3
	leaf	65.0±2.6	132.4±2.1	27.6±2.6	9.6±0.6	13.2±2.8
North	seed	83.0±3.5	243.1±4.5	8.6±0.6	nd	nd
	pulp	132.5±2.2	488.3±13.0	21.1±1.4	36.5±0.6	16.8±0.4
	peel	93.0±2.6	507.0±3.5	6.6±0.2	20.3±0.7	12.7±0.2
	leaf	72.5±2.1	113.6±7.6	9.8±0.6	3.4±0.3	4.5±0.3
Crab	seed	98.2±3.7	464.6±34.5	31.5±0.7	61.1±2.4	22.3±0.5
	pulp	164.1±2.7	1653.2±47.4	8.7±0.4	30.9±0.8	14.0±1.1
	peel	107.8±5.8	1339.5±1.4	28.3±3.1	10.3±1.1	13.0±2.6
	leaf	77.1±1.9	161.8±5.3	32.5±4.6	14.8±2.2	14.0±0.7
Cranberry	seed	100.4±1.0	357.7±5.3	9.2±0.2	3.3±0.5	4.0±0.2
	pulp	159.1±3.8	1204.7±3.4	26.8±0.4	55.8±0.7	18.5±0.4
	peel	108.5±2.1	985.5±1.4	7.5±0.2	28.9±0.6	14.4±0.4
	leaf	80.0±2.4	149.2±23.1	31.1±0.7	10.9±3.8	12.4±0.8

[a] Each value is the mean of triplicates ± standard deviation. [b] nd, not detected

Table 5.4. Individual Phenolic Compounds in Fruit Parts and Leaves (mg/100 g FW)[a]

cultivar	part	hydrolyzable tannins[b]	caffeic acid	*p*-coumaric acid	ferulic acid	catechin	epicatechin	quercetin
R19	seed	36.3±2.3	2.8±0.3	1.4±0.9	0.8±0.3	nd[c]	6.5±0.3	10.8±1.4
	pulp	103.1±1.2	14.4±1.4	8.1±1.3	2.0±0.1	101.2±6.7	11.7±1.9	77.1±6.5
	peel	5759.5±61.3	19.7±2.7	4.3±0.9	17.7±2.3	118.3±6.8	26.1±3.4	94.3±8.3
	leaf	6147.9±89.5	22.3±3.6	16.7±0.4	11.5±0.7	42.7±3.3	61.4±6.2	33.8±2.6
R26	seed	31.8±1.0	2.5±0.5	1.3±0.3	0.7±0.3	nd	6.2±0.5	11.2±1.2
	pulp	99.5±0.9	13.8±1.3	7.7±0.3	1.7±0.3	96.4±6.4	10.9±1.7	75.8±5.8
	peel	5185.9±66.2	19.2±2.8	4.1±0.1	17.5±3.1	115.8±2.2	26.7±2.8	94.8±8.5
	leaf	6772.0±39.0	21.8±3.5	16.3±1.1	11.2±0.3	41.9±8.3	61.8±5.4	32.9±2.4
Cvg-Eve	seed	26.6±0.5	3.1±1.2	2.1±0.2	0.5±0.0	nd	5.8±0.3	10.6±1.3
	pulp	80.9±3.5	12.5±1.1	7.1±0.3	1.4±0.3	88.6±2.3	10.±1.1	69.8±7.8
	peel	6076.3±24.8	19.8±2.0	4.7±0.9	17.8±0.3	120.6±5.7	27.7±2.4	95.8±8.6
	leaf	6240.6±34.7	22.7±2.3	16.8±0.9	10.9±1.1	42.3±2.4	60.3±5.2	32.7±2.1
North	seed	22.8±1.3	2.1±0.4	2.8±0.2	0.5±0.3	nd	6.0±0.6	10.6±1.0
	pulp	71.2±2.2	12.3±1.2	6.6±0.6	1.3±0.2	82.7±5.3	9.6±1.6	66.7±5.7
	peel	4792.3±35.5	18.9±2.7	3.8±0.3	17.1±0.3	110.7±7.7	25.4±2.8	92.1±8.5
	leaf	6060.6±34.6	22.1±2.3	17.2±0.1	11.1±1.7	42.8±2.9	60.7±4.3	32.4±2.6
Crab	seed	30.7±1.3	3.4±0.3	3.2±0.1	1.1±0.3	nd	6.1±0.3	11.1±1.4
	pulp	84.3±1.4	13.6±1.2	7.5±0.5	1.8±0.3	92.3±1.2	10.5±2.1	72.1±5.9
	peel	6894.8±12.6	21.4±3.8	5.1±0.3	18.9±1.6	126.7±7.1	29.5±3.1	99.2±7.2
	leaf	6954.3±14.4	23.5±2.4	16.1±0.8	10.8±0.9	41.7±1.1	61.3±5.9	33.4±2.3
Cranberry	seed	36.6±0.6	3.2±0.3	3.6±0.8	1.3±0.3	nd	6.1±0.4	10.9±1.7
	pulp	100.6±1.3	14.1±1.4	8.0±0.1	2.0±0.3	98.5±4.5	11.7±0.7	76.3±5.3
	peel	6780.9±18.4	20.7±2.2	5.2±0.3	18.6±3.0	125.6±6.3	28.3±3.5	97.8±7.2
	leaf	6782.7±50.1	23.3±3.3	17.1±0.5	11.3±0.3	42.1±3.3	61.5±5.3	33.2±3.3

