IMPACT OF MATURATION ON THE BIO-PROPERTIES OF SABA BANANA: AN *IN VITRO* STUDY

FLORENCIO COLLADO REGINIO JR.

GRADUATE SCHOOL OF HORTICULTURE

CHIBA UNIVERSITY

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ABSTRACT

Owing to its abundance, low cost, nutritional benefits, convenience, and wide range of applications, Saba became one of the most important banana cultivars in the Philippines in terms of production and trade. This banana variety is also gaining popularity in other countries; however, information about its functional components and the digestibility of its bioactive substances are still insufficient. This study was conducted to investigate the bio-properties of Saba banana fruit as affected by maturation. Five maturity stages were identified based on peel color index (stage 1, all green; stage 2, green with trace of yellow; stage 3, more yellow than green; stage 4, yellow with green tip; stage 5, yellow with brown flecks). Bio-properties were assessed in terms of chemical composition (starch and sugar contents, proximate composition, and pectin content) and functional components (total content of bioactive compounds, antioxidant activities, and individual phenolic compounds). The study also examined the changes undergone by these components during *in vitro* gastrointestinal digestion. Moreover, the potential of Saba banana peel, in comparison to the pulp, as source of health-beneficial agents were evaluated. Results showed that maturation significantly influenced the bio-properties of Saba banana, particularly of the peel. With few exceptions, both peel and pulp showed high values in unripe stages and then decreased as ripening proceeded; however, data of pulp were mostly insignificant. *In vitro* digestibility studies revealed that the changes in the composition of the fruit accompanying maturation in combination with physical properties of the digesta (i.e. viscosity and physical structure) could account for the decreasing trend of starch hydrolysis as ripening proceeded and the slower release of bioactive components in ripe fruits than unripe counterpart. The study suggests that determination of proper maturation could ensure an optimal exploitation of biological activities which could have an effect on their absorption efficiency during digestion.

ABSTRACT (日本語)

サババナナは，量の豊富さ，生産コストの低さ，栄養上の利点，利便性，幅広い用途などにより，貿易面でフィリピンにとって最も重要なバナナ品種の一つである．サババナナはまた他の国でも人気を得ている．しかしその機能性や生理活性物質の消化性に関する情報は不十分なままである．本研究は，熟度によって影響を受けるサババナナ食用部のバイオ特性を調査するために実施した．熟度は果皮の色指標に基づいて５段階で設定した（第１段階：緑色果，第２段階：黄色痕跡を伴う緑色果，第 3 段階：緑色部よりも黄色部が多い状態の果実，第 4 段階：緑色部がごく小範囲の黄色果，第 5 段階：茶色の斑点がある黄色果）．バイオ特性として，化学組成（デンプンおよび糖含有量，粗成分含有量，ペクチン含有量）および機能性（生理活性物質量，抗酸化活性，および個々のフェノール化合物量）の観点から評価した．本研究ではまた，in vitro での模擬消化中におけるそれらバイオ特性の変化も検討した．さらに，健康に有益な物質の供給源としての果皮の可能性についても，果肉と比較，検討の上評価した．その結果，熟度が果皮のバイオ特性に大きく影響を与えることが示された．これに対して果肉のバイオ特性はそれほど大きな変化を示さなかった．しかしバイオ特性が最も高い値を示す未熟果の果肉は，いくつかの例外を除いて果皮が示す傾向と同様に登熟とともにわずかながらバイオ特性の減少を示した．一方，in vitro での模擬消化試験を適用した結果，登熟に伴う果肉の成分変化が消化途中の物性（すなわち，粘度と物理的な構造）と組み合わさることで，より熟した果肉のデンプン加水分解率の減少傾向や生理活性物質のより緩慢な放出現象を説明できることが明らかとなった．本研究により，適切な熟度の判断が消化中のバイオ特性利用効率に影響を及ぼす生物活性の最適化に寄与することが示唆された．

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CHAPTER 1

General Introduction

1.1 Background of the Study

The increasing demand for nutritious, safe, and healthy food due to rapidly rising population combined with the growing concern to maintain biodiversity and protect the environmental resources have posed a major challenge to agriculture production. This paved the way for the promotion of diverse, sustainable, and healthy diets aiming to reduce nutrition-related illnesses which are strongly associated to plant-derived foods (Dwivedi et al., 2017). Plant, their products, and processing by-products have always been extensively utilized not only as valuable source of food but also as a natural component of drugs and excipients in different pharmaceutical formulations (Srivastava & Malviya, 2011). The wide array of nutritionally important constituents and the presence of secondary metabolites in plants that are known to contain various biological effects led to numerous investigations linking each component in the prevention and management of certain diseases. These secondary metabolites are often produced in a phase of subsequent to growth and believed to help plant to increase their overall ability to survive besides primary metabolites which are essentially aimed at growth and development. Some of the substances produced via secondary metabolism affect the biological systems which are considered as bioactive (Azmir et al., 2013). This provides the scientific basis for the use of plants such as herbs in the traditional systems of medicine (Hussein & El-Anssary, 2018). Thus, knowledge of the chemical composition of plant-derived foods is necessary to understand their value and optimize their functionality.

1.1.1 Bioactive compounds

The shifting trend of current researches from the traditional basic nutritional benefits of foodstuffs to disease prevention and health-promoting ingredients resulted in increasing interest in bioactive components that have their origin in plant sources (Ullah & Khan, 2008). Bioactive compounds are typically produced as secondary metabolites eliciting pharmacological or toxicological effects in animals or humans (Bernhoft, 2010). Such biological effects may be positive or negative depending on the nature of substance, concentration, and bioavailability. Most plants have an almost limitless ability to synthesize such compounds which serve partly as a response to ecological and physiological pressures such as UV radiation, wounding (Khoddami, Wilkes, & Roberts, 2013), and predation mainly by microorganisms and insects (Ullah & Khan, 2008).

Bioactive compounds of plants are divided into three main categories based on the biosynthetic origins: (a) terpenoids (approximately 25,000 types), (b) alkaloids (approximately 12,000 types), and (c) phenolic compounds (approximately 8000 types) (Croteau, Kutchan, & Lewis, 2000). These compounds are synthesized in four major pathways (Figure 1.1). Terpenoids are derived from mevalonic acid and methyerythritol phosphate pathways, which produced isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), respectively, being the basic units for terpene synthesis (Olivoto et al., 2017). Alkaloids are nitrogen-containing compounds derived from aromatic (from shikimic acid pathway) and aliphatic amino acids (from tricarboxylic acid cycle). Through the two basic metabolic pathways: shikimic acid and malonic acid pathways, the biosynthesis of phenolic compounds has been associated (Azmir et al., 2013; Taiz & Zeiger, 2006). Phenylalanine, a product of shikimic acid pathway, is the precursor of numerous phenolic compounds (Olivoto et al., 2017).

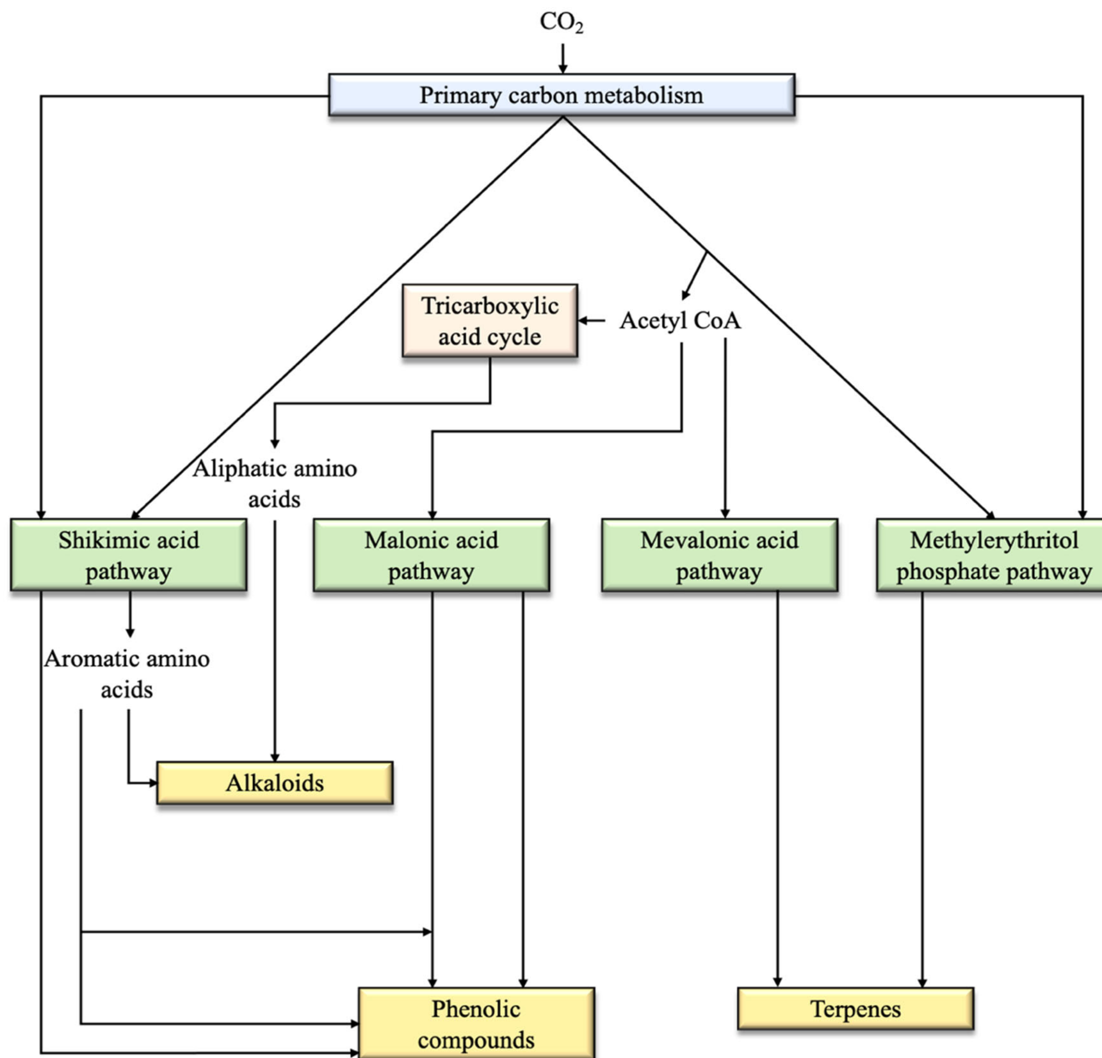

Figure 1.1 Simplified view of pathways for the synthesis of three major groups of plant bioactive compounds (adapted from Azmir et al. (2013)).

1.1.2 Phenolic compounds and their classifications

Among the secondary metabolites, phenolics probably constitute the largest group of plant secondary metabolites with one or more phenol groups as a common characteristic and range from simple structures (having one aromatic ring) to highly complex polymeric compound (Hussein & El-Anssary, 2018). Phenolics can be classified according to their structure, the most common are flavonoids, tannins, and phenolic acids. Flavonoids constitute an enormous class of phenolic compounds often found in vacuoles of plant tissues (Croteau et al., 2000) and exist in conjugated forms, the most common being glycosides (Rice-Evans, Miller, & Paganga, 1997). Structural variations subdivide the flavonoids into several families which include flavonols (e.g. quercetin, rutin, and kaempferol), flavanones (e.g. hesperidin and naringenin), flavones (e.g. apigenin and luteolin), flavanols (e.g. catechins, epicatechins, epigallocatechins), isoflavones (e.g. genistein, biochanin A), and anthocyanidins (e.g. delphinidin and malvidin) (Rice-Evans et al., 1997; Ullah & Khan, 2008). Phenolic compounds that have the ability to precipitate protein are tannins which have two distinct types – hydrolyzable and condensed tannins (Hussein & El-Anssary, 2018). Condensed tannins are large polymers of flavonoids and hydrolysable tannins which are polymers composed of a monosaccharide core (most often glucose) and organic acids (e.g. gallic and ellagic acids) (Bernhoft, 2010; Hussein & El-Anssary, 2018). The other main classes of phenolics are phenolic acids which occur rarely in free form and usually occur as esters, glycosides, or amides. Two-parent structures of phenolic acids are hydroxycinnamic and hydroxybenzoic acids which differ in the number and location of hydroxyl groups on the aromatic ring. Hydroxycinnamic acid derivatives include ferulic, caffeic, *p*-coumaric, and sinapic acids while hydroxybenzoic acid derivatives consist of gallic, vanillic, syringic, protocatechuic, and *p*-hydroxybenzoic acids (Khoddami et al., 2013).

Phenolic compounds in plants can also be classified according to their solubility features: soluble and insoluble-bound fractions. Solvents such as water, methanol, ethanol, and acetone are used to extract the soluble fractions (Yu et al., 2019) which can be further divided into free, esterified, and glycosylated forms. Free phenolics, present as phenolic aglycone (Shahidi & Peng, 2018), are the most extensively studied fraction of phenolic in the past years (Pereira, Arruda, de Morais, Eberlin, & Pastore, 2018) because of its easy extractability. Both esterified and glycosylated are conjugated to low-molecular mass components and sugars which are extracted under mild saponification conditions to hydrolyze and release the existing phenolics (Salawu, Bester, & Duodu, 2014). In contrast, insoluble-bound phenolics are covalently bound to indigestible matrices such as polysaccharides (pectin, hemicellulose, cellulose, and

arabinoxylan), rod-shaped structural proteins, and highly-polymerized phenolics (condensed tannin and lignin) (Shahidi & Peng, 2018) which play important roles in providing both chemical and physical barrier and protecting against pathogen invasion (Chen, Zhang, Chen, Han, & Gao, 2017). These bound phenolics are released by acid, alkaline, or enzyme hydrolysis (Q. Li et al., 2018). Research studies have shown that bound phenolics have demonstrated a significantly higher antioxidant capacity compared to free phenolics in numerous *in vitro* antioxidant assays carried out (Liyana-Pathirana & Shahidi, 2006). Therefore, there is an increasing interest in the determination of bound phenolics as neglecting this could underestimate the amount the real phenolic content of the fruit and their corresponding antioxidant activities.

1.1.3 Antioxidant activity and its measurement

The health-protective properties of phenolic compounds have been attributed mainly to their antioxidant activity. The protection mechanism is generally by inhibiting the formation of free radical species and repairing oxidative damage, thus preventing the development of various chronic and degenerative diseases (Isabelle et al., 2010; Sulaiman et al., 2011). More specifically, the activity of antioxidants is determined by their reduction potential either as hydrogen or electron donor, ability to stabilize the unpaired electron, reactivity with other antioxidants, and metal-chelating potential (Rice-Evans et al., 1997).

In different food products such as fruits, the antioxidant capacity is determined by a mixture of various antioxidant compounds with different action mechanisms. Therefore, combining more than one assay is necessary to provide a better assessment and estimation of the antioxidant capacity of food (Almeida et al., 2011). A wide range of spectrophotometric assays has been adopted, the two most widely used being 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. Both assays use chromogen compounds of a radical nature in which the presence of antioxidants leads to the disappearance of radical chromogens (Arnao, 2000). Among others such as oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assay which involve either hydrogen and electron transfer, respectively (Floegel, Kim, Chung, Koo, & Chun, 2011). Metal ion chelating (MIC) assay has also been used as a measure of antioxidant activity based on how effective the compounds in competing with ferrozine for ferrous ion (Y. Y. Lim, Lim, & Tee, 2007).

1.1.4 *In vitro* digestibility

Digestibility is the most important biological measure of foodstuffs. It can be studied using *in vivo* and *in vitro* methods. The former is the first and most widely method; however, this is laborious, costly, and time-consuming as it requires human participation or animal subject to allow the holistic understanding of the actual effects of nutritional ingredients on the living body (Ogawa et al., 2018). Thus, *in vitro* testing has been proposed as a faster and more cost-effective method to simulate the natural ruminant digestive process and rapidly screen food products for their estimated biological activity (Germaine et al., 2008). Though *in vitro* digestion cannot perfectly actualize the highly complex physiological events during digestion, the model may serve as a useful tool to study the structural changes and release of food components under simulated gastrointestinal conditions (Hur, Lim, Decker, & McClements, 2011).

One of the most common food components being studied for digestibility is starch. Ingestion of starchy food and its subsequent degradation during digestion has received considerable attention in the context of worldwide health concerns. Understanding the digestibility of starch offers a significant contribution in predicting and controlling the breakdown and absorption of glucose, thereby offering assistance in regulating postprandial blood glucose levels. Previous studies have been conducted reporting various factors that could affect the starch digestion rate and its consequent glycemic impact (Bi et al., 2017; Jiang et al., 2015; Tamura, Singh, Kaur, & Ogawa, 2016). Among them are the physical characteristics of the food matrix which could influence the motility and mixing of food particles and enzymes during *in vitro* starch hydrolysis. Several researches had been published relating the impact of food particle geometry on changes in hydrolysis of food components through their influence on the surface area available for digestion, thus affecting bioavailability (Dhital, Bhattarai, Gorham, & Gidley, 2016; Ellis et al., 2004; Thuengtung, Niwat, Tamura, & Ogawa, 2018; Tydeman et al., 2010).