[a] Each value is the mean of triplicates ± standard deviation. [b]Hydrolyzable tannins include gallotannins, ellagic acid derivatives, and gallagyl tannins which comprises mainly of punicalagin isomers and punicalin. [c] nd, not detected

Table 5.5. Total Lipid Content and Fatty Acid Compositions of Pomegranate Seed (%)[1]

lipid	R19	R26	Cvg-Eve	North	Crab	Cranberry
total lipid	18.1±0.3b	18.3±0.4b	20.7±0.4a	21.5±1.1a	18.3±0.3b	18.2±0.3b
myristic	0.3±0.1	0.3±0.3	0.4±0.0	0.5±0.2	0.2±0.0	0.4±0.0
palmitic	4.2±0.2	4.8±0.2	4.4±0.2	4.1±0.4	3.2±0.1	2.8±0.4
palmitoleic	0.1±0.0	0.1±0.0	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0
stearic	3.3±0.2	3.6±0.3	3.1±0.1	2.8±0.0	2.4±0.3	2.1±0.6
oleic	3.6±0.3	4.1±0.1	5.4±0.2	7.5±0.1	5.8±0.2	7.7±0.2
linoleic	3.2±0.2	3.8±0.3	3.5±0.2	4.9±0.0	3.3±0.1	5.8±0.3
linolenic	0.3±0.1	0.1±0.0	0.2±0.0	0.1±0.0	0.3±0.0	0.2±0.0
punicic	83.4±0.5	81.8±0.4	80.7±0.5	78.3±0.7	83.1±0.3	78.8±0.7
arachidic	0.6±0.1	0.8±0.0	0.6±0.0	0.3±0.0	0.5±0.1	0.4±0.1
lignoceric	0.8±0.1	0.7±0.1	0.8±0.3	0.8±0.1	0.6±0.2	1.3±0.2
nervonic	0.2±0.0	0.2±0.0	0.2±0.0	0.4±0.0	0.4±0.1	0.2±0.0
∑saturated	9.3	10.3	9.4	8.6	7	7.1
∑unsaturated	90.8	90.1	90.2	91.3	93	92.8
saturated/unsaturated	0.1	0.1	0.1	0.1	0.1	0.1

[1] Each value is the mean of triplicates ± standard deviation. Values with the same letter are not significantly different at $p \leq 0.05$.

Table 5.6. Total Lipid Content and Fatty Acid Compositions of Pomegranate Fruit (%)[1]