1.1.5 Banana industry

Banana is one of the world's most important fruit crops in terms of commercial production (Raut & Ranade, 2004) ranking next to rice, wheat, and maize and is considered as the 5th most traded agricultural produce (B. Singh, Singh, Kaur, & Singh, 2016). Banana is grown in 150 countries across the world (Prabha & Kumar, 2015) with an estimated gross production of 120 million tons each year (Reay, 2019). The global exports of banana last 2019, excluding plantain, was 20.2 million tons, an increase of 5% compared to 2018. The strong

supply growth in Ecuador and the Philippines is chiefly accountable for this rise (Food Agriculture Organization of the United Nations, 2020). Additional reason for the steady growth of banana export is its low cost. It is the least expensive among the most-consumed fruit crops pricing around 1.22 USD per kg (Kirk, Melloy, Iyer, & Jaacks, 2019). Since banana is one of the most popular fruit crops, it also generates approximately 36 million tons of peel waste every year. The peel accounts for about 35% of the whole fruit weight and is usually discarded directly into landfill or with general waste (Vu, Scarlett, & Vuong, 2018) contributing to massive amounts of organic materials to be managed. Thus, it is noteworthy to investigate the potential of banana peel as a source of high-added value compounds to minimize its contributed wastes and maximize its use.

1.1.6 Maturation and maturity indices

Maturity can be defined as physiological and horticultural. Physiological maturity is the stage of development when a plant part will continue development even if detached whereas horticultural is the stage of development when a plant part possesses the necessary characteristics at which it can be marketed and used by consumers (Reid, 1992). The degree of maturity and ripeness of fruit is of utmost importance in determining its suitability for consumption or processing since the quality characteristics of end products are dependent on the quality of horticultural produce (Singhal, Kulkarni, & Rege, 1997). Underlying the various noticeable physical changes during postharvest ripening is a series of biochemical processes in which the chemical composition of the unripe fruit is transformed (Rhodes, 1978).

Banana, being a climacteric fruit, is harvested at physiological maturity and then allowed to ripen until it reaches the desired yellow color and flavor characteristics. Maturity of the banana bunches dictates the quality of fruit during ripening and overall marketability. One of the most prominent changes during maturation of banana is the transformation of peel color. The color of the peel is considered as the first quality parameter evaluated by traders and consumers (Prabha & Kumar, 2015). Another maturity index is the pulp firmness which is important in the evaluation of fruit susceptibility to physical and mechanical damage. The texture of banana is a physical attribute resulting from a combination of several factors including water turgor and structural components of cells and tissues (Dadzie & Orchard, 1997). The total soluble solids of banana also showed a direct relationship with fruit ripening and thus can be a useful index of maturity. Sugar forms the main component of soluble solid of ripe banana and its increase could be associated with the enzymatic degradation of starch (Adi, Oduro, & Tortoe, 2019).

The conversion of starch into sugars is the most-striking post-harvest chemical change in banana (Dadzie & Orchard, 1997). Extensive research studies about the hydrolysis of starch during ripening of bananas were reported (Cordenunsi & Lajolo, 1995; Lustre, Soriano, Morga, Balagot, & Tunac, 1976; Marriott, Robinson, & Karikari, 1981). Banana, with its innumerable varieties, is the usual climacteric fruit when starch reserves accumulation and degradation during maturation and ripening, respectively, are particularly investigated. Moreover, when sourcing starches from nonconventional botanical sources, banana starch has the potential to be a commodity starch as the green banana pulp contains up to 70-80% starch (dry weight basis) which is comparable to other starch crops (Zhang, Whistler, Bemiller, & Hamaker, 2005).

1.1.7 Saba banana [*Musa 'saba'*(*Musa acuminata* × *Musa balbisiana*)]

Among the large number of banana cultivars in the Philippines that have not yet been fully studied in terms of biochemical properties, Saba banana is the most promising. The banana variety belongs to the ABB genome group and is the 2nd most-produced variety next to cavendish accounting for about 28% of the country's banana production. Saba banana is said to be native in Southeast Asia which is recognized in the following names: *giant pisang kepok* (Indonesia), *pisang abu nepah* (Malaysia), *kluai hin* (Thailand), and *chuoi mat* (Vietnam) (T. K. Lim, 2012). The abundance and the relatively low cost of Saba banana in the Philippines make it one of the most popular among the many banana cultivars in terms of production and trade because of its wide range of applications in the local market. It is one of the important food sources in rural areas where it is often used as an alternative food staple to rice because of its less-sweet character compared to other dessert bananas. The fully ripe fruit can be eaten raw but is more commonly cooked by boiling and frying in syrup (Lustre et al., 1976). Likewise, the green unripe stage is consumed like a vegetable in making savory dishes and can be processed into several starch-based food products such as chips, ketchup, and sauces. Due to its cheaper price when compared to dessert bananas and high possibility of formulation into a wide range of products, an increase in both consumption and utilization of Saba banana has been observed over the year (Olawoye, Kadiri, & Babalola, 2017). Nutritional information about this variety of banana showed that the ripe Saba banana contains 190 µg beta-carotene, 32 mg ascorbic acid, 36 mg phosphorus, and 0.9 mg iron per 100 g edible portion. The published values were higher than the composition of red and green varieties of apple per 100 g comparison. In addition, it has higher carbohydrates than potato and almost equal calories and dietary fiber as that of boiled milled rice and brown rice, respectively (Food and Nutrition Research Institute - Department of Science and Technology, 2018). With these innumerable

remarkable characteristics, an extensive study about its intrinsic properties is necessary which could possibly add a higher value to this particular variety of banana and could also be an opportunity to fully recognized in the international market.

1.2 Significance and Objective of the Study

With the increasing concerns in public health issues, it is necessary to fully exploit the intrinsic features of plant-derived foods which might have the potential to offer natural remedies for nutrition-related diseases. This warrants the need to examine their chemical composition to provide information about their nutritional applications and functionality. The abundance, low cost, economical, and nutritional value of Saba banana fruit led to its investigation via *in vitro* tests and assays. This banana variety is one most important fruit crops in the Philippine food industry due to its wide range of applications and is also gaining popularity in other countries. Though it is extensively used in the local food market, details about its biochemical properties are still insufficient. Thus, research topics about Saba banana are essential to obtain more information about its potentially emerging beneficial effects. In general, the objective of this study was to examine the impact of maturation on the bio-properties of Saba banana. This was the first work that dealt with the detailed phenolic composition of this banana variety in different phenolic fractions, as well as the digestive fate of the bioactive components. This could serve as a reference for future studies about other indigenous varieties of banana. Moreover, knowledge on the proper maturity could ensure optimal exploitation of biological activities which could have an effect on their absorption efficiency during digestion.

1.3 Content of the Study

The bio-properties of Saba banana were investigated in terms of chemical composition and functional components. Chemical composition encompassed starch and sugar contents, proximate composition, and pectin content, whereas functional components included *in vitro* assays of total content of bioactive compounds and antioxidant activities and quantitative evaluation of individual phenolic compounds. The study also included the assessment of these bio-properties through simulated *in vitro* digestion process. The maturity of Saba banana was determined through evaluation of peel color by visual observation, image analysis using graphic software (Adobe Photoshop CC 2019) (Chapter 2), and by using a chromameter (Chapter 4).

In Chapter 2, the maturity of Saba banana was further confirmed using other indices e.g. pH, total soluble solids, and titratable acidity. The initial determination of starch and sugar contents was done as baseline data to determine the effect of maturity on *in vitro* digestibility of starch. The basis for the estimation of starch hydrolysis during *in vitro* digestion was the amount of sugars accumulated, specifically glucose. In addition, to elucidate the food matrix effects on starch digestibility, two different physical structures of Saba banana were employed at different stages of maturity (cut and slurry samples). Unhomogenized cut samples represented minimally processed food product that retained the tissue structure of food and could also be as a result of incomplete comminution during chewing. In contrast, homogenized slurry samples were regarded as structure-less food materials as a result of processing approaches such as crushing and blending or thorough mastication that fully disrupt the cellular structure. These two physical structures also showed significant differences in consistency, the reason for the inclusion of viscosity measurement in the study. Research studies using banana flour as raw material to determine the starch digestibility were extensive; however, little to no information was available on the fresh state of banana as different factors could synergistically affect its digestibility.

The effect of two different physical structures of Saba banana at different stages of maturity was continued to observe in Chapter 3 with total phenolics and antioxidant activities as the parameters being investigated. The changes in the contents of these parameters were examined during *in vitro* digestibility to determine the influence of maturity and physical structure on their release behaviors. The study also analyzed the functional components (total phenolic, flavonoid, and antioxidant activities) of fresh Saba banana at varying maturity stages. The extraction method included the separation of free from bound phenolics. To summarize the results of the study, a correlation analysis was conducted to determine the strength of relationship between the variables used including the effect of maturity.

One reason for the observed significant differences in starch hydrolysis values of different maturity stages of Saba banana was the modifications in the content of its pectic polysaccharides, specifically water-soluble pectin. Up to date, the study of pectic polysaccharide fractions from this variety of banana has not been reported. Chapter 4 encompasses the fractionation of the pectic polysaccharides into water-, chelate-, acid-, alkali-soluble pectins. Quantification of pectin was done by spectrophotometric (3,5-dimethylphenol and carbazole) and gravimetric (calcium pectate) analyses. Moreover, the viscosity of water-soluble pectin solutions from different extract was observed simulating the conditions during *in vitro* digestibility. The data obtained in this study could be used not only to justify the results

of the previous study but also to have a baseline data of the characteristics of pectin at different maturity stages of Saba banana which could influence its intrinsic properties, e.g. electrical impedance. Hence, the study also investigated the relationship of impedance characteristics on the modifications of pectic polysaccharides in Saba banana during maturation.

Chapter 5 attempts to investigate the potential of Saba banana peel as a source of health-beneficial agents. In comparison to the pulp, bio-properties of Saba banana peel were examined. The chemical composition and antioxidant activities of peel and pulp were compared at different stages of maturity. In addition to free and insoluble-bound phenolics reported in Chapter 3, esterified and glycosylated phenolics were also extracted and evaluated in this chapter. This was the first time the phenolic fractions of unripe and ripe peel and pulp of Saba banana were characterized and quantified using high-performance liquid chromatography coupled with diode array detector and electrospray ionization, alongside tandem mass spectrometry (HPLC-DAD-ESI-MS). Moreover, antioxidant activities of each fraction were assessed using different *in vitro* assays, the same methods used in Chapter 3. To summarize the results of the study, variations in the bio-properties of unripe and ripe peel and pulp were assessed using principal component and correlation analyses.

CHAPTER 2

In vitro examination of starch digestibility of Saba banana [*Musa 'saba'*(*Musa acuminata* × *Musa balbisiana*)]: impact of maturity and physical properties of digesta

2.1 Introduction

Saba' [*Musa 'saba'*(*Musa acuminata* × *Musa balbisiana*)] is one of the banana cultivars considered to have good potential in the export industry. The fruit crop is usually grown in Southeast Asia with local names such as *giant pisang kepok* (Indonesia), *pisang abu nepah* (Malaysia), *kluai hin* (Thailand), *chuoi mat* (Vietnam), and Saba or *cardaba* (Philippines) (T. K. Lim, 2012). In the Philippine setting, this variety is the most important among the many banana cultivars in terms of commercial production and trade because of its wide range of applications in the domestic market. Most people in the rural areas regarded Saba banana as one of their food sources often consumed as an alternative food staple to rice and corn. The fully ripe fruit can be eaten raw but is more commonly cooked by boiling and frying in syrup (Lustre et al., 1976). Likewise, the green unripe stage is consumed like a vegetable in making savory dishes and can be processed into several starch-based food products such as chips, ketchup, and sauces. Due to its more affordable price when compared to dessert bananas and high possibility of formulation into a variety of products, an increase in both consumption and utilization has been observed over the year (Olawoye et al., 2017).

Aside from being a staple food and as a raw material in various processed products, banana, in general, is known to offer great health benefits and nutritional value (Seymour, 1993; Verma, Kumar Mani, & Mishra, 2017). In the green stage, bananas stand out for having high resistant starch contents (Bezerra, Rodrigues, Amante, & Silva, 2013). Substantial researches had been conducted linking resistant starch in enhancing colonic health, acting as a vehicle to increase the total dietary fiber content of food, and aiding diabetes and weight management (Nugent, 2005). Conversely, previous studies also suggested the restriction of banana as desserts or snacks as it contains approximately twice the amount of carbohydrate compared to other fruits. The conversion of starch into free sugars during the ripening process may influence its postprandial blood glucose response (Hermansen, Rasmussen, Gregersen, & Larsen, 1992). Thus, studying the digestibility of banana, particularly its starch content, at different maturity stages is necessary to understand its nutritional implications and functionality as an energy source.

Research and development efforts on banana have been ongoing. To date, information on the digestibility of banana starch and food products with banana flour as a raw material is extensive. Jiang et al. (2015) reported that the starch from different banana cultivars showed differences in structural, physicochemical, and digestibility characteristics. Bi et al. (2017) concluded that the digestibility of green banana flour and starch was affected by chemical composition, particularly pectin, and granule morphology, crystalline structure, amylose content, branched-chain length distributions of amylopectin, and starch molecular weight. However, little to no information is available on the digestibility of fresh banana fruit, specifically the Saba banana variety, and the influence of maturation on its starch digestibility. When assessing the rate of starch digestion, it is also essential to elucidate the food matrix effects on the extent to which the food component is hydrolyzed. The complex food matrix of fresh Saba banana may influence the catalytic efficiency of digestive enzymes in breaking down starch and thus affecting its bioavailability. One way to establish the structure of such matrix is through food processing or oral mastication, with or without disruption of the tissue structure. This can have a critical role on starch digestibility by affecting the accessibility of digestive medium to starch in the food or through its influence on the surface area available for digestion (Mishra, Hardacre, & Monro, 2012). As reported in previous *in vivo* and *in vitro* studies about almonds (Ellis et al., 2004), legumes (Dhital et al., 2016), carrots (Tydeman et al., 2010), and rice (Thuengtung et al., 2018), intactness of cell wall structure could be a controlling factor in the bioaccessibility of nutrients from plant-based foods. For these reasons, a simulated gastrointestinal *in vitro* digestion model was employed to examine the digestibility of starch in Saba banana as affected by maturity and physical characteristics of the digesta, which involved viscosity and physical structure. The impact of viscosity and gross structure on starch digestibility was evaluated through the preparation of unhomogenized cut and homogenized slurry samples. The former represented minimally-processed food product which retained the tissue structure and could also be as a result of limited comminution. The latter, on the other hand, represented structure-less food materials as a result of processing approaches such as crushing and blending or thorough mastication that fully disrupt the cellular structure.

2.2 Materials and Methods

2.2.1 Materials and chemicals

Commercial green mature Saba bananas were purchased from Diamond Star Agro-Products Inc., Taguig City, Philippines. The fruit was allowed to ripen until five different maturity stages developed as shown in Figure 2.1 — all green (stage 1), green but turning yellow (stage 2), greenish-yellow (stage 3), yellow with green tips (stage 4), and yellow with brown flecks (stage 5). Enzymes used in simulated digestion such as pepsin (porcine gastric mucosa, >250 U mg^{-1} solid), pancreatin (hog pancreas, 4x USP), and invertase (grade VII from baker's yeast, >300 U mg^{-1} solid were bought from Sigma-Aldrich (St. Louis, Mo., U.S.A.). Amyloglucosidase (3260 U mL^{-1}) and resistant starch assay kit (K-RSTAR) were purchased from Megazyme International (Wicklow, Ireland). Sugar standards (sucrose, glucose, and fructose) and ultrapure water were supplied by Wako Pure Chemical Industries (Osaka, Japan).

Figure 2.1 Five maturity stages of Saba banana used in the study.

2.2.2 Sample preparation

A minimum of 7 Saba banana fingers from 6 bunches was randomly selected every sampling period. The samples were peeled, cut into small round sections, and divided into three portions. One part was freeze-dried (FDU-1100, Eyela, Tokyo, Japan), ground, and passed through a 0.5 mm mesh sieve (Sanpo, Tokyo, Japan) before analysis of starch content. Another portion was allotted for the simulated digestion. The slurry state was homogenized using a household blender (NM200, Yamazen, Tokyo, Japan) for 2 min while cut sample (with

dimension of ca. ≤ 3 mm) was prepared using a combination of manual cutting and food chopper (Tefal, Rumilly, France). Both physical states were mixed with water to have the same initial starch content of approximately 4%. The remaining raw Saba banana samples were used for the determination of pH, titratable acidity (TA), and total soluble solids (TSS) using supernatant from homogenized pulp (10 g banana pulp:30mL distilled water).

2.2.3 Physicochemical analyses

Physicochemical analyses were used as indices to determine the maturity of Saba banana. Peel color was assessed by capturing an RGB image using a digital camera (E-510 Olympus, Tokyo, Japan) with 1/125 shutter speed, F4.0 exposure, and ISO 100. The captured RGB image was converted to CIE L*a*b* to obtain L* (lightness), a* (–greenness to +redness), and b* (–blueness to +yellowness) values using Adobe Photoshop CC (Adobe, San Jose, CA, U.S.A.). Chroma [$C = (a^{*2}+b^{*2})^{1/2}$] and hue angle [$h° = \tan^{-1}(b^*/a^*)$] were calculated from the chromaticity coordinates. Moisture content was analyzed by gravimetric heating using an oven dyer (Oven 8150, Labserv, Longford, Ireland) following the method of AOAC (AOAC International, 2000) with slight modification. pH, TSS, and TA were determined using the protocols of Dadzie and Orchard (1997) with minor modifications. A pH meter (AS800, As One, Osaka, Japan) and a digital refractometer (PR-101, Atago, Tokyo, Japan) were used to measure pH and TSS, respectively, while TA was obtained by titration to a pH of 8.1 and was expressed as malic acid (%).

2.2.4 Sugar analysis

A reversed-phase high-performance liquid chromatography (RP-HPLC) was used for the separation and determination of sugars (sucrose, glucose, and fructose) in the digested fractions. The supernatants collected from simulated digestion were first filtered through 0.45 μm membrane filter (Advantec, Tokyo, Japan). The chromatographic separation was performed using HPLC system (Shimadzu, Kyoto, Japan) equipped with pump (LC-20 AD), refractive index detector (RID-20A), column (Shim-pack SCR-101N), oven (CTO-20 AC), and degassing unit (DGU-20A3). The mobile phase, ultrapure water, was pumped at a flow rate of 0.8 mL min^{-1} under isocratic elution at a constant oven temperature of 60 °C. Analytical data were collected and processed by LabSolutions software (Shimadzu, Kyoto, Japan).

2.2.5 Total and resistant starch analysis

Total starch (TS) and resistant starch (RS) contents were determined using an assay kit (Megazyme International, 2019) following AOAC Official Method 2002.02. Briefly, 100 ± 5 mg of freeze-dried sample was added with 4 mL pancreatic α-amylase (10 mg mL^{-1}) containing

amyloglucosidase (3 U mL^{-1}). The sample was incubated at 37 °C with continuous shaking (200 strokes/min) for exactly 16 h to allow the solubilization of non-resistant starch and hydrolyzation to D-glucose. After incubation, the reaction was terminated by the addition of 4 mL ethanol (99% v/v), and the supernatant was separated with the pellet by centrifugation at 1500 × g for 10 min. The resistant starch in the pellet was re-suspended twice in 8 mL of 50% ethanol and centrifuged again at 1500 × g for 10 min. The recovered pellet was then dissolved in 2 M KOH for approximately 20 min in an ice-water bath with vigorous stirring. The solution was neutralized with 8 mL of 1.2 M sodium acetate buffer (pH 3.8) followed by the addition of 0.1 mL of amyloglucosidase (3,300 U mL^{-1}) and incubation at 50 °C for 30 min with intermittent mixing. The volume of the sample was adjusted to 100 mL with distilled water. An aliquot of the solution was centrifuged at 1500 × g for 10 min. Free glucose from resistant starch was determined using 0.1 mL aliquots (in duplicate) of diluted supernatant and 3 mL glucose oxidase/peroxidase (GOPOD) reagent after incubation at 50 °C for 20min. The absorbance of the sample and standard (D-glucose) were measured at 510 nm in UV-Vis spectrophotometer (V-630Bio, Jasco, Tokyo, Japan) against a reagent blank consisting of 0.1 mL of distilled water. For the determination of non-resistant starch, the supernatants obtained from washings were pooled and adjusted to 100 mL with 100 mM sodium acetate buffer (pH 4.5). An aliquot (0.1 mL) was incubated with 10 µL of diluted AMG solution (300 U mL^{-1}) in 100 mM sodium maleate buffer (pH 6.0) for 20 min at 50 °C. GOPOD reagent (3 mL) was added and further incubation of the sample was done at 50 °C for 20 min. The absorbance of free glucose from non-resistant starch was measured the same method as resistant starch. Resistant and non-resistant starches were calculated by multiplying the measured free glucose using a conversion factor of 0.9 (factor to convert from free D-glucose, as determined, to anhydro-D-glucose as occurs in starch), which is shown below:

$$\text{Resistant starch/ Non} - \text{resistant starch (\%)} = \Delta E \times \frac{F}{W} \times V \times 0.9$$

where ΔE is the absorbance of the reaction against the reagent blank, F is the conversion from absorbance to micrograms (100 µg of D-glucose divided by the GOPOD absorbance for the 100 µg of D-glucose), W is the dry weight of sample analyzed, and V is the volume of extract.

2.2.6 Simulated *in vitro* gastrointestinal digestion

A two-stage simulated *in vitro* gastrointestinal digestion model was employed following the method of Dartois, Singh, Kaur, and Singh (2010) with modifications by Thuengtung et al. (2018). Fresh Saba banana in each maturity stage (170 g) were directly transferred into a jacketed-glass reactor maintained at 37 ± 1°C with continuous stirring. Simulated gastric

digestion was initiated by adjusting the pH to 1.2 ± 0.1 using different molar concentrations of HCl and addition of simulated gastric fluid containing 0.12 g pepsin and buffer with 0.2% (w/v) NaCl (pH adjusted to 1.2). After 30 min of gastric digestion, the pH was changed to 6.8 ± 0.1 by the addition of different molar concentrations of NaOH to deactivate pepsin and simulate the digestion in the small intestine. The intestinal digestion process began when the simulated intestinal fluid, containing 0.1 g pancreatin, 7.5 mg invertase, 2 mL amyloglucosidase, and buffer with 0.68% (w/v) monobasic potassium phosphate (KH_2PO_4) (pH adjusted to 6.8), was added to the reaction mixture. Aliquots of supernatants were collected from the following time periods: after 5 (G5) and 30min (G30) of gastric digestion; and after 5 (I5), 30 (I30), 60 (I60), 120 (I120), 180 (I180), and 240 min (I240) of small intestinal digestion for slurry samples while cut samples were continued until 480 min (I480). The digested fractions were mixed with 3 mL of 95% ethanol to stop the enzymatic reactions, centrifuged at 1800 × g for 10 min, and stored at −20 °C until further analysis.

2.2.7 Digesta viscosity measurement

The viscosity of supernatant from digesta was measured using a torsional oscillation viscometer (Viscomate VM-10A, Sekonic, Tokyo, Japan). Four sampling points were selected during the course of *in vitro* digestion representing initial value at time 0, after gastric digestion (G30), during intestinal digestion (I60), and at the end of digestion (I240 and I480 for slurry and cut samples, respectively). The supernatant was withdrawn from the digestion reactor and viscosity was measured under static conditions at 37 ± 1°C.

2.2.8 Total starch and starch hydrolysis

The total starch (TS) content from the freeze-dried Saba banana is defined as follows:

$$\text{TS (\%)} = \text{Resistant starch} + \left[\text{Non} - \text{resistant starch} - \left(\text{Initial Glucose} + \frac{\text{Initial Sucrose}}{2}\right)\right]$$

The starch hydrolysis (%) was computed based on the amounts of sucrose, glucose, and fructose accumulated during *in vitro* digestion. It was estimated by deducting the initial glucose content of the sample and the glucose hydrolyzed from sucrose. The latter was expected to be equal to the amount of fructose formed during sucrose hydrolysis and the value was deducted to the total amount of glucose accumulated. Thus, the formula is defined as follows:

$$\text{Starch hydrolysis (\%)} = \frac{(\text{Glucose} - \text{Glucose at G30}) - (\text{Fructose} - \text{Fructose at G30})}{\text{TS}} \text{ x } 100$$

2.2.9 Scanning electron microscopy

Banana starch was obtained using the extraction method described by (Espinosa-Solis, Jane, & Bello-Perez, 2009) with some modifications. Samples were fixed on aluminium stubs

using double-sided tape and viewed under a scanning electron microscope (SEM) (SU1510, Hitachi high-technologies, Tokyo, Japan) using an acceleration voltage of 15 kV.

2.2.10 Statistical analysis

The results were presented as mean values ± standard deviation. The data were analyzed using one-way analysis of variance and the means of results for each experiment were compared using Tukey's test ($p < 0.05$). Statistical analyses were run using R software version 3.5.2 (R Development Core Team, 2018).

2.3 Results and Discussion

2.3.1 Physicochemical properties

Saba banana, same as other climacteric fruits, is harvested at mature green stage and then allowed to ripen naturally or artificially. One of the most noticeable changes during ripening process is in the peel color which acts as a simple indicator of fruit maturity. During ripening of Saba banana, lightness (L*) values slightly increased while hue angle (h°) significantly decreased as the peel changed from mature green in unripe stage to brownish-yellow color in ripe stages (Table 2.1). The presence of brown flecks at the last stage resulted in a further drop in the values of h° and L*. No definite trend in the values of chroma (C) means that during ripening, different colors are present simultaneously since the green color of chlorophyll pigment is degraded and at the same time, the characteristic yellow color of carotenoid is synthesized (X. Fu et al., 2018), coupled with a build-up of diverse types of anthocyanins (Adebayo, Hashim, Abdan, Hanafi, & Mollazade, 2016). The enzyme, chlorophyllase, is responsible for the breakdown of chlorophyll which is known to increase in activity on the rise of climacteric phase (Verma et al., 2017). Aside from color, other physicochemical properties used to confirm the stages of banana were pH, TA, moisture content, total soluble solids (TSS), and sugar and starch contents. With the formation of organic acids, specifically malic and citric acids, pH and titratable acidity decreased and increased, respectively, during ripening (Lustre et al., 1976) . The moisture content was found to increase (Table 2.1) during ripening due to respiratory breakdown of starches and osmotic transfer from peel to pulp (Mohapatra, Mishra, & Sutar, 2010), which could be attributed to sugar accumulation in the pulp. The formation of sugar was manifested in the increasing value of TSS as ripening occurred as well as the changes in sugar contents. Sucrose, glucose, and fructose are the most common sugars accumulated during fruit development and ripening (Seymour, 1993). In this study, it was evident that sugars were present even at the initial stage of ripening. Although no significant changes in sugar

Table 2.1 Physicochemical properties of five maturity stages of Saba banana.

Maturity	MC (%)	TSS (°Brix)	pH	TA (% malic acid)	Gluc (%)	Fruc (%)	Suc (%)	RS (%)	Total starch (%)	L*	C	h°
1	62.31 ± 0.19^{c}	4.20 ± 0.40^{e}	6.66 ±.02^{a}	0.037 ± 0.003^{e}	0.22 ± 0.03^{c}	0.22 ± 0.03^{c}	2.34 ± 0.34^{c}	17.37 ± 0.57^{a}	26.05 ± 0.13^{a}	52.19 ± 2.67^{c}	43.75 ± 3.71^{a}	105.73 ± 1.79^{a}
2	62.29 ± 0.32^{c}	5.15 ± 0.23^{d}	6.41 ± 0.02^{b}	0.043 ± 0.004^{d}	0.39 ± 0.02^{c}	0.39 ± 0.01^{c}	2.55 ± 0.16^{c}	17.08 ± 0.64^{a}	24.28 ± 0.94^{b}	56.38 ± 2.00^{ab}	38.33 ± 2.09^{b}	99.84 ± 2.38^{b}
3	62.27 ± 0.24^{c}	7.80 ± 0.26^{c}	5.89 ± 0.03^{c}	0.060 ± 0.001^{c}	1.87 ± 0.46^{b}	2.15 ± 0.69^{b}	4.68 ± 0.89^{b}	14.00 ± 0.51^{b}	21.82 ± 0.94^{c}	57.50 ± 2.93^{a}	42.22 ± 6.14^{ab}	93.21 ± 1.99^{c}
4	63.19 ± 0.35^{b}	11.70 ± 0.40^{b}	4.96 ± 0.01^{d}	0.157 ± 0.003^{b}	3.57 ± 0.59^{a}	3.98 ± 0.63^{a}	5.35 ± 0.91^{b}	7.61 ± 0.13^{c}	15.49 ± 0.40^{d}	57.69 ± 0.80^{a}	37.81 ± 1.05^{b}	80.83 ± 1.23^{d}
5	64.33 ± 0.27^{a}	16.45 ± 0.23^{a}	4.70 ± 0.01^{e}	0.218 ± 0.002^{a}	3.65 ± 0.37^{a}	3.96 ± 0.37^{a}	7.99 ± 0.91^{a}	5.05 ± 0.40^{d}	14.75 ± 0.61^{d}	53.81 ± 2.67^{cb}	38.48 ± 3.50^{ab}	77.93 ± 1.17^{e}

Moisture content (MC), titratable acidity (TA), glucose (Gluc), fructose (Fruc), sucrose (Suc), resistant starch (RS), and total starch are reported as fresh weight (%). Lightness (L*) is from 0 for black to 100 for white; chroma (C) is calculated as [$C = (a^{*2} + b^{*2})^{1/2}$]; and hue angle (h°) is calculated from the arctangent of b*/a*. Values are means ± standard deviations of three replicates except for moisture content $n = 7$, color, $n = 8$, and resistant and total starch, $n = 4$. Mean values with different letters in the same column indicate significant differences ($p < 0.05$).

contents of stages 1 and 2 were detected, the concentration was found to be increasing and became significant at the onset of stage 3. For all stages, sucrose was found in the greatest amount with more than 3-fold increase in concentration from stage 1 to 5. In contrast, from around 0.2% in the unripe stage, glucose and fructose increased by more than 10-fold at the last stage. The increased percentage of sugars in banana pulp during fruit ripening was primarily due to degradation of starch (Aquino et al., 2016). Starch is the principal component of mature green bananas, which undergoes important chemical changes during postharvest ripening (Aquino et al., 2016; Dadzie & Orchard, 1997; Zhang et al., 2005). The total starch content of Saba banana showed a reduction of more than 40% from stage 1 to 5 which was comparable to the results of previous studies. Mohapatra et al. (2010) reported that in triploid hybrid group of bananas, starch content decreased from 20–30% to 1–2% during ripening. Moreover, Aquino et al. (2016) studied starch contents of different banana varieties including one from the ABB genomic group, in which Saba banana is categorized, and showed starch contents of 27.93% and 13.33% for unripe and ripe stages, respectively. Though the pattern of

starch degradation may vary significantly among banana varieties, soluble sugars, mainly sucrose, accumulate following the starch breakdown during ripening (Cordenunsi-Lysenko et al., 2019). In the present study, more than 70% of resistant starch was rapidly degraded enzymatically at the end of storage. The transformation of starch to sugars is catalyzed by the increased activity of several starch degrading enzymes during ripening such as alpha- and beta-amylases, phosphorylases, and debranching enzymes (H. Gao, Huang, Dong, Yang, & Yi, 2016; Seymour, 1993; Verma et al., 2017; Zhang et al., 2005).

2.3.2 Sugar contents and their behaviors during simulated digestion

The study observed the changes in sugar contents of homogenized slurry and unhomogenized cut Saba banana during simulated *in vitro* digestion in terms of glucose, sucrose, and fructose concentrations (Figure 2.2). The variations in the structure of slurry and cut samples showed an effect in the sugar profile even at the initial phase of simulated digestion. From 0 to the end of gastric digestion, an increase in the concentrations of glucose and fructose while a sharp decrease in sucrose content were observed in all maturity stages. Glucose (Figure 2.2a) and fructose (Figure 2.2e) of slurry samples were found to increase ranging from 97–165% and 89–147%, respectively, in stage 1 to 5. This trend was also noticed in cut samples (Figure 2.2b,f); however, comparing from slurry counterpart, lesser increment was observed with percent difference values ranging from 40–96% in glucose and 10–88% in fructose. Sucrose, on the other hand, showed a percent decrement ranging from 17–69% in slurry (Figure 2.2c) and 19–36% in cut (Figure 2.2d). During the gastric conditions, acid catalyzes the hydrolysis of sucrose chemically (Miloski, Wallace, Fenger, Schneider, & Bendinskas, 2008). The mechanism behind acid-catalyzed sucrose hydrolysis involves protonation to the oxygen of the glycosidic bond followed by fructosyl-oxygen bond cleavage forming D-glucose and a fructose carboxonium ion, which can react with water to form D-fructose (Edye & Clarke, 1998). The varying contents of sugars between slurry and cut samples could be due to their different physical structures; the former having damaged or disrupted cells brought about by homogenization process while the latter had intact tissue/ cell structure. The disrupted cells in slurry samples facilitated the immediate release of sugars making them more available for reaction or hydrolysis, whereas in cut samples, sugars are enclosed in cell walls which may possibly resist acid disruption in the gastric phase of digestion. The addition of small intestinal enzymes such as invertase, which is responsible for the breakdown of sucrose to glucose and fructose monosaccharide units in the intestinal phase of digestion (White, 2014), showed more prominent changes in the sugar contents of slurry and cut Saba banana. More than 94% of