lipid	R19	R26	Cvg-Eve	North	Crab	Cranberry
total lipid	0.2±0.0a	0.3±0.0b	0.3±0.0b	0.2±0.0a	0.3±0.0b	0.3±0.0b
myristic	0.5±0.1	0.6±0.3	0.7±0.1	0.8±0.1	0.6±0.3	0.8±0.3
palmitic	27.6±0.5	28.1±0.2	27.8±0.3	26.8±0.3	28.3±0.5	28.6±0.6
palmitoleic	0.4±0.3	0.4±0.1	0.5±0.2	0.4±0.0	0.5±0.1	0.5±0.1
stearic	3.1±0.3	3.2±0.3	3.4±0.4	3.3±0.1	3.2±0.3	3.3±0.3
oleic	18.8±0.7	18.4±0.6	18.9±0.3	18.3±0.3	19.2±0.4	18.7±0.5
linoleic	46.7±0.3	47.2±0.5	45.7±0.4	45.3±0.5	45.8±0.7	45.0±0.6
linolenic	0.2±0.1	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
punicic	nd[2]	nd	nd	nd	nd	nd
arachidic	0.3±0.0	0.2±0.0	0.3±0.1	0.3±0.0	0.3±0.1	0.3±0.1
lignoceric	1.4±0.2	1.6±0.1	1.5±0.0	1.3±0.0	1.5±0.3	1.6±0.1
nervonic	0.8±0.2	0.7±0.2	0.8±0.1	0.8±0.1	0.7±0.2	0.8±0.0
∑saturated	33.1	33.9	33.9	32.7	34.1	34.8
∑unsaturated	66.9	66.9	66.1	65	66.4	65.2
saturated/unsaturated	0.5	0.5	0.5	0.5	0.5	0.5

[1] Each value is the mean of triplicates ± standard deviation. [2] nd, not detected. Values with the same letter are not significantly different at $p \leq 0.05$.

Table 5.7. Tocopherol, Phytosterol, and Phospholipid Content of Seeds and Fruits (mg/100 g)[a].

cultivar	part	tocopherols			phytosterols				phospholipids	
		α	γ	δ	brassicasterol	campesterol	stigmasterol	β-sitosterol	PC	PE
R19	seed	161.2±1.4	81.1±0.2	20.7±0.4	2.1±0.9	22.8±1.6	32.9±3.6	333.4±11.2	5.8±3.6	15.6±2.7
	fruit	1.1±0.1	nd[b]	0.5±0.0	0.5±0.1	nd	18.1±2.1	32.7±9.6	5.7±2.8	11.5±1.0
R26	seed	170.1±0.4	80.2±1.1	20.3±0.4	2.2±0.2	17.9±2.1	38.1±1.3	314.3±13.4	18.2±1.4	10.2±0.9
	fruit	3.4±0.2	0.1±0.0	1.1±0.1	0.6±0.3	nd	14.9±1.6	38.1±10.2	4.8±1.1	nd
Cvg-Eve	seed	168.1±1.5	88.2±0.8	23.1±2.4	1.5±0.1	19.5±2.0	27.8±4.8	290.1±11.5	23.1±2.7	62.2±4.7
	fruit	0.6±0.1	nd	0.4±0.0	nd	nd	11.4±5.6	35.8±5.7	4.8±1.0	9.4±3.8
North	seed	173.7±1.3	92.8±0.3	23.8±1.2	0.9±0.2	39.3±1.8	28.5 ±1.5	243.5±10.2	16.3±3.2	40.8±2.6
	fruit	4.1±1.1	0.1±0.0	0.1±0.0	0.3±0.2	nd	9.3±1.1	49.4±8.3	15.1±4.1	33.5±3.9
Crab	seed	168.2±1.2	82.4±0.7	21.4±1.5	nd	19.1±1.1	46.3±8.1	345.8±11.0	26.1±2.1	74.2±3.7
	fruit	4.0±1.0	0.2±0.0	0.7±0.1	nd	nd	10.9±1.8	47.4±10.2	5.8±1.9	nd
Cranberry	seed	162.4±0.8	81.4±0.5	20.6±0.9	nd	38.5±2.5	35.8±5.1	338.3±12.3	17.3±2.2	43.4±2.1
	fruit	2.8±0.2	0.2±0.0	0.1±0.0	nd	nd	11.7±2.9	41.2±6.8	3.7±0.2	nd

[a] Each value is the mean of triplicates ± standard deviation. [b] nd, not detected. PC, phosphatidylcholine; PE, phosphatidylethanolamine