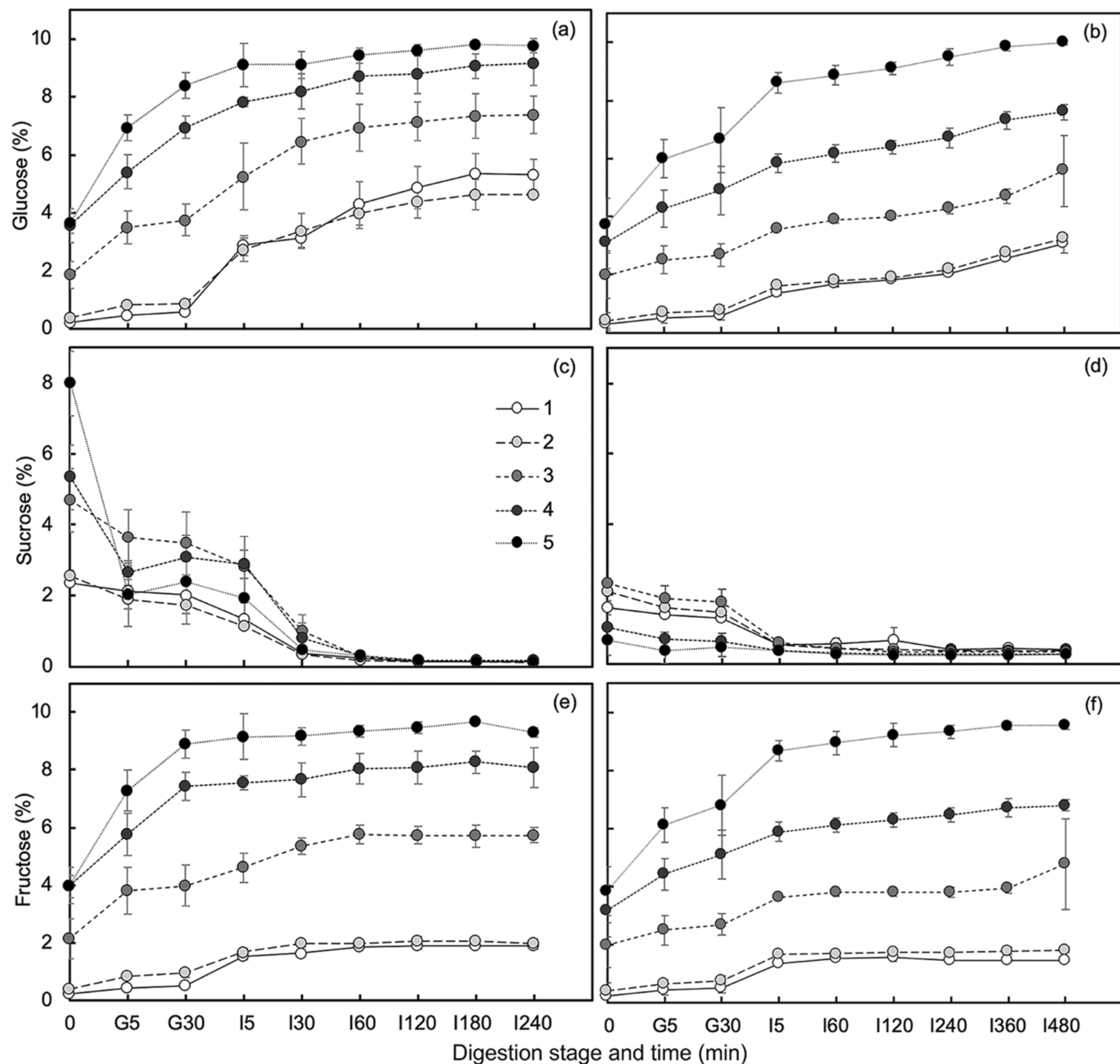

Figure 2.2. Release behaviors of sugars (%) in different maturity stages of slurry (a, c, e) and cut (b, d, f) Saba banana during simulated digestion. Error bars represent standard deviation (*n* = 3).

sucrose was hydrolyzed in all stages of slurry sample. In contrast, after 240 min of intestinal digestion, cut samples exhibited a lower percent decrement in sucrose concentration ranging from 63–85% (stage 1 to 5). Almost the same values were observed when cut samples continued until 480 min of intestinal digestion. The lower percent decrement could be accounted to slow release rate of sugars and hindered penetration of digestive enzymes into the intact tissue of the sample. Based on the initial and final values of glucose and fructose, the green mature Saba banana (stage 1) showed the highest percent increment in both slurry (25-fold in glucose and 8.5-fold in fructose) and cut (10-fold in glucose and 6.5-fold in fructose)

states, and a decreasing trend was observed at increasing maturity. Aside from sucrose, starch is also hydrolyzed which contributed to the increased glucose accumulation during the simulated intestinal phase of digestion.

2.3.3 Combined effect of maturity and physical properties of digesta on starch hydrolysis

A significant decrease in starch hydrolysis (%) was observed in both homogenized slurry and unhomogenized cut samples as Saba banana proceeded ripening (Figure 2.3). The green unripe stages (stage 1 and 2) showed the highest starch hydrolysis with values of 56% and 44%, respectively, for slurry samples (Figure 2.3a) after 240 min of simulated digestion. Lesser values were obtained in cut samples (Figure 2.3b) with 44% and 40% starch hydrolysis for stages 1 and 2, respectively, even at a longer digestion time of 480 min. The last stage was found to have the lowest starch hydrolysis (%) but was not significantly different from stages 3 and 4. The values of ripe stages (stages 3, 4, and 5) varied ranging from 18–30% in slurry samples while cut showed a hydrolysis value of around 30% (after 480 min of intestinal digestion). Low percent hydrolysis was obtained in cut samples at intestinal digestion time of 240 min which ranged from 12–15% with stage 1 having the highest value. The interplay of different factors in fresh Saba banana could account for the decreasing rate of starch hydrolysis as the fruit ripens. One factor that contributed to the outcome was the differences in the physical characteristics of the digesta. Prior to simulated *in vitro* digestion, digesta viscosity of Saba banana showed increasing values with an increase in fruit maturity. However, as the homogenized slurry passed through the successive stages of the simulated gastrointestinal process, there was a progressive decrease in the viscosity values of the ripe stages. A percent decrement of 73%, 67%, and 3% was observed in viscosity values of stages 5, 4, and 3, respectively, after simulated digestion. This trend could be brought about by the dilution of samples upon addition of gastric and intestinal fluids, possible effect of severe acidic conditions on the digesta, and enzymatic liquefaction of starch. In contrast, all maturity stages of unhomogenized cut samples showed increasing viscosity values due to the continuous release of food components during the simulated digestion process. Nevertheless, the trend of increasing viscosity as fruit ripening proceeded was consistent even after 4 and 8 hr of intestinal digestion for slurry and cut samples, respectively. Though this may be accounted for the different dilution values applied to each stage to come up with initial digesta having the same starch content; however, this may also be explained by the presence of substances in fresh from of Saba banana that under proper conditions could increase the viscosity of the samples such as pectic polysaccharides. A number of reports showed the conversion of water-insoluble

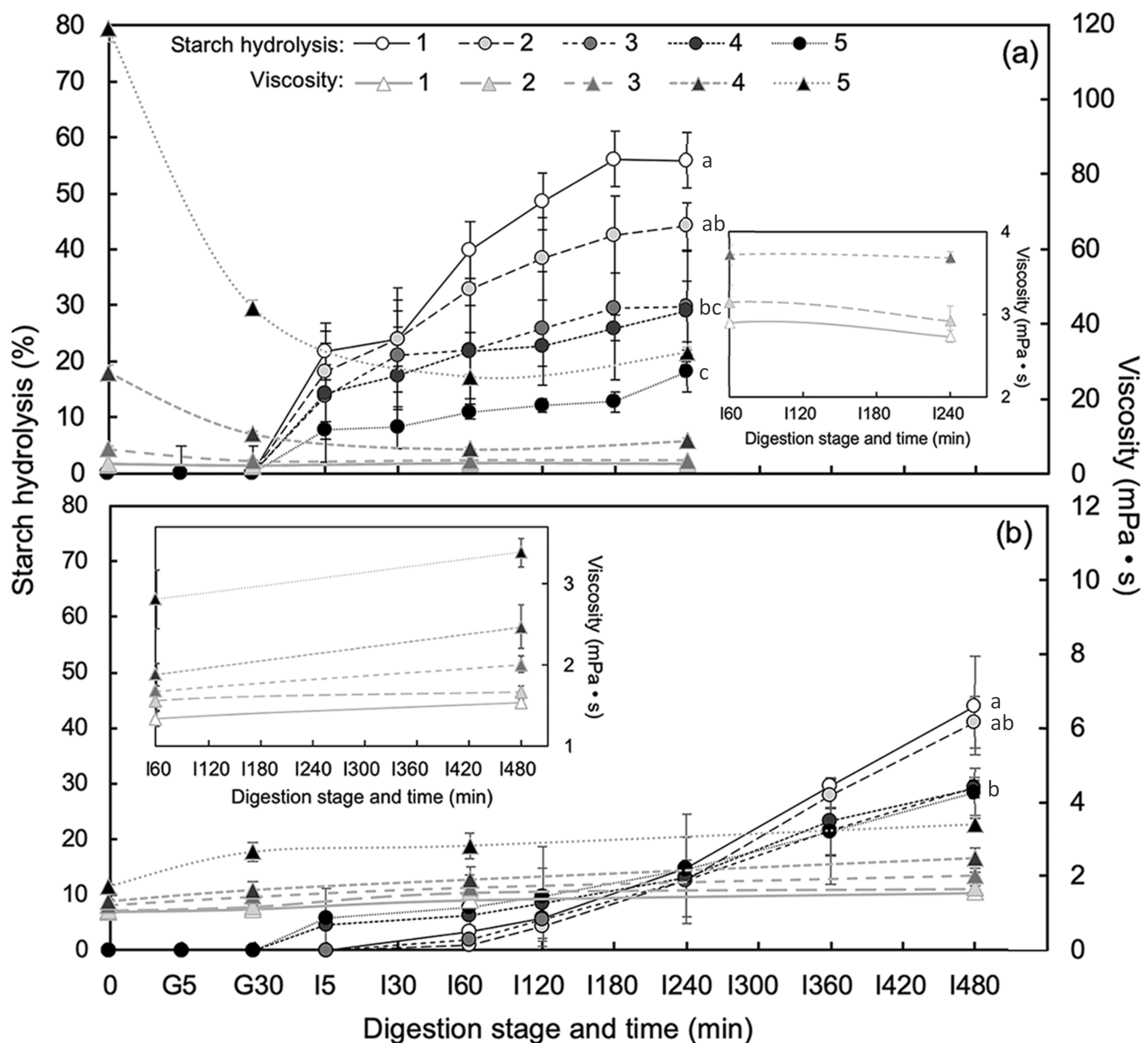

Figure 2.3 Starch hydrolysis (%) and viscosity of digesta (mPa·s) in different maturity stages of slurry (a) and cut (b) Saba banana during simulated digestion. Error bars represent standard deviation (*n* = 3). Mean values with different letters in the same parameter indicate significant differences ($p < 0.05$).

pectin to water-soluble pectin (WSP) during fruit ripening (Duan et al., 2008). Hence, increasing amounts of water-soluble pectins were observed in fruits such as guava (Yusof, Mohamed, & Bakar, 1988), cherry tomatoes (Inari, Yamauchi, Kato, & Takeuchi, 2002), peach (L. Li, Guan, Feng, Ji, & Sun, 2009), plums (Taylor, Rabe, Jacobs, & Dodd, 1995), and banana (Duan et al., 2008) during maturity storage. This was further confirmed by the study of Lustre et al. (1976) which reported increasing pectin content (as calcium pectate) in naturally and chemically-ripened Saba banana pulp during storage.

The concentrations of pectic polysaccharides in the digesta of each sample were determined, ranging from 64–72 mg per 170 g, with stage 1 having the highest value while stage 2 had the lowest. Since the recorded values were almost the same, it can be deduced that their differences could lie on the fractions of pectin present. Based on the results of pectin extraction experiment, the highest water-soluble pectin was observed in ripe stages (3, 4, and 5). This water-soluble pectin from ripe fruit is capable of swelling in water and form gel (Brown, 2018). Corroborating the results of this work, previous study about carrot pectin (Mierczyńska, Cybulska, Pieczywek, & Zdunek, 2015) characterized WSP by having the highest viscosity, particularly in the fourth term of storage, among other fractions (chelate and alkali). In the current study, the increasing sugar and acid during ripening could trigger the gelling reaction with pectin resulting in an increase in viscosity of the digesta and thus low rate of hydrolysis in the ripe stages of Saba banana. The ability to form gels in the presence of sugar and acid and viscosity build-up are the most unique and outstanding properties of pectin (Flutto, 2003; Thakur, Singh, Handa, & Rao, 1997). These independent networks formed by pectin could also embed cellulose/xyloglucan network enhancing its viscoelastic properties (Gawkowska, Cybulska, & Zdunek, 2018). The inhibition of propulsive and mixing effects, which results in less frequent interactions between substrates and digestive enzymes, is the main impact of increased viscosity on starch digestibility (J. Singh, Dartois, & Kaur, 2010). This is turn could affect the glycemic response by reduction in the rate of glucose released and accumulated. It was found that the high resistant starch content of unripe stages of Saba banana had little or no effect on starch digestibility due to the inverse relationship of maturity and starch hydrolysis rate. Though resistant starch contributes to the proportion of starch that escapes from digestion in the small intestine and seems to reduce the accessibility of the substrates to amylases (H. Gao et al., 2016), this type of starch lacks gel-forming effect which is present in non-starch polysaccharides like pectin (Cummings & Englyst, 1995). Therefore, the physical properties of digesta is also a factor that could exhibit an impact on starch digestion rate.

The presence of pectin could also significantly reduce the rate of digestion by its association with starch components and digestive enzymes. The interaction of pectin with amylose and its protective effect on starch swelling and enzymatic hydrolysis were cited as the possible reasons for the increased concentrations of slowly digestible and resistant starches in corn starch, respectively (Ma et al., 2019). Moreover, an association between pectin and amyloglucosidase was observed which changed the digestive enzyme's conformation and thus hindered its access to starch (Bai et al., 2017).

The morphological structure of starch granule could also influence digestibility (Espinosa-Solis et al., 2009; Kaur & Singh, 2016). Scanning electron micrographs of Saba banana starch granule showed small and large spherical and elongated starch granules with lenticular shape for unripe stages (Figure 2.4). As maturity advanced, a decrease in the number of small granules was observed and starch was contorted to an elongated structure which was apparent in the last stage of maturity. These findings were similar to those found by Soares et al. (2011) which reported that starch granules of dessert bananas were predominantly small and leaf-like while plantain had both small and elongated granules at the green unripe stage. As a result of starch degradation during ripening, the small granules in plantain disappeared but the elongated shape of starch granules remained in both types. This particle size and shape of starch may also play an important role for digestibility as small and round granule sizes have been reported to have higher enzymatic susceptibility (Cordenunsi & Lajolo, 1995; Romano et al., 2016).

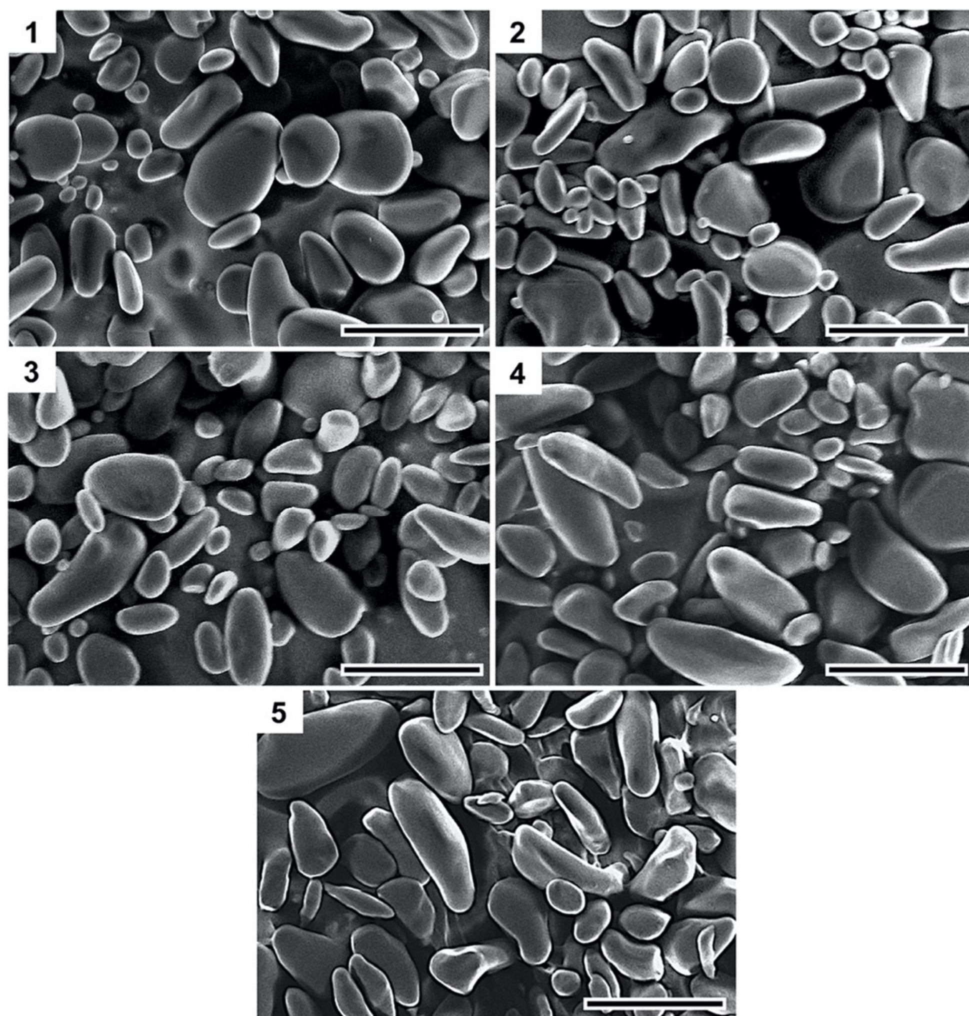

Figure 2.4. Morphological observation of starch granules from different maturity stages of Saba banana. Scale bar=50µm.

Additionally, it has been reported that food structure is one of the major determinants of the rate of starch digestion (Hoebler et al., 1998; Ogawa et al., 2018; Wong & O'Dea, 1983). The physical structure could be an intrinsic factor that hindered the access of digestive enzymes and slow down the degradation of starch (Cummings & Englyst, 1995) from the food matrix of Saba banana. Therefore, modifications in its structure and composition through processing might be reasonably expected to have a significant effect on starch digestibility. The same condition applies during the oral phase of digestion, wherein mastication could break down the particles of food into small pieces. The process increases the surface area of food particles, promoting the ingress of digestive enzymes and liberating starch that is then rapidly digested. However, there are limits to the capacity of humans to reduce the particle sizes of foods during mastication (Dhital et al., 2016) and no endogenous enzymes present in the human upper gut can degrade plant cell walls (Tydeman et al., 2010). Thus, depending on the initial structure of food (Hoebler et al., 1998), the degree of mastication (Ogawa et al., 2018), and the particle size of food materials (Dhital et al., 2016), lesser physical degradation (i.e. incomplete comminution and larger particles) occurring during oral digestion could increase the probability of survival of some cells with structures still remained intact and components encapsulated by cell walls. When cells are not thoroughly disrupted, the intact cell wall could act as barrier to decomposition during gastric and small intestine passage and thus the starch that remained inside the structure is not immediately hydrolyzed. The only way enzymes could penetrate the food is by diffusion through the intact cell walls; however, this penetration effects of digestion is relatively a slow process (Mishra et al., 2012). Consistent with this expectation, in this study, it was found that green mature Saba banana (stage 1 and 2) of slurry had significantly higher starch hydrolysis at a short intestinal digestion time of 240 min than cut samples which were digested at 480 min. This trend was also observed in the ripe Saba banana samples (stage 3, 4, and 5) when compared at the same digestion time of 240 min (18–30% for slurry and 13–15% for cut). The extension in the digestion time of cut samples to 480 min brought an increment of 14%, 16%, and 17% in stages 5, 4, and 3, respectively. This increase in starch hydrolysis may also be observed in slurry samples if digestion time was extended; however, the possibility of the effect of high digesta viscosity would limit the enzyme accessibility to starch polymers and thus may result in a low hydrolysis value.

The starch hydrolysis values presented in the current study were in fresh form of Saba banana. Processing methods by applying heat such as boiling could also have an effect on the digestibility of starch. In a previous *in vitro* study about digestibility of raw and cooked banana starch and flour, Bi et al. (2017) reported a significant increase in digestibility after cooking

for 10 min in a boiling water bath. The cooked starches showed digestion rates of nearly 80% which were much higher than those of flours with approximately around 40–60%. Same reason with that of the fresh form, the presence of other components in banana flour could prevent the starch gelatinization process, resulting in their low digestion rates.

2. 4 Conclusion

The combined effect of physical properties and physicochemical changes during maturation could account for the variations in starch digestibility of different stages of Saba banana. Among the physical properties discussed in this study that significantly affect digestibility were viscosity and physical structure. The observed increase in viscosity could be attributed to food component that is capable of forming a gel, such as water-soluble pectin, and its interaction with water, sugar, and acid, which all have shown to increase as the fruit proceeds ripening. The differences in physical structure could correspond to the degree of particle breakdown during mechanical processing or oral mastication. Highly viscous digesta was found to have a low impact on starch digestibility due to the immobilization of digestive components. The same effect was observed in food samples with intact cell structure as this hindered the action of enzymes. Both high viscosity and presence of intact cell walls may offer physiological advantages in preventing the sudden surge in *in vivo* postprandial blood glucose level upon ingestion of banana. However, this study needs further investigation before conclusive evidence can be obtained as various factors are synergistically involved in the starch digestibility of this variety of banana.

CHAPTER 3

Bio-properties of Saba banana (*Musa 'saba'*, ABB Group): influence of maturity and changes during simulated *in vitro* gastrointestinal digestion

3.1 Introduction

Owing to its increasing demand and whole-year availability, banana is considered as one of the world's most important fruit crops in terms of commercial production and one of the most consumed staple food commodities after major cereals (Raut & Ranade, 2004; B. Singh et al., 2016). An estimated 19.2 million tons of banana had been exported globally in 2018 which accounted for the strong supply growth from the two leading exporters, Ecuador and the Philippines (Food Agriculture Organization of the United Nations, 2019). Among the economically important banana cultivars in the Philippines, foremost of which is Saba (*Musa 'saba'*[*Musa acuminata* × *Musa balbisiana*]), an ABB genome group, which accounts to more than 25% of the country's banana production. This banana variety can be utilized at all stages of maturity either raw or cooked and is used primarily for manufacturing various food products such as condiments (banana ketchup), snacks (banana chips), viands, and desserts (Doloiras-Laraño et al., 2018). Saba banana is also relished in other Southeast Asian countries (T. K. Lim, 2012) but is gaining popularity in Latin America (B. Singh et al., 2016) and Southern Nigeria (Olawoye et al., 2017).

Banana is not only an important source of starchy staple food but could also exert a beneficial effect on human health. It contains phytonutrients, including vitamins and different classes of phenolic compounds (Bennett et al., 2010; B. Singh et al., 2016). In plants, phenolic compounds may exist in free, soluble conjugated (esterified), and insoluble-bound forms (Y. Gao, Ma, Wang, & Feng, 2017; Shahidi & Yeo, 2016). Research studies have shown that bound phenolics have demonstrated a significantly higher antioxidant capacity compared to free phenolics in numerous *in vitro* antioxidant assays carried out (Liyana-Pathirana & Shahidi, 2006). Insoluble-bound phenolics are localized in the cell wall matrix of plant cells which are covalently bonded to cell wall components such as cellulose, pectin, and structural proteins (Shahidi & Yeo, 2016) and can be released after acidic or alkaline hydrolysis (Y. Gao et al., 2017). The beneficial effect of these phenolic compounds as a source of natural antioxidants is associated with health-protective properties. The protection mechanism is generally by inhibiting the formation of free radical species and repairing oxidative damage, thus preventing

the development of various chronic and degenerative diseases (Isabelle et al., 2010; Sulaiman et al., 2011).

The antioxidant capacity of fruit is determined by a mixture of various antioxidant compounds with different action mechanisms; therefore, it is necessary to combine more than one method to provide a broader picture of the antioxidant capacity of foodstuffs (Almeida et al., 2011). The most widely used methods being 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays, among others such as oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP) assay (Floegel et al., 2011), and metal ion chelating (MIC) activity. A well-established parameter to express the antioxidant activity of biological sample as equivalents of standard antioxidant in this respect being Trolox Equivalent Antioxidant Capacity (TEAC) (Arnao, 2000) and Vitamin C Equivalent Antioxidant Capacity (VCEAC) (Floegel et al., 2011).

The bioaccessibility of antioxidant compounds during gastrointestinal digestion is crucial for their absorption and bioavailability. Thus, *in vitro* studies, even though typically constituting of only a static model of digestion, have been developed to allow the holistic understanding of the actual effects of nutritional ingredients on the living body and the changes and release of antioxidant compounds from the food matrix (Hur et al., 2011) as affected by the composition and structural features of food under simulated gastrointestinal conditions. It is a faster and more cost-effective method to simulate the natural digestive process and rapidly screen food products for their estimated biological activity (Germaine et al., 2008). Though it cannot perfectly actualize the highly complex physiological events during digestion, it has been demonstrated that the evaluation of bioaccessibility through *in vitro* models can be well correlated with results from *in vivo* studies and animal models (Bouayed, Hoffmann, & Bohn, 2011), which was patterned after the gastrointestinal digestion conditions of a healthy adult human.

Nutrient bioaccessibility during gastrointestinal digestion process varies for the same food depending on processing conditions and presence of other components (Parada & Aguilera, 2007). Mechanical processes such as grinding or cutting could either disrupt or retain the cellular structure of food (Mishra et al., 2012) which may have an impact on the release and absorption of nutrients. The presence of intact cells in the food matrix has been reported to survive digestion in the upper gastrointestinal tract and that mastication could bring damage to the cells of plants which made nutrients bioaccessible (Parada & Aguilera, 2007). On the other hand, the rise and loss of fruit components or attributes (cell integrity, acids, sugars, pectin) during ripening (Maieves et al., 2015; Tareen, Abbasi, & Hafiz, 2012) may also bring

a significant effect on the transition of food compounds during digestion. However, not many studies have determined how varying physical structures and maturation changes in fruits affect the release of bioactive compounds during digestion.

The bio-properties of Saba banana have been the subject of limited studies that focused mainly on the ripe stage of maturity (Borges et al., 2014) and the content of few extractable free phenolics, ignoring the bound fractions (Sulaiman et al., 2011). Subsequently, a comprehensive review by B. Singh et al. (2016) summarized the previous researches on bioactive compounds of different banana cultivars including ABB genome group of Saba banana. However, insufficient data exist on the bio-properties of mature unripe counterpart and the content of bound phenolics. The analysis of bound phenolics will provide a better estimate of the food's actual contribution on biological activities, and the determination of the antioxidant capacity of mature green stage will present a basis in the selection of optimum maturity stage that may have great potential as a source of bioactive compounds for eventual production of functional products. Moreover, since Saba banana has become one of the important fruit crops for consumption, observation of the digestive fate of its compounds is important to assess their chemical and physical stability in the varying conditions of the gastrointestinal tract.

Given the above, this study aimed to determine the variations in free and bound phenolics, flavonoid, and antioxidant activities of different maturity stages of Saba banana and investigate the changes undergone by phenolics and antioxidant activities during simulated *in vitro* gastrointestinal digestion. The effect of varying physical structures of food was also evaluated through the preparation of homogenized slurry and unhomogenized cut samples representing structure-less and intact cellular structure, respectively. Additionally, the comparability of DPPH and ABTS assays, expressed as TEAC and VCEAC values, was also evaluated. The study provided information on the quality attributes of Saba banana in terms of its biochemical properties and its potential health benefits upon subjecting to the physical, enzymatic, and chemical processes of simulated digestion.

3.2 Materials and Methods

3.2.1 Chemicals and reagents

All chemicals and reagents were of analytical grade and were obtained from various commercial sources. Sigma-Aldrich Ltd. (St. Louis, MO, USA) provided the enzymes used in simulated *in vitro* gastrointestinal digestion such as pepsin (porcine gastric mucosa, >250 U mg^{-1} solid), pancreatin (hog pancreas, 4x USP), and invertase (grade VII from baker's yeast, >300 U mg^{-1} solid); chemicals used in antioxidant activity determination which included 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate (FerroZine); $FeCl_3$, and standards such as gallic acid monohydrate and (+)-catechin hydrate. Wako Pure Chemical Industries, Ltd. (Tokyo, Japan) provided $FeCl_2$, $FeSO_4$, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) and Trolox. Megazyme International Ltd. (Wicklow, Ireland) provided amyloglucosidase (3260 U mL^{-1}) while Dojin Chemical Laboratory Co., Ltd. (Tokyo, Japan) supplied ethylenediaminetetraacetic acid disodium salt (EDTA).

3.2.2 Sample and preparation

Saba bananas were purchased from Diamond Star Agro-Products Inc., Taguig City, Philippines. The fruit was received in mature green stage and was kept for ripening at 23 ± 1 °C in an incubator (MIR-153, Sanyo Electric Co., Ltd., Japan). Five maturity stages were selected based on the physicochemical properties reported in the previous study (Reginio, Ketnawa, & Ogawa, 2020). At least 7 Saba banana fingers from each of the following stages: (1) green; (2) green but turning yellow; (3) greenish yellow; (4) yellow with green tips; and (5) yellow with brown flecks, were peeled, sliced, and divided into different portions. One part was frozen in liquid N_2, freeze-dried (Eyela FDU-1100, Tokyo Rikakikai Co. Ltd., Japan), ground, and passed through a 0.5 mm mesh sieve (Sanpo, Sanyo, Japan) for analyses of bio-properties. The remaining samples were used for simulated *in vitro* gastrointestinal digestion with varying physical states, homogenized slurry and unhomogenized cut samples. The former was homogenized using a household blender (NM200, Yamazen, China) for 2 minutes while the latter was prepared by manual and mechanical cutting (dimension of ca. $\leq$3 mm) using a food chopper (Tefal, Rumilly, France). Both states were combined with water prior to simulated digestion to have the same amount of starch content (4%).

3.2.3 Extraction of free and bound phenolics

Free and bound phenolics were extracted using the method reported by Sumczynski, Kotásková, Družbíková, and Mlček (2016) with slight modification. Briefly, 1 g of freeze-dried sample was treated twice with 8 mL of 80% aqueous methanol in a water bath at 37 °C for 1 h. The supernatants obtained from the initial extraction and the subsequent washing were collected, combined after centrifugation at 4000 × *g* for 25 min, and adjusted to pH 4.5–5.5 using 6 M HCl. The resulting solution was used to directly determine free phenolics. For bound phenolics, the residues obtained from extracting free phenolics were used. The samples were rewashed using 20 mL of distilled water. After removing water, samples were homogenized twice with 20 mL of 4 M NaOH and sonicated for 2 h in an ultrasonic bath. The pH of the mixture was then adjusted to 4.5–5.5 using 6 M HCl. The supernatant was collected after centrifugation at 4000 × *g* for 25 min.

3.2.4 Determination of bio-properties

All spectrophotometric determinations were done using a Multiskan FC microplate reader (Thermo Fisher Scientific, MA, USA). Assays were performed and read in 96-well microplates. The plate contained 3 to 4 repetitions per sample and blank with 5 to 6 levels of standards.

3.2.4.1 Total phenolic content (TPC)

TPC was determined using Folin-Ciocalteu reagent according to ISO 14502–1 (International Organization for Standardization, 2005) with some modifications. Briefly, 25 µL of the extract was mixed with 125 µL of 10% (v/v) Folin-Ciocalteu reagent and 100 µL of 7.5% (w/v) Na_2CO_3. The mixture was allowed to stand for 1 hour in the dark at room temperature. After incubation, the absorbance was measured at 740 nm versus a prepared blank. The blank consisted of distilled water instead of sample. Gallic acid was used as a reference standard for plotting the calibration curve (0–125 ppm). The total phenolic contents were determined from the linear equation of the standard curve, expressed as mg gallic acid equivalents per 100 g of fresh weight (mg GAE 100 g^{-1} FW).

3.2.4.2 Total flavonoid content (TFC)

TFC was determined using aluminum chloride colorimetric method (Zhishen, Mengcheng, & Jianming, 1999) with some modifications. Briefly, 33 µL of the extract was diluted with 133 µL of distilled water, and 10 µL of 5% (w/v) $NaNO_2$ was added. After 5 min, 10 µL of 10% (w/v) $AlCl_3$ was reacted to the mixture and incubated for another 6 min. Then, 67 µL of 1 M NaOH was added and the total volume was adjusted to 333 µL by mixing 80 µL

of distilled water. The mixture was mildly shaken and the absorbance was measured at 520 nm against the blank (distilled water). A calibration curve was constructed with different concentrations of catechin as standard (0–100 ppm). TFC was expressed as mg catechin equivalents per 100 g fresh weight (mg CE 100 g^{-1} FW).

3.2.4.3 Ferric-reducing antioxidant power (FRAP)

FRAP capacity was carried out using the method of Benzie and Szeto (1999) with some modifications. The sample (20 μL) was mixed with 130 μL FRAP reagent containing acetate buffer (300 mM pH 3.6), TPTZ solution (10 mM), and $FeCl_3$ (20 mM) in a 10:1:1 (v/v/v) ratio. The mixture was shaken and incubated at 37 °C for 30 min away from light. The absorbance was measured at 595 nm with distilled water as blank. A calibration curve was constructed with standard solutions of $FeSO_4$ (0–500 μmol/L) and the results were expressed as μmol $FeSO_4$ equivalent per 100 g FW.

3.2.4.4 Metal ion chelating (MIC) activity

MIC activity on Fe^{2+} was estimated using the method of Dinis, Madeira, and Almeida (1994) with some modifications. The diluted extract (300 μL) was mixed with 5 μL of 2 mM $FeCl_2$ and 10 μL of 5 mM FerroZine. The mixture was gently shaken and left standing at room temperature for 10 min. Absorbance was measured at 560 nm with distilled water as blank. A standard curve of EDTA (0–30 μmol/L) was prepared and the chelating activity was expressed as μmol EDTA equivalent per 100 g FW.

3.2.4.5 $DPPH^{\bullet}$ scavenging activity (DPPH)

$DPPH^{\bullet}$ scavenging activity was determined according to Molyneux (2004). Sample (5 μL) was mixed with 195 μL methanolic solution of $DPPH^{\bullet}$ (60 μM). The mixture was gently shaken, kept in the dark, and left to stand at room temperature for 30 min. Thereafter, the absorbance was measured at 520 nm against a methanol blank. The $DPPH^{\bullet}$ scavenging activity was calculated based on the calibration curves of Trolox (0–1000 μmol/L) and Vitamin C (0–100 ppm). The results were expressed as μmol Trolox equivalent (TE) and mg Vitamin C equivalent (VCE) antioxidant capacities (TEAC and VCEAC, respectively) per 100 g FW.