Table 5.8. Comparison of Different Parts of Pomegranate Cultivars

part	cultivar	total organic acid (mg/100 g FW)[1,2]	total hydrolyzable tannins (mg/100 g FW)[1,3]	total polyphenols (mg GAE/100 g FW)[1,4]
seed	R19	419.3±1.2bc	36.3±2.3a	91.1±1.1a
	R26	605.7±7.8a	31.8±1.0ab	90.4±0.8a
	Cvg-Eve	406.5±6.7c	26.6±0.5c	87.4±1.3ab
	North	334.7±33.9d	22.8±1.3d	84.9±1.0b
	Crab	580.5±18.1a	30.7±1.3b	90.1±1.5a
	Cranberry	474.6±6.4b	36.6±0.6a	91.1±1.4a
pulp	R19	1073.2±10.1d	103.1±1.2a	173.4±0.7a
	R26	2235.3±13.0 a	99.5±0.9a	167.5±0.6b
	Cvg-Eve	1089.5±30.0d	80.9±3.5b	161.5±1.3c
	North	695.1±8.2e	71.2±2.2c	151.3±1.4d
	Crab	1932.2±30.1b	84.3±1.4b	161.6±1.5c
	Cranberry	1464.8±5.5c	100.6±1.3a	170.9±0.9ab
peel	R19	910.7±2.4d	5759.5±61.3c	311.4±0.8b
	R26	1857.9±28.5a	5185.9±66.2d	303.7±3.5c
	Cvg-Eve	749.1±11.8e	6076.3±24.8b	314.0±1.1b
	North	639.6±13.9f	4792.3±35.5e	285.8±2.5d
	Crab	1500.9±50.9b	6894.8±12.6a	329.1±1.3a
	Cranberry	1144.8±3.7c	6780.9±18.4a	323.7±1.6a
leaf	R19	255.4±1.8d	6147.9±89.5bc	344.2±0.6e
	R26	324±5.9a	6772.0±39.0a	355.6±1.1d
	Cvg-Eve	247±2.2de	6240.6±34.7b	344.0±1.1e
	North	237.6±2.0e	6060.6±34.6c	370.4±1.3b
	Crab	300.2±7.0b	6954.3±14.4a	380.9±0.5a
	Cranberry	283.6±3.2c	6782.7±50.1a	363.0±1.0c

[1]Values are average and standard deviation of triplicates. [2]Sum of all organic acids analyzed.[3]Hydrolyzable tannins include gallotannins, ellagic acid derivatives, and gallagyl tannins which comprises mainly of punicalagin isomers and punicalin.[4]Sum of hydrophilic and lipophilic fractions. Values with the same letter for each part in each column are not significantly different at $p \leq 0.05$.

Table 5.9. Comparison of Pomegranate with Other Georgia-grown Crops and Other Fruits and Fruit Juices

fruits	total polyphenols (mg GAE/100 g FW)	TEAC (μM TE/g FW)	ref
pomegranate seed[b]	89.2±7.1 [a]	15.0±1.8 [a]	
pomegranate pulp[b]	164.4±6.4 [a]	26.5±2.1 [a]	
pomegranate peel[b]	311.3±10.8 [a]	34.3±1.9 [a]	
Georgia-grown other main crops			
rabbiteye blueberries	556.1±216.9	27.6±5.3	*(28)*
southern highbush blueberries	399.3±149.1	14.8±8.2	*(28)*
blackberries	486.5±97.1	20.4±3.3	*(28)*
muscadine-purple (whole fruit)	247.7±100.5	17.6±7.1	*(12)*
Vidalia onion (var Nirvana)	73.3±1.1	1.1 ±0.0	*(29)*
other fruits			
apple (red delicious)	-	1.6	*(30)*
banana	-	0.6	*(30)*
pineapple	-	9.9	*(30)*
cherry	-	2.7	*(30)*
watermelon		0.7	*(30)*
strawberry (cultivated)		11.0	*(30)*
fruit juices			
apple juice[d]	-	4.3±0.3[c]	*(31)*
red wine[e]	-	19.8±0.4[c]	*(31)*
pomegranate juice[f]	-	41.6±1.8[c]	*(31)*
acai juice[g]	-	12.8±0.4[c]	*(31)*

[a]Sum of hydrophilic and lipophilic fractions. [b]Average of all six cultivars ± standard deviation. [c]TEAC (μM TE /mL). [d]Dole Apple Juice (Pepsico, NY). [e]Merlot Beringer (Beringer Vineyards, Napa, CA). [f]POM Wonderful (POM Wonderful LLC, Los Angeles, CA). [g]Bolthouse Bom Dia Acai-Mangosteen (Bolthouse Juice Products LLC, Bakerfield, CA).