3.2.4.6 $ABTS^{\bullet+}$ scavenging activity (ABTS)

$ABTS^{\bullet+}$ scavenging activity was determined according to the method of Ketnawa, Suwannachot, and Ogawa (2019) with some modifications. $ABTS^{\bullet+}$ solution was prepared by the reaction of 7 mM $ABTS^{\bullet+}$ dissolved in 2.45 mM potassium persulfate and left standing in the dark at room temperature for 12 h before use. The $ABTS^{\bullet+}$ solution was diluted with distilled water to obtain an absorbance of 0.7 ± 0.02 at 740 nm. The actual assay was initiated

by combining 10 μL of the sample with 320 μL of diluted $ABTS^{•+}$ solution. The $ABTS^{•+}$ scavenging activity was measured at 740 nm after 10 min incubation at 30 °C in the dark with distilled water as blank. Same as $DPPH^{•}$ activity, reference substances (Trolox and Vitamin C), were allowed to react with the $ABTS^{•+}$ solution to determine TEAC and VCEAC values, respectively.

3.2.5 Simulated *in vitro* gastrointestinal digestion

Two-stage simulated *in vitro* gastrointestinal digestion model as described by Ketnawa et al. (2019) with some modifications was employed (Figure 3.1). The sample, weighing 170 g, was directly poured into a jacketed glass reactor connected to a circulating water bath (Eyela NTT-20S, Tokyo Rokakikai Co., Ltd., Japan) maintained at 37 ± 1 °C. The sample was digested for 60 min in the simulated gastric phase which was initiated by adjusting the pH to 1.2 ± 0.1 using different molar concentrations of HCl and addition of simulated gastric fluid containing 0.12 g pepsin and buffer with 0.2% (w/v) NaCl (pH adjusted to 1.2). Then, simulated small intestinal digestion phase proceeded by changing the pH to 6.8 ± 0.1 using different molar concentrations of NaOH and addition of simulated intestinal fluid containing 0.1 g pancreatin, 7.5 mg invertase, 2 mL amyloglucosidase, and buffer with 0.68% (w/v) monobasic potassium phosphate (KH_2PO_4) (pH adjusted to 6.8). Aliquots of mixture (1 mL) were collected at time 0 (before the start of digestion), 5 (G5), 30 (G30), and 60 min (G60) in the gastric phase; and after 5 (I5), 60 (I60), 120 (I120), 180 (I180), and 240 min (I240) in the small intestinal phase for slurry samples while cut samples were continued until 480 min (I480). Supernatants were added with 2 mL of 95% ethanol to stop the enzymatic reactions, centrifuged at 1800 × *g* for 10 min, and stored at −40 °C until further analysis. TPC and antioxidant activities were determined, the same methods as reported above.

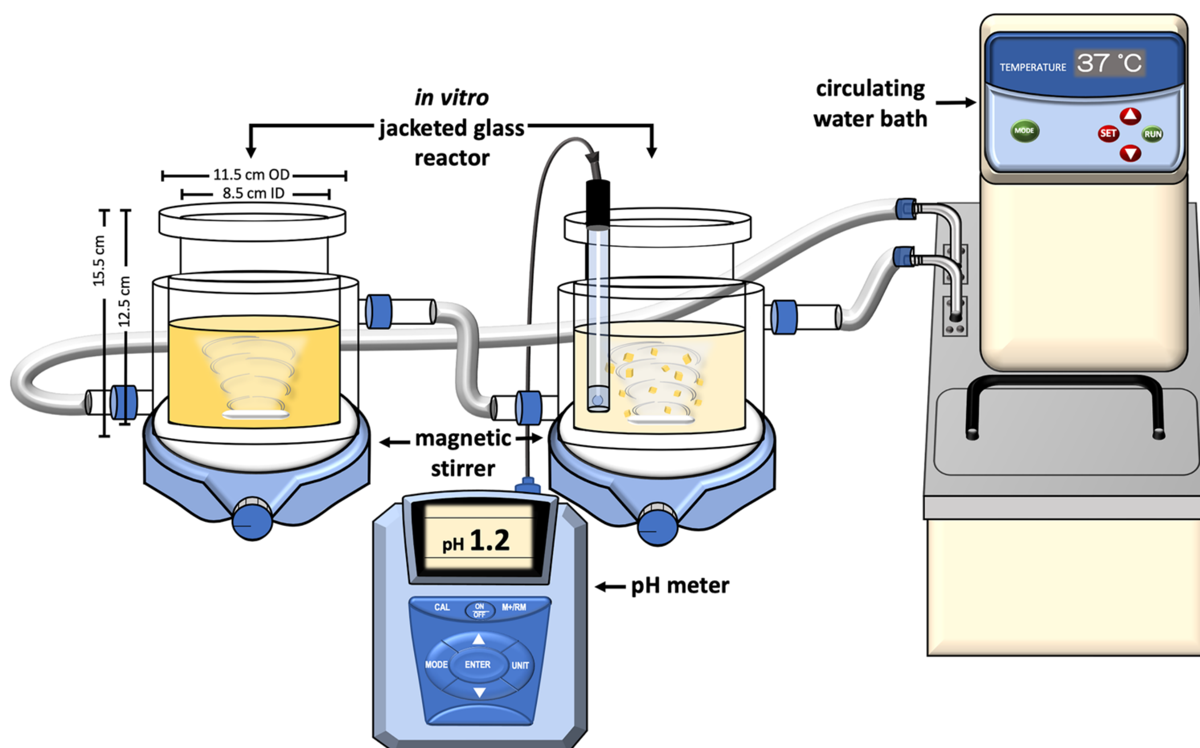

Figure 3.1 Simulated *in vitro* gastrointestinal digestion system set-up.

3.2.6. Statistical analysis

Statistical analysis was performed using R software version 3.3.3 GUI 1.69 Mavericks (R Development Core Team, 2018). Results were presented as mean ± standard deviation. Data were analyzed using t-test and one-way analysis of variance (ANOVA) and significant differences existed between mean scores were determined using Tukey's procedure set at $p < 0.05$. Correlation analysis based on Pearson's method was performed among the variables.

3.3 Results

3.3.1 Bio-properties of fresh Saba banana during maturation

A decrease in total phenolic content (TPC) and total flavonoid content (TFC) was observed as maturity progressed, with significant differences between the first and last stages (Figure 3.2a). From 109.06 ± 2.11 mg in the mature green stage, TPC decreased to 103.47 ± 1.02 mg in stage 5; however, no significant difference was observed between stage 1 and middle stages (2, 3, and 4). TFC also showed no significant difference among the initial stages (1, 2, and 3) and accounted on average for 18–26% of TPC ranging from 19.42 ± 1.55 mg to 28.70 ± 1.41 mg. Bound phenolics were on the average around 3-fold higher than free fractions. Similarly, around 90% of TFC was obtained from bound fractions.

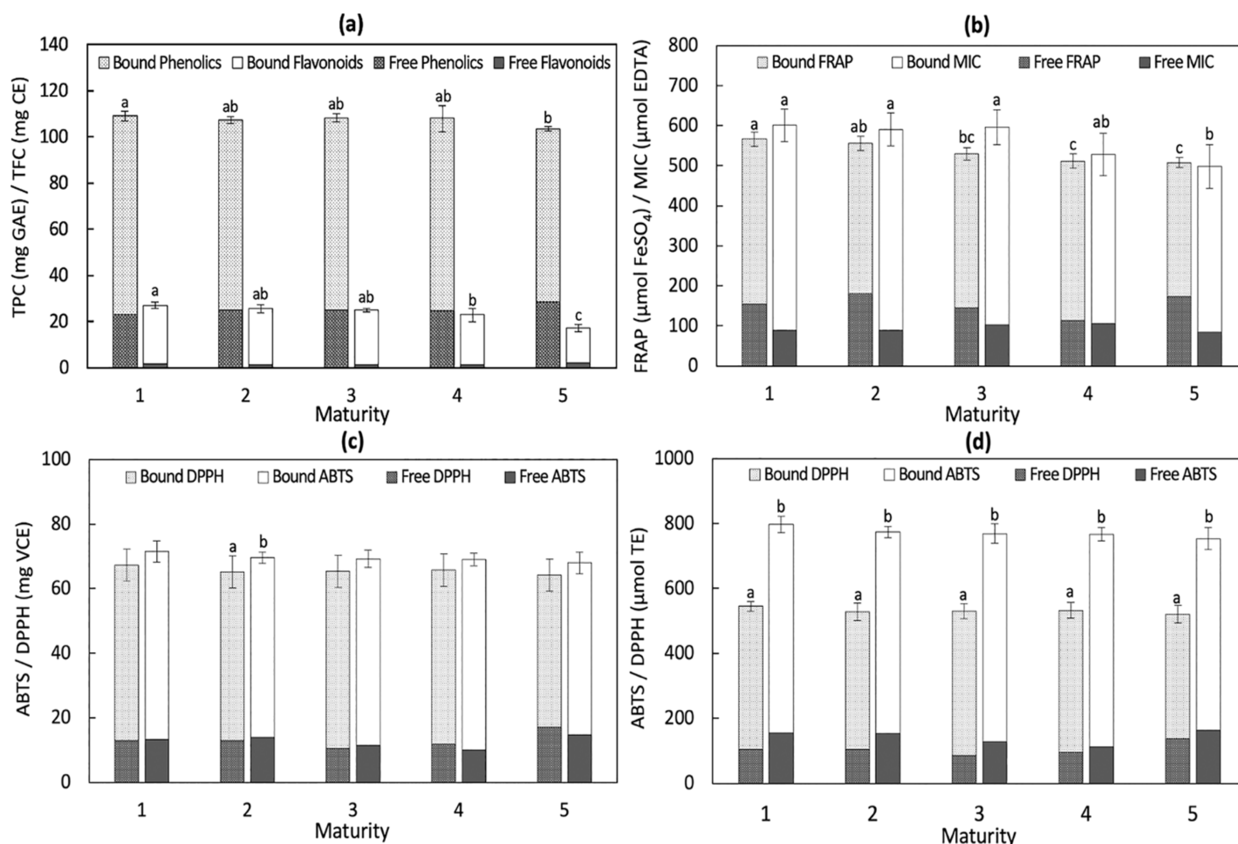

Figure 3.2 Bio-properties of fresh Saba banana at different maturity stages ($n = 5$). For a and b, mean values with different lowercase letters for the same parameter indicate significant differences between maturity stages ($p < 0.05$). For c and d, mean values with different lowercase letters indicate significant differences between antioxidant assays ($p < 0.05$).

Antioxidant activities followed a similar trend to that of TPC as ripening proceeded (Figure 3.2b-d). The antioxidant activity of free phenolic was also found to be lower compared to that of bound phenolic. The Saba banana showed FRAP and MIC values ranging from 498.33 ± 54.35 μmol to 601.04 ± 40.19 μmol. Comparing the two radical scavenging assays, antioxidant capacities by ABTS (753.60 ± 34.24 μmol to 797.5 ± 24.9 μmol TEAC and 68.02 ± 3.39 mg to 71.53 ± 3.24 mg VCEAC) was consistently higher than antioxidant capacities by DPPH (519.98 ± 27.19 μmol to 545.12 ± 14.80 μmol TEAC and 64.11 ± 3.40 mg to 67.25 ± 1.86 mg VCEAC). Significant differences between TEAC of ABTS and DPPH assays were observed while, in general, no significant difference was determined in VCEAC.

3.3.2 Bio-properties of different structural states of Saba banana during simulated *in vitro* digestion

At time 0, stages 1 and 2 of both slurry and cut states of digested Saba banana samples showed the same behavior as that of fresh which found to have higher values of TPC and antioxidant activities than other maturity stages (Figures 3.3 & 3.4). From the initial (time 0) to gastric phase (G60) of simulated digestion, there was on the average a 1.5- to 3-fold increase in the amount of total phenolics in both states with the highest increment observed in ripe stages (Figure 3.3a,b). Following gastric digestion, TPC, in general, had a continuous increase throughout digestion and was released more rapidly in slurry than cut state.

A comparable trend of increasing antioxidant activity values with increasing digestion time during the gastric phase was observed, except for MIC. From time 0 to G60, FRAP values of slurry samples increased more than 2-fold while values for cut samples varied from 1.8- to 4.9-fold (Figure 3.3c,d). Both slurry and cut states were found to have a higher percent increment in ripe stages (4 and 5) than in mature green counterpart. The TEAC values of slurry samples based on the ability to scavenge $DPPH^{\bullet}$ were around 2- to 4-fold higher than that of $ABTS^{\bullet+}$ in the gastric phase while cut samples showed a much wider gap (3- to 46-fold) (Figure 3.4). This trend was consistent even in VCEAC exhibiting a 3- to 6-fold and a 6- to 28-fold greater antioxidant capacity of DPPH than ABTS for slurry and cut samples, respectively. Interestingly, MIC values from time 0 to G60 of slurry samples dropped as maturity advanced (7% decrement in stage 1 to 57% in stage 5) (Figure 3.3e). The cut samples, on the other hand, showed no definite trend (Figure 3.3f).

In contrast with TPC, antioxidant activities were shown to decrease at the onset of intestinal digestion (I5), except for MIC. From G60 to I5, a percent decrement ranging from 12–30% was observed in FRAP and more than 60% in DPPH assay. Surprisingly, different from other maturity stages, ABTS assay values of stage 5 showed a percent increment of 2–12% from gastric to intestinal phase. Similarly, MIC exhibited a reverse trend of results compared to those observed in other antioxidant activity measurements. A sharp increase ranging from 1.6- to 7-fold was detected with the last stage of maturity having the highest increment.

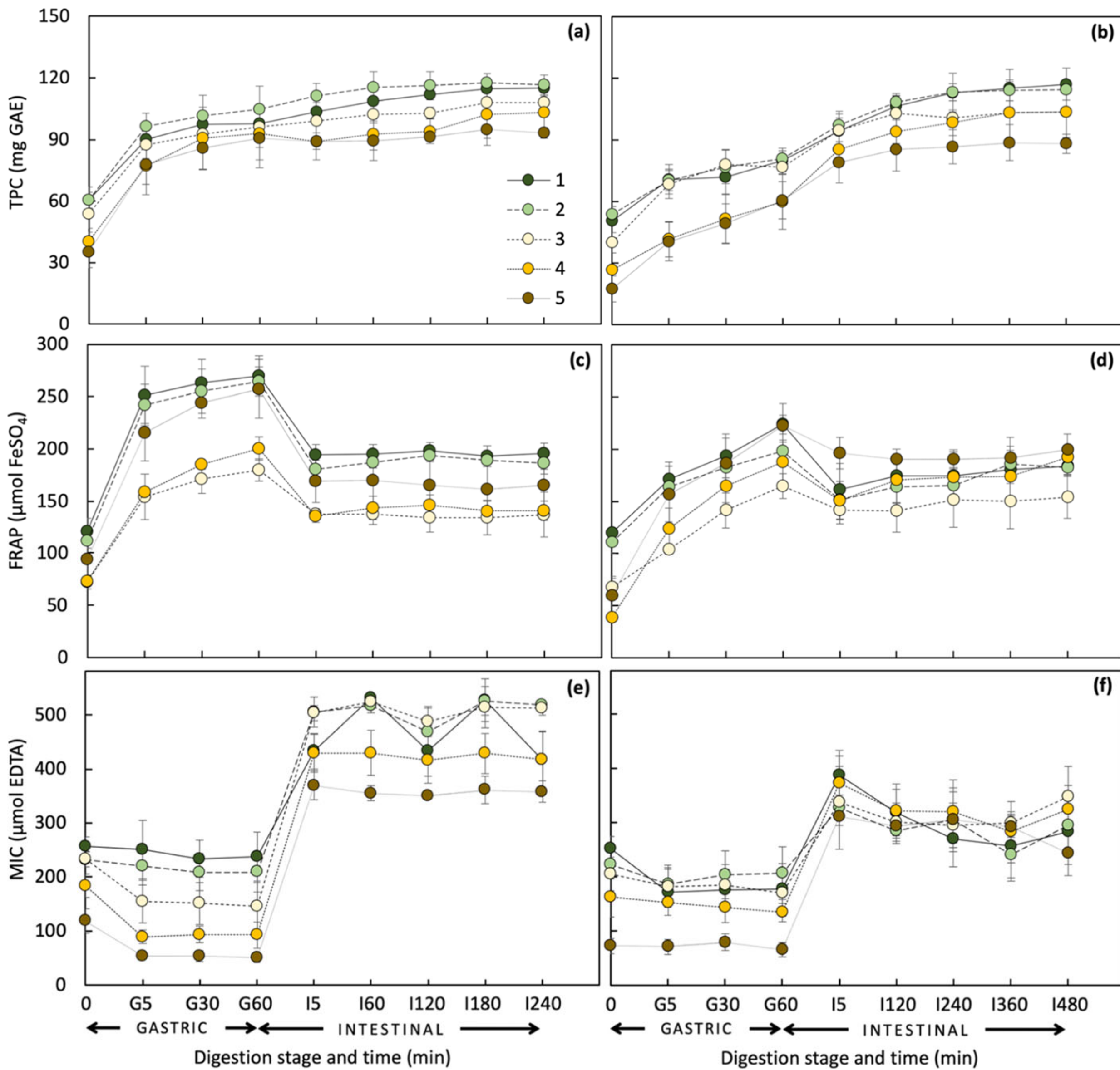

Figure 3.3 TPC, FRAP, and MIC values of digested fractions of slurry (a,c,e) and cut (b,d,f) Saba banana at different maturity stages ($n = 3$).

At the end of simulated digestion (I240 and I480 for slurry and cut samples, respectively), similar to the results of fresh Saba banana, antioxidant capacities detected by ABTS assay of slurry samples from time 0 showed on the average a higher percent TEAC increment (44–305%) than DPPH assay (24–177%) while no significant difference was observed in VCEAC. Both assays had the highest increment in the last stage of maturity. The cut samples, on the other hand, showed a different trend having higher percent increment values of both TEAC and VCEAC in DPPH assay.

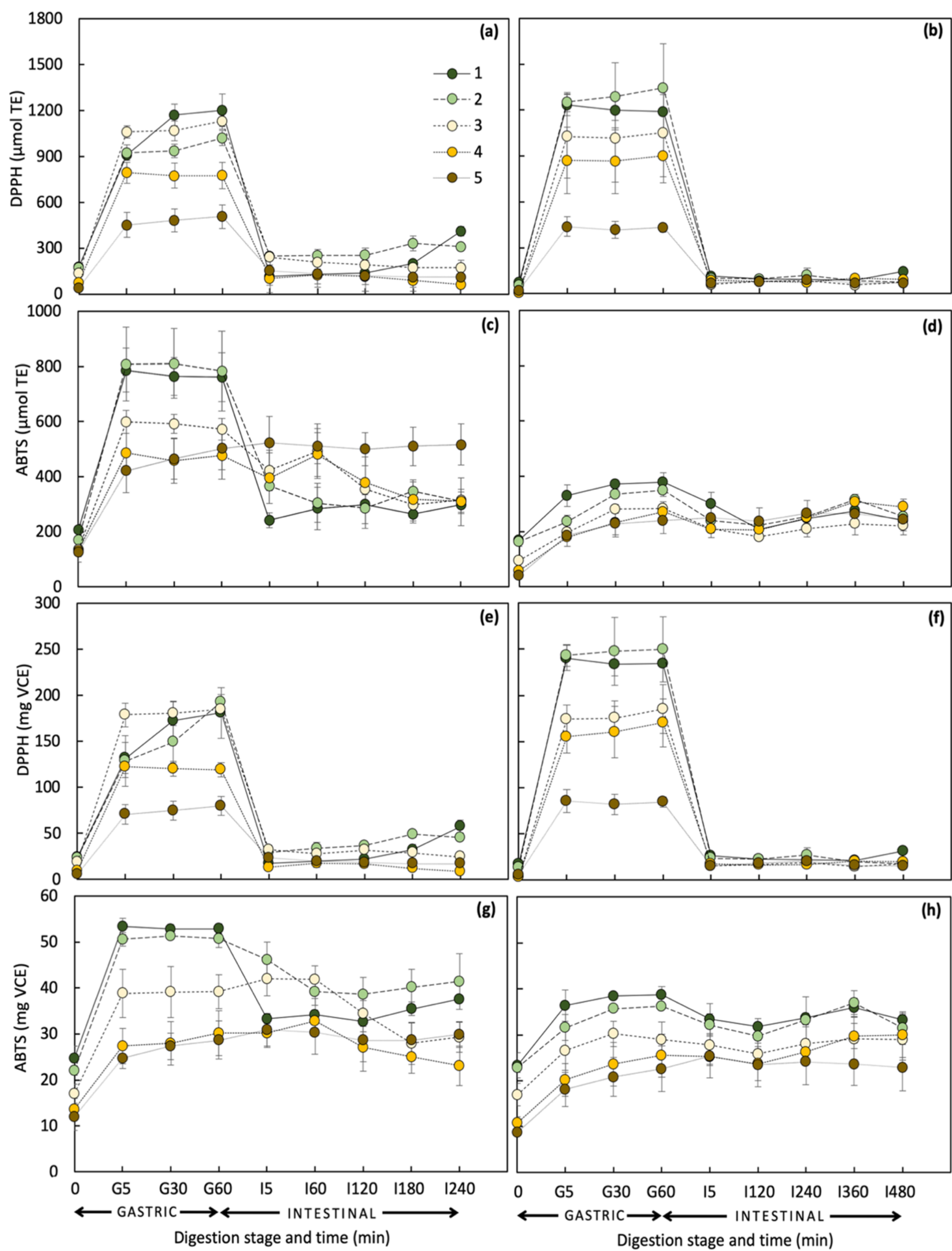

Figure 3.4 TEAC and VCEAC values of digested fractions of slurry (a,c,e,g) and cut (b,d,f,h) Saba banana ($n = 3$).

3.3.3 Correlation analysis

The maturity of fresh Saba banana showed significant negative linear correlation ($p < 0.05$) with TPC, TFC, and antioxidant activities, except for DPPH (Figure 3.5a). The correlation between the release of antioxidant compounds and maturity during *in vitro* digestion in homogenized slurry sample showed almost the same result as fresh with no significant relationship for both TEAC and VCEAC of DPPH assay (Figure 3.5b). However, in unhomogenized cut state, only TPC and TEAC and VCEAC of ABTS were found to have a significant correlation with maturity (Figure 3.5c). In both fresh and digested fractions, only FRAP was found to have a moderate to strong correlation with TPC among the antioxidant activity assays. There were more linear correlations of TPC with antioxidant activity in digested samples than in fresh state; the highest of which was obtained in cut samples. In addition, antioxidant activities showed a positive correlation to each other, except for MIC. TEAC and VCEAC measured by ABTS assay were significantly correlated to antioxidant activities by DPPH assay in digested fractions while no correlation was observed in fresh sample.

3.4 Discussion

The determination of total phenolic content as free and bound forms in Saba banana revealed that, between the two, a major fraction was contributed from bound phenolics. A previous evaluation of banana pulp showed that it contained significant levels of cell wall-bound phenolics such as anthocyanidins (Bennett et al., 2010), quercetin, and cyanidin-3-*O*-glucoside chloride (Dong, Hu, Hu, & Xie, 2016). Since bound phenolics are bound to insoluble macromolecules, they have different pathways and absorption mechanisms in the gastrointestinal tract when compared to that of free phenolics. Free phenolics have partial release in the mouth and absorb in the small intestine; however, bound phenolics move directly to the colon where they undergo fermentation by the gut microbiota, thereby releasing the bound phenolics (Shahidi & Yeo, 2016). Nevertheless, both free and bound fractions are sources of natural antioxidant compounds, as shown by their reactions and activities in different antioxidant assays performed. The observed high phenolic contents and antioxidant activities of mature green stage which then declined as ripening proceeded were comparable to the previous studies (Bennett et al., 2010; Fatemeh, Saifullah, Abbas, & Azhar, 2012; Macheix, Fleuriet, & Billot, 1990). This could be accounted for the different biochemical, physiological, and structural reactions and modifications happen as the fruit undergoes important changes

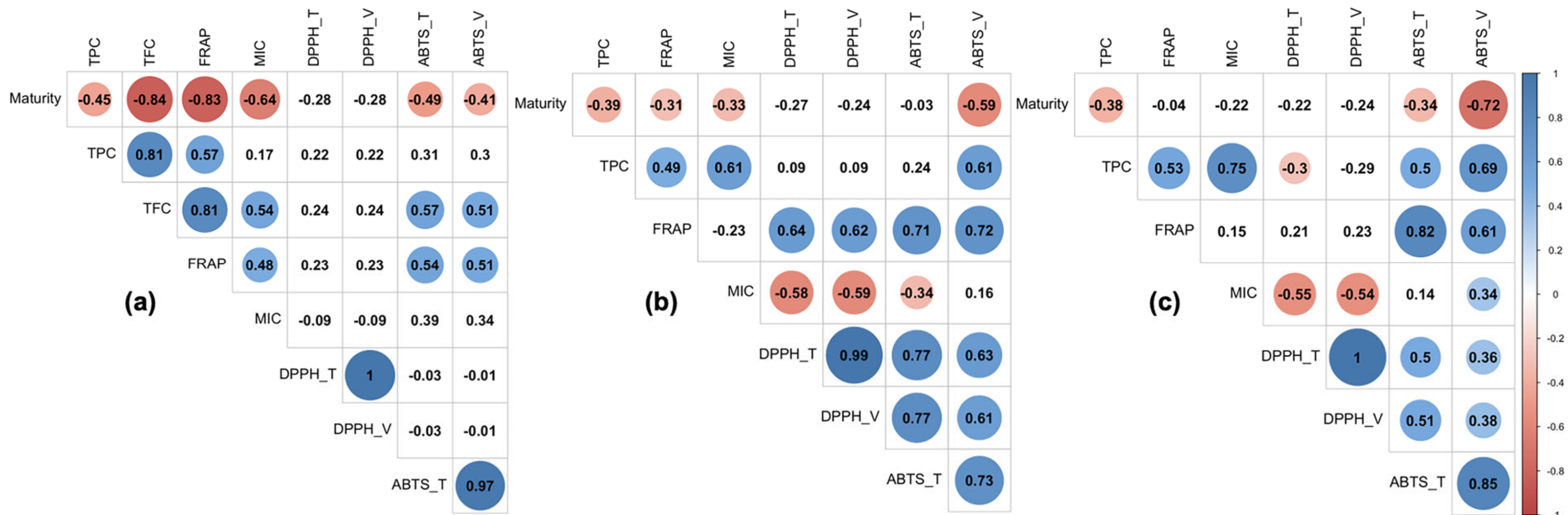

Figure 3.5 Correlation analysis between different parameters ($p < 0.05$) of fresh (a) and digested (slurry [b] and cut [c]) Saba banana. T = TEAC; V = VCEAC.

during maturation process, thus affecting the contents of polyphenols and their antioxidant activities. Such modification could be associated with the oxidation of polyphenols by polyphenol oxidase (PPO) (Rodríguez et al., 2016) which results in polyphenols cross-linked with other polyphenols, carbohydrates, or proteins (Kennedy, Matthews, & Waterhouse, 2000). The activity of PPO during banana ripening has been widely studied and shows varying trends of results in previously conducted researches. Montgomery and Sgarbieri (Montgomery & Sgarbieri, 1975) found a 35% decrease in PPO activity in ripening banana pulp. Giami and Alu (1994), reported around 4-fold increase in PPO with ripening while Young (1965) observed no change in the activities of several banana enzymes as ripening proceeded. Though differences in the previous results are evident, this cannot deny the fact that PPO is still active even during the late stages of ripening which could affect the contents of phenolic compounds in Saba banana during storage.

Fruit maturation involves high metabolic activity which usually requires physiological mechanisms of defense (Maieves et al., 2015). This is important as ripening in most fruits like banana encompasses the conversion of insoluble protopectin into water-soluble pectin, which softens the texture of the fruit and eventually results in cell wall deterioration (Tareen et al., 2012). An important mechanism by which the plants could strengthen their cell walls, providing both physical and chemical barriers, and defend themselves against invasion of pathogens during this period is through the integration of phenolic esters into the cell walls. Additionally, these phenolic esters could also prevent the action of reactive oxygen species on cell membrane damage. These caused the observed decrease in the phenolic acid content of date palm during ripening (Amira et al., 2012), which could also account for the trend of results of this study.

Our study measured antioxidant activities by the ability to scavenge free radicals ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$), chelate ferrous ions (MIC), and reduce ferric iron (FRAP). The results obtained comparing the two free radical assays showed the same trend of change during simulated digestion; however, values of DPPH assay possibly underestimated the antioxidant capacity and ABTS assay showed better values, which was in agreement with previous findings (Almeida et al., 2011; Floegel et al., 2011). The solubility of $ABTS^{\bullet+}$ in aqueous solution allows it to measure hydrophilic compounds (Arnao, 2000), which made up >90% of the total antioxidant capacity in most fruits (X. Wu et al., 2004). Aside from the difference in reaction media, the variations in the composition of radicals used and their reaction mechanisms could contribute to the observed discrepancies in the readings of the two scavenging assays. ABTS assay is an electron transfer reaction while DPPH is based on the normal hydrogen atom

transfer between antioxidants and nitrogen radicals. The creation of a more stable and less transient nitrogen radicals, instead of peroxyl radical which is highly reactive, caused many antioxidants to react slowly or may even become inert to $DPPH^{\bullet}$ (Huang, Ou, & Prior, 2005), unlike $ABTS^{\bullet+}$ in which different antioxidant compounds donate one or two electrons to reduce the radical cation (Chen et al., 2015). This could be the reason for the stronger correlation of ABTS assay with maturity and TPC of the digested samples, which was in agreement with the previous study (Floegel et al., 2011). However, the obtained antioxidant capacity values in the present study were found to be higher compared to the previously published data about banana owing to the difference in cultivar used and the extraction method applied. Proteggente et al. (2002) reported a TEAC value of 181 µmol/ 100 g fresh weight (FW) of banana varieties from Caribbean region using ABTS assay. Zang et al. (2017) classified banana as having low antioxidant activity with VCEAC reading of <30 mg/100 g FW. Both studies did not determine bound phenolics; the reason for the lower TEAC and VCEAC values. This was also true for the result of FRAP activity which was higher by more than 3-fold than the reading of the previous study (Proteggente et al., 2002). On the other hand, banana was found to have the highest ferrous ion chelating power (>50% in 100 mg fruit/mL) among the tropical fruits studied by Y. Y. Lim et al. (2007).

Regarding the low correlation between antioxidant activities and TPC of fresh Saba banana extract, this could indicate that phenolic compounds were not the sole contributors to the antioxidant capacities of the fruit crop. It is worth noting that other secondary metabolites with antioxidant potential might be accountable in enhancing the antioxidant activities of Saba banana. This includes vitamin C, β-carotene, and vitamin E (Sulaiman et al., 2011). On average, banana pulp at the ripening stage contains ascorbic acid in the range of 6.9–10 mg/100 g FW, a wide variation of β-carotene levels ranging from 92 to 636 µg/100 g dry weight (B. Singh et al., 2016), and vitamin E content in the range of 0.06–0.52 mg/100 g FW (Thakur et al., 1997).

Based on the results of simulated digestion, gastric and intestinal conditions affected the release of TPC and antioxidant activities of Saba banana. Though the two digestive phases showed a different trend of results, it is evident that the action of digestive enzymes contributed to the release of bioactive compounds, which could make them bioaccessible, and therefore potentially bioavailable. In general, TPC and antioxidant activities were increasing during the gastric phase, except for MIC. The acidic pH environment in the gastric stage may induce the release, hydrolysis, and/or transformation of phenolic compounds (Velderrain-Rodríguez et al., 2014), and these compounds are stable under acid conditions (Olivas-Aguirre et al., 2016). This was supported by previous *in vitro* studies which reported high stability and released rate of

flavonoids and phenolic acids under stomach conditions (Bermúdez-Soto, Tomás-Barberán, & García-Conesa, 2007; Flores, Singh, Kerr, Pegg, & Kong, 2014) resulting in an increase in total phenolics. However, during the intestinal digestion, a decreasing trend in the values of antioxidant activities was observed, as also shown in previous *in vitro* digestibility studies such as apples (Bouayed et al., 2011), lettuce (Ketnawa et al., 2019), and some edible flowers (Chen et al., 2015). While the small intestine is considered as the major site for free phenolics absorption, the change in pH conditions during intestinal digestion affected the amounts of released phenolic compounds. There was a high possibility that a proportion of the phenolic compounds, which are sensitive to mild-alkaline conditions of the small intestine, were transformed into different structural forms with different chemical properties, and thus affecting their biological activity (Bermúdez-Soto et al., 2007). An example is the biochemical transformation of flavylium cation of anthocyanidin to less stable chalcone and carbinol pseudobase forms even at near-neutral pH (Thuengtung et al., 2018), which eventually leads to degradation of anthocyanin (Bermúdez-Soto et al., 2007; Nagar, Okun, & Shpigelman, 2019). Similarly, flavonols, such as quercetin, have been reported to undergo oxidation, hydroxylation, and ring cleavage at medium pH values in a time-dependent manner resulting in the formation of complex product profiles (J. Wang & Zhao, 2016). The low chemical stability of these compounds, as a function of their chemical structure, in the mild alkaline pH condition of the small intestine could cause their degradation and inactivity (Nagar et al., 2019). In addition, pH could affect the racemization of molecules, possibly changing the composition of the compounds and altering their biological reactivity, as pH at the intestinal stage could increase racemization in some compounds, thus affecting their antioxidant activity (Chen et al., 2015). In contrast with the results of reducing ability of antioxidant compounds, iron-chelating properties showed high stability in near-neutral pH concentrations. Most flavonoids were observed to have high chelating activity in pH 6.8 and 7.5 while low to no activity at acidic pH (4.5). This could be accounted for the different proton dissociation reactions of hydroxyl groups of flavonoids in various pH which found to have high efficiency at neutral than acidic conditions (Mladěnka et al., 2011).