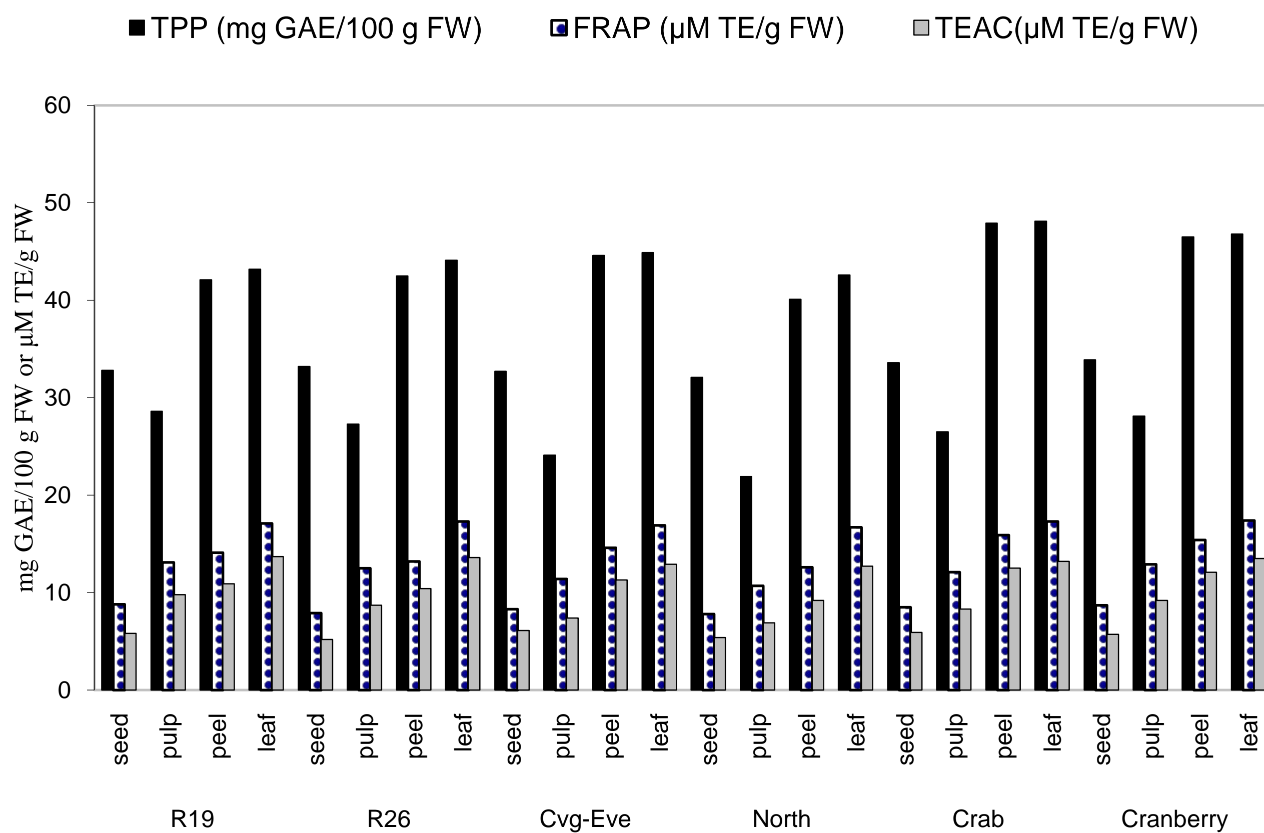

Figure 5.1

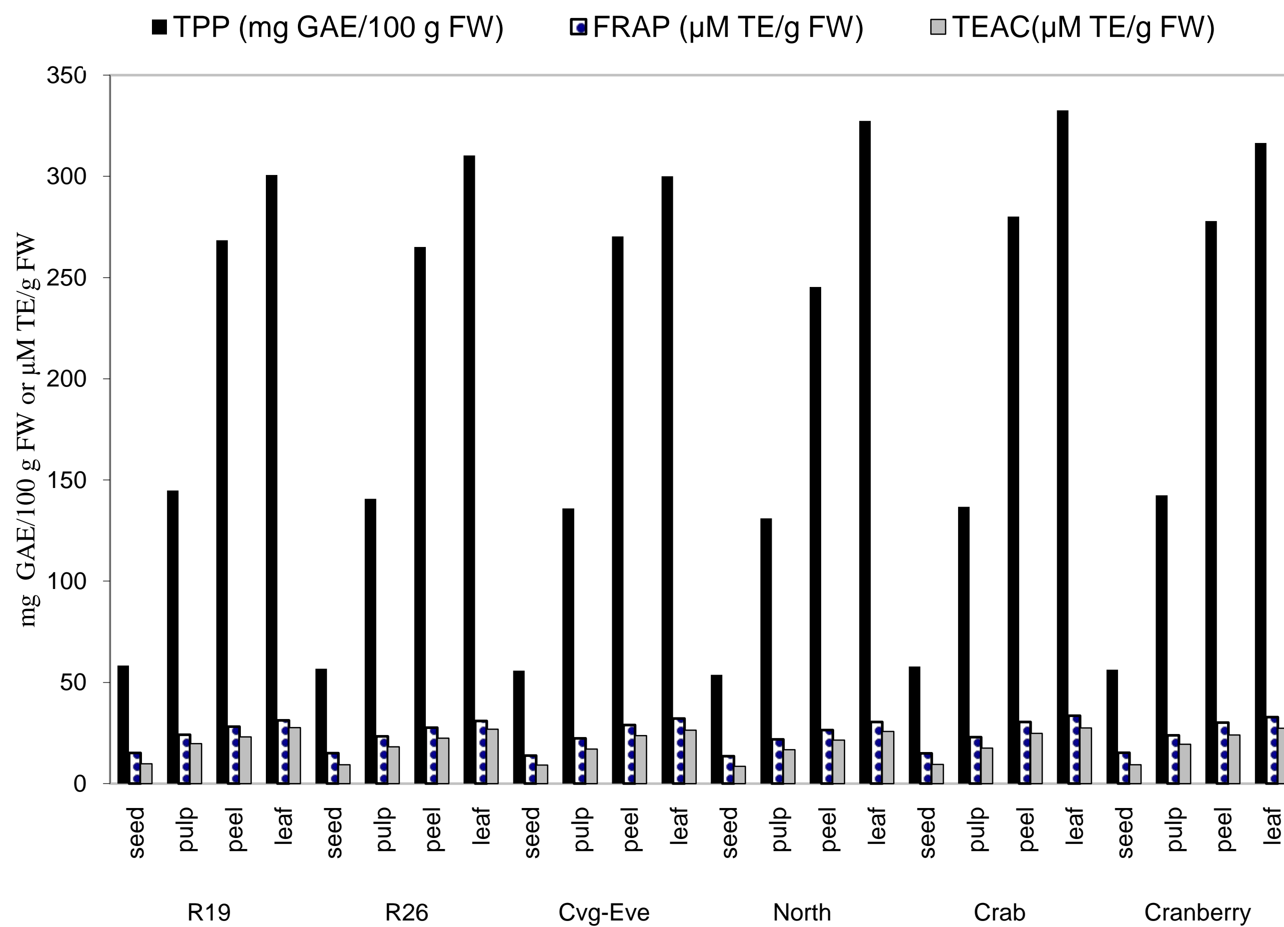

Figure 5.2

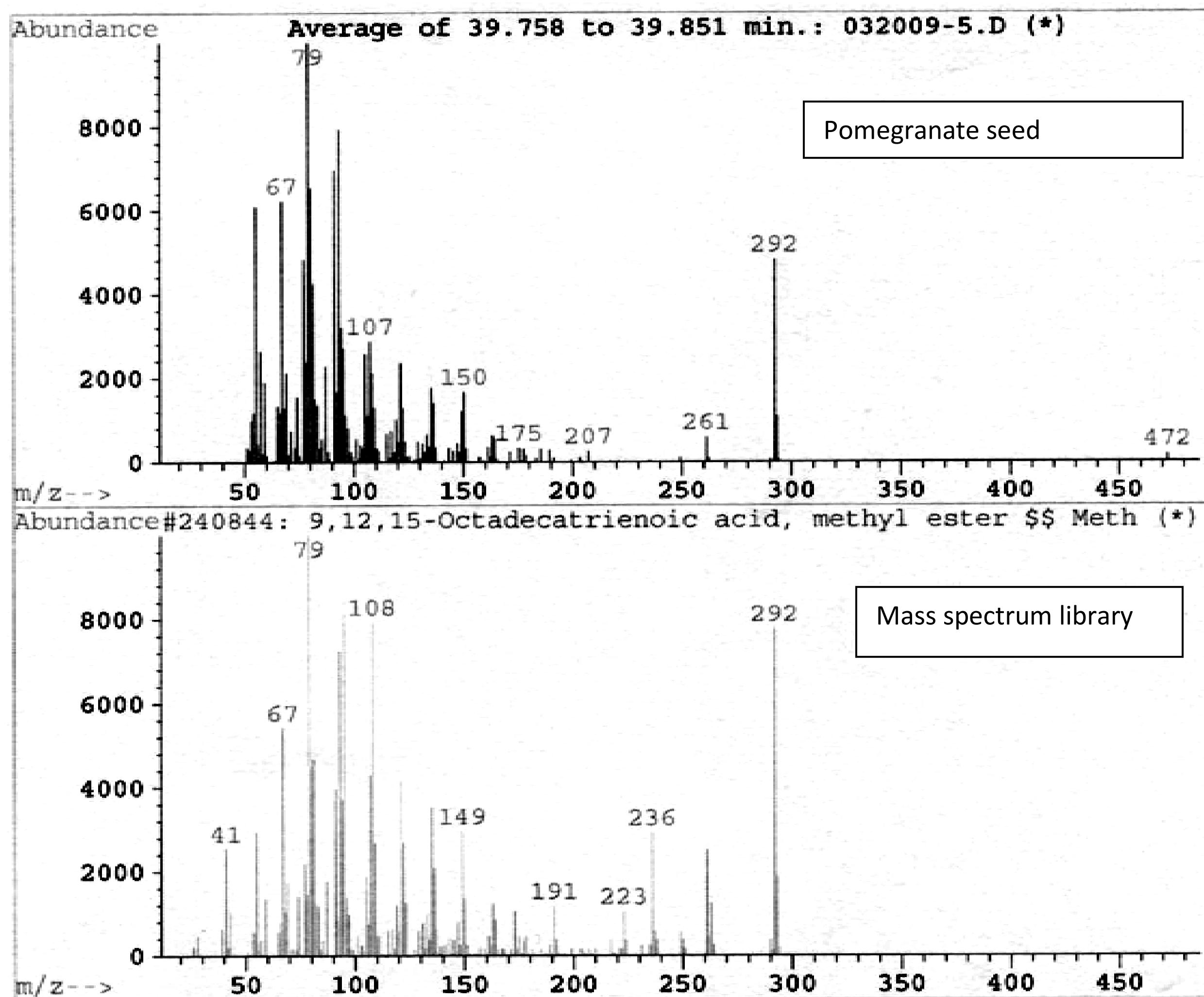

Figure 5.3

CHAPTER -6

Conclusion

Georgia-grown underutilized fruits contain certain phytochemicals which may have commercial applications. The organic acids identified were malic, citric, oxalic, ascorbic, and succinic acids. High malic and citric acids were found in all the fruits. Oxalic acid was found mostly in leaves and also in fig and pomegranate fruits. Ascorbic acid content varied from not being detected in Cvg-Eve and North seeds to 65.9 mg/100 g FW in R26 pulp.

Hydrophilic fractions showed much higher antioxidant capacity than lipophilic fractions. In terms of antioxidant capacity and phenolic compounds the following trend was observed in loquat, mayhaw, pawpaw, and fig: Leaf > seed > peel > pulp whereas, in pomegranate the order was leaf > peel > pulp> seed. Pomegranate was found to be rich in hydrolyzable tannins (punicalagin), especially in the peel fractions which resulted in its high antioxidant capacity.

The seeds of these underutilized fruits can be used as a source of various phytochemicals and lipids. The total lipids content in seeds of loquat, mayhaw, pawpaw, and pomegranate were 18.5, 16.0, 21.5, and 19.2%, respectively. Linoleic acid was the major fatty acid in all the fruits except pomegranate. Pomegranate seed contained a distinctive fatty acid, punicic acid, which accounted for 81% of its fatty acids. Among the phytosterols, β-sitosterol was found in the highest concentration in all the samples. Pomegranate seeds were rich in tocopherol content, especially α- and β-tocopherols.

The result of this present work indicates that these fruits contain several bioactive compounds with possible physiological and industrial benefits and may find use in nutraceutical industries.

Suggestions for future work:

(1) More intervarietal analysis for better variety selection.

(2) Effect of different extraction procedures on antioxidant content and capacity.

(3) Effect of processing on the bioactive compounds while preparing products of these fruits.

(4) In vivo studies to confirm the antioxidant/prooxidant activity in complex biological system.

(5) Screening for anti-nutritional or toxic compounds, if any.