The sudden decrease of antioxidant activity during intestinal digestion still requires detailed investigation, as discussion about this phenomenon is deficient, which according to some studies may also be brought by digestive enzyme interaction with polyphenols (Nagar et al., 2019). Nevertheless, TPC values remained statistically the same after simulated intestinal digestion as compounds such as gallic acid can tolerate neutral to harsh alkaline conditions in the small intestine (Thuengtung et al., 2018). Other possibilities that could explain the observed

trends are: (a) the phenolics detected during simulated intestinal digestion were only metabolites without antioxidant property (Flores et al., 2014) and (b) the antioxidant activity values were affected by the sensitivity of the assays to pH changes which could also explain the surprisingly higher values of DPPH than ABTS during the gastric phase. Unlike ABTS assay, DPPH assay is more sensitive to pH (Shalaby & Shanab, 2013) and is found to have high percent inhibition at pH less than 4; since at pH higher than 4.5, the possibility of deprotonation of gallic acid could occur resulting in decreased inhibition of DPPH$^{\bullet}$ by having no ability to donate hydrogen atom (Noipa, Srijaranai, Tuntulani, & Ngeontae, 2011). DPPH assay is also sensitive to oxygen which could explain the lower TEAC and VCEAC values of slurry than cut state. The diradical property of oxygen incorporated during homogenization could react with DPPH$^{\bullet}$ directly with the presence of light energy and thus decrease the absorbance of DPPH (Ozcelik, Lee, & Min, 2003).

About the differences in the observed trend between cut and slurry samples, their varying physical structures could affect the release of bioactive compounds. The structure of food is known to have an important role in the bioaccessibility of nutrients (Ogawa et al., 2018). Manipulating the structure of food to control the release of compounds and digestibility of food components could be done through processing and during mastication (Mishra et al., 2012; Parada & Aguilera, 2007). Our study observed the changes in two physical states, homogenized slurry and unhomogenized cut. The former could represent products subjected to crushing and blending or thoroughly masticated food which involved forces strong enough to disrupt the plant cell structure and release the containing bioactive compounds. On the other hand, the latter could represent the minimally processed products which retained the tissue structure and could also be as a result of incomplete comminution during chewing. The homogenization process brought damage to the cells of slurry samples which increased the surface area for immediate interactions with digestive enzymes, leading to the liberation of antioxidant compounds and possibly enhancing their bioaccessibility in the gut. Unlike in unhomogenized cut samples, the intact cell wall structure could act as an effective barrier that hinders both the access of enzymes and the diffusion of the compounds out of the fragment and therefore could lead to lower recovery of antioxidant compounds. The possibility of undetectable fracture on cell wall is high in cut sample, which means that the only way enzymes could penetrate the food is by diffusion through the cell walls. This penetration effects of digestion through layers of intact cells underlying the cut surfaces is relatively a slow process (Mishra et al., 2012). This slow release of bioactive compounds could also account for the much wider gap of increment range of cut samples than slurry state. The low recovery initially of the cut sample would mean

high residual concentrations which could be released on the latter part of digestion, resulting in a higher increment range. Additionally, as reported in our previous research (Reginio, Ketnawa, et al., 2020), digesta viscosity of Saba banana showed increasing values with an increase in fruit maturity and this could also affect the released rate of phenolics and antioxidant activities during simulated digestion. In the present study, a higher percent increment in bio-properties of ripe stages was also observed when compared to unripe stages throughout simulated digestion. It seems likely that the highly viscous composition of digesta in ripe stages, particularly slurry state, could inhibit the mixing process and, as a consequence, restrict the interaction of substrates and enzymes resulting in the partial release of phenolic compounds during the initial phase of digestion. The high viscosity was accounted for the different dilutions applied for each maturity stage and the presence of water-soluble pectins in ripe fruits. As already mentioned, the conversion of water-insoluble to water-soluble pectin happens during ripening. This pectin from ripe fruit is capable of forming a gel with water and thus increasing the viscosity of the food matrix (Brown, 2018). The effect of maturity on *in vitro* release of bioactive compounds during simulated digestion was supported by the result of correlation analysis which showed significantly negative correlation with TPC for both slurry and cut samples ($p < 0.05$).

3.5 Conclusion

The study determined the bio-properties of Saba banana in free and bound forms which varied considerably during maturation. The different scavenging radicals used in estimating antioxidant capacities of fresh and digested samples also showed varying antioxidant potentials. The action of digestive enzymes and the changing pH conditions in the gastrointestinal tract affected the release and stability of phenolic compounds. The results of this study further demonstrate that modification in the physical structure (i.e. processing or chewing) and changes in the composition of Saba banana accompanying maturation played an important role in disintegrating plant tissue structure and regulating substrate-enzyme interaction, respectively; thus, facilitating the extraction and release of bioactive compounds from the food matrix, which in turn may have an impact on their bioaccessibility and bioavailability. For future studies, correlation of the obtained results using *in vivo* methods is necessary to better model the digestive fate of bioactive compounds in Saba banana as different physical, enzymatic, and chemical reactions and transformations are occurring during gastrointestinal digestion process.

CHAPTER 4

Modifications of pectic polysaccharides during maturation of Saba banana and its relation to electrical impedance characteristics

4.1 Introduction

Plant and their products have always been extensively utilized as a source of various drugs and excipients in different pharmaceutical formulations (Srivastava & Malviya, 2011). The wide array of nutritionally important components and diverse secondary constituents in plants that are known to have health-promoting effects led to various explorations linking each constituent in the prevention and management of certain diseases. Pectin, one of the plant cell wall components, is considered not only as a valuable functional food ingredient widely employed in food manufacturing (Taboada et al., 2010) but also as an emerging bioactive food polysaccharide (Maxwell, Belshaw, Waldron, & Morris, 2012; Yabe, 2018) because of its important positive effects on human health. Consumption of pectin has been associated with dietary fiber's biochemical benefits. It acts as a soluble fiber which can increase gut viscosity, increase the synthesis of bile acids from cholesterol, and thereby reducing circulating blood cholesterol (Brouns et al., 2012). More attention has been given for the potential role of modified pectin in the reduction of carcinogenesis. The underlying mechanism by which it can help in cancer prevention and progression is still unknown; however, evidence suggests that the small molecular weight pectin fragments called galactans can inhibit the ability of an important regulator of tumor metastasis to induce apoptosis (Maxwell et al., 2012). Another study correlates the specific structural elements within pectin for the apoptosis-inducing activity in cancer cells (Jackson et al., 2007). These are only a few of the numerous reports that investigated the potential of dietary pectin to impact human nutrition and health.

Pectin is a structural polysaccharide in plants with a backbone comprised of α 1-4 linked galacturonic acid residues (Luzio, 2004). It is synthesized in the golgi and delivered to the cell wall, specifically in the middle lamella, by secretory vesicles (Daher & Braybrook, 2015). It provides structural integrity and mechanical resistance to tissues by forming a hydrated cross-linked three-dimensional network (Nurdjanah, Hook, Paton, & Paterson, 2013; Taboada et al., 2010). Pectic polysaccharides contain three main structural regions, the most prominent is homogalacturonan occupying about 65% of typical pectin and is a linear polysaccharide of D-galacturonic (Mierczyńska et al., 2015; Yabe, 2018). The decrease in fruit firmness and disassembly of cellulose and hemicellulose network have been associated with pectin

degradation during ripening (Duan et al., 2008). Pectic substances refer to pectin or group of polysaccharides that are classified into three general types depending on the degree of maturity of fruit. In immature or raw fruits, protopectin is the pectic acid present which is insoluble in water. Pectinic acid is a soluble substance derived from protopectin by enzymatic action in mature or slightly ripened fruits which is composed of more than a negligible proportion of methyl ester groups (methoxy group). Lastly, pectic acid is a polymer of galacturonic acid, a constituent sugar, nearly free or without any methyl ester group (Bonner, 1936; Doesburg, 1965; Yabe, 2018). Extensive studies have been conducted on the possibility of producing pectin from fruit sources; however, most pectin extracted have poor gelling ability characteristics as compared to the commercially available citrus peel and apple pomace (Nurdjanah et al., 2013). Therefore, it is of great importance to continuously explore fruits and their wastes as potential sources of pectin to contribute to its increasing demand for food and pharmaceutical applications.

The amount of galacturonic acid residues is an important parameter to consider in the quantitative analysis of pectin (Luzio, 2004). This can be done through colorimetric quantification using widely known chromogens viz. carbazole and 3,5-dimethylphenol (Kumar, Nagar, & Tripathi, 2014). Both assays use concentrated sulfuric acid which when subjected to heat with galacturonic acid will produce a product that reacts specifically with the chromogens (Yapo, 2012). Pectin content can be also estimated by calcium pectate method which is considered as an old technique of precipitating pectin. This is carried out by hydrolysis with sodium hydroxide, acidification with acetic acid, and addition of calcium chloride (Carré & Haynes, 1922). Previous study concluded that the yield of calcium pectate may be used as a criterion of purity and measure of true pectin content (Grassino et al., 2016).

The changes in tissue composition and structure during storage and ripening influenced the dielectric properties of fruits which can be used to assess fruit quality and deterioration (Juansah, Budiastra, Dahlan, & Seminar, 2012). Under a current excitation, the conducting liquids (e.g. intra- and extracellular fluids) and membrane of biological cells behave like capacitance which produces a complex bioelectric impedance (Chowdhury, Bera, Ghoshal, & Chakraborty). This can be measured through electrical impedance spectroscopy (EIS) which is used to provide information about the physiological state of various biological tissues, including fruits (L. Wu, Ogawa, & Tagawa, 2008).

In this paper, electrical impedance data were used as maturity indices to confirm the stages of Saba banana. Pectic polysaccharide fractions were extracted and quantified in each stage and pectin yield from Saba banana was estimated by spectrophotometric (3,5-

dimethylphenol and carbazole) and gravimetric (calcium pectate) analyses. The characteristic of pectin extracted at different maturity stages was evaluated for the degree of esterification. Moreover, the relationship between the modification of pectic polysaccharides and the impedance properties during maturity was investigated. It is noteworthy to obtain the quantitative data of the pectic polysaccharides fractions of Saba banana at varying maturity since, in our previous study, we reported that pectic substances were one of the factors that could affect the digestibility of Saba banana (Reginio, Ketnawa, et al., 2020). Thus, additional investigation about the viscosity of water-soluble pectin extract was conducted.

4.2 Materials and Methods

4.2.1 Materials and chemicals

Saba banana was purchased from Diamond Star Agro-Products Inc., Taguig City, Philippines in green mature stage and was brought at the Postharvest and Food Engineering Laboratory, Graduate School of Horticulture, Chiba University for analysis. Thermostable α-amylase and amyloglucosidase (3260 U mL^{-1}) were provided by Megazyme International Ltd. (Wicklow, Ireland). Galacturonic acid monohydrate, carbazole, 3,5-dimethylphenol, calcium chloride, and sodium hexametaphosphate were provided by Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

4.2.2 Sample preparation

Saba banana fruits were allowed to ripen in an incubator (MIR-153, Sanyo Electric Co., Ltd., Japan) at 23 ± 1 °C. The maturity stages of the fruit were chosen based on color analysis ($L*$, chroma, and hue angle) of the peel measured using a chromameter (CM-600d, Konica Minolta, Tokyo, Japan) as shown in Table 4.1. The color measurements were taken at three different sections of each fruit and the mean values were computed to represent the color values of different maturity stages. At least 15 banana fingers from 10 bunches were randomly taken at each maturity stage. The fruits were soaked first in 200 ppm of sodium hypochlorite solution for 1 min, rinsed in running water, and allowed to stand on absorbent paper until dry before separating the peel from the pulp by cutting. After the determination of electrical impedance data, the samples were cut into small round sections, immediately submitted to freezing in liquid nitrogen, and conditioned in freezer at -40 °C. The frozen samples were then freeze-dried, ground, and sieved (particle size <0.5 mm) and the resulting powder was kept in freezer at -40 °C.

Table 4.1 Color values of different maturity stages of Saba banana used in the study.

Maturity	Visual color	Lightness	Chroma	Hue angle
1	all green	59.67 ± 2.44^{b}	31.82 ± 3.51^{b}	101.29 ± 1.71^{a}
2	green with trace of yellow	61.56 ± 2.71^{b}	32.12 ± 2.95^{b}	96.04 ± 1.62^{b}
3	more yellow than green	64.80 ± 1.89^{a}	36.65 ± 2.56^{a}	88.83 ± 3.18^{c}
4	yellow with green tip	64.96 ± 1.92^{a}	37.15 ± 4.23^{a}	76.85 ± 2.27^{d}
5	yellow with brown flecks	53.19 ± 3.94^{c}	27.02 ± 4.08^{c}	70.02 ± 3.60^{e}

Different letters for the same parameter indicate significant differences among the stages ($p < 0.05$).

4.2.3 Electrical impedance spectroscopy

The electrical impedance characteristics were measured using an LCR meter (HIOKI, IM3533-01, Japan) with two parallel steel needle electrodes positioned 1 cm apart. Briefly, a 1 cm thick circular block of banana was excised from the pulp at three different positions along the long axis of the banana ([1] 1.5 cm from the top, [2] center, and [3] 1.5 cm from the stem or bottom) (Figure 1). The impedance data were measured at 200 frequency points (logarithmic frequency interval) using sweep function from 1 mHz to 200 kHz under a voltage of 1V. The complex impedance of fruit, Z (Ω), as a function of frequency was defined as:

$$Z = R + jX$$

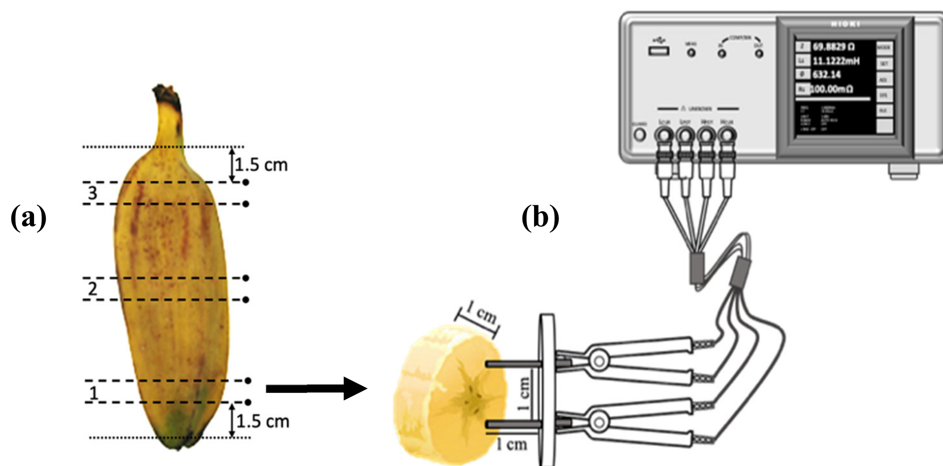

Figure 4.1. Sampling points for electrode placement in banana (a) and experimental setup of the impedance measurement (b).

where R and X represented the magnitude of real (resistance [Ω]) and imaginary parts (reactance [Ω]), respectively, of Z and j was the imaginary unit. The real and imaginary parts

of impedance were calculated from the measured impedance amplitude |Z| and phase angle (θ) of fruit sample using the equations:

$$R = |Z| \cos\theta; X = |Z| \sin\theta$$

4.2.4 Extraction of alcohol insoluble solid (AIS)

The freeze-dried samples (35g) were suspended in distilled water (200 mL), added with 0.35 mL heat-stable α-amylase, and then heated at 80 ˚C for 30 min with continuous stirring to hydrolyze the starch and inactivate the endogenous enzymes, respectively (Figure 4.2). After cooling in a water bath at 50 ˚C for 5 min, amyloglucosidase (0.35 mL) was added and subsequently incubated at 50 ˚C for 30 min. Following enzyme treatment, the mixture was diluted with water to sample ratio of 10:1 and was stirred for additional 1 h. The AIS was obtained by treating the mixture with absolute ethanol to a final concentration of 70%. The mixture was stirred for another 1 h and then filtered. The solid phase was collected, washed with 90% (v/v) ethanol, air-dried overnight inside fume hood to remove ethanol, and freeze-dried. The dried powder was considered as the alcohol-insoluble solid which was stored at -40 °C until use.

4.2.5 Sequential extraction of pectin fractions

Different experimental conditions were employed for the sequential extraction of pectic substances from Saba banana AIS: (1) distilled water stirred at room temperature for 30 min and left overnight; (2) 0.8% (w/v) sodium hexametaphosphate at 90 ˚C for 1 h; (3) 0.1 N HCl at 100 ˚C for 1 h; and (4) 0.2 N NaOH at room temperature left overnight (Figure 4.2). Each AIS sample (5 g) was extracted with 100 mL solvent. The extracts were centrifuged at 3000 × *g* for 15 min, filtered through Advantec 101, and the residue was washed with distilled water. The extracts were freeze-dried, ground, and then stored at -40 ˚C until further analysis.

4.2.6 Measurement of anhydrogalacturonic acid (%)

A modified version of 3,5-dimethylphenol (DMP) method by Scott (1979) in a 96-well plate was done to estimate the amount of uronic acids in the pectin fractions. A serial dilution of sample (12.5 μL) was mixed with 12.5 μL of 2% (w/v) sodium chloride. Concentrated sulfuric acid (200 μL) was added followed by vigorous mixing and heating in an oven at 70 °C for 10 min. After cooling for 15 min at room temperature, 10 μL of 0.1% (w/v) 3,5-dimethylphenol in glacial acetic acid was added and incubated at room temperature for 10 min. The plate was read in Multiskan FC microplate reader (Thermo Fisher Scientific, MA, USA) at 405 and 450 nm. The uronic acid concentration was calculated from the difference of absorbance taken at two wavelengths to correct the interference from hexoses. The estimated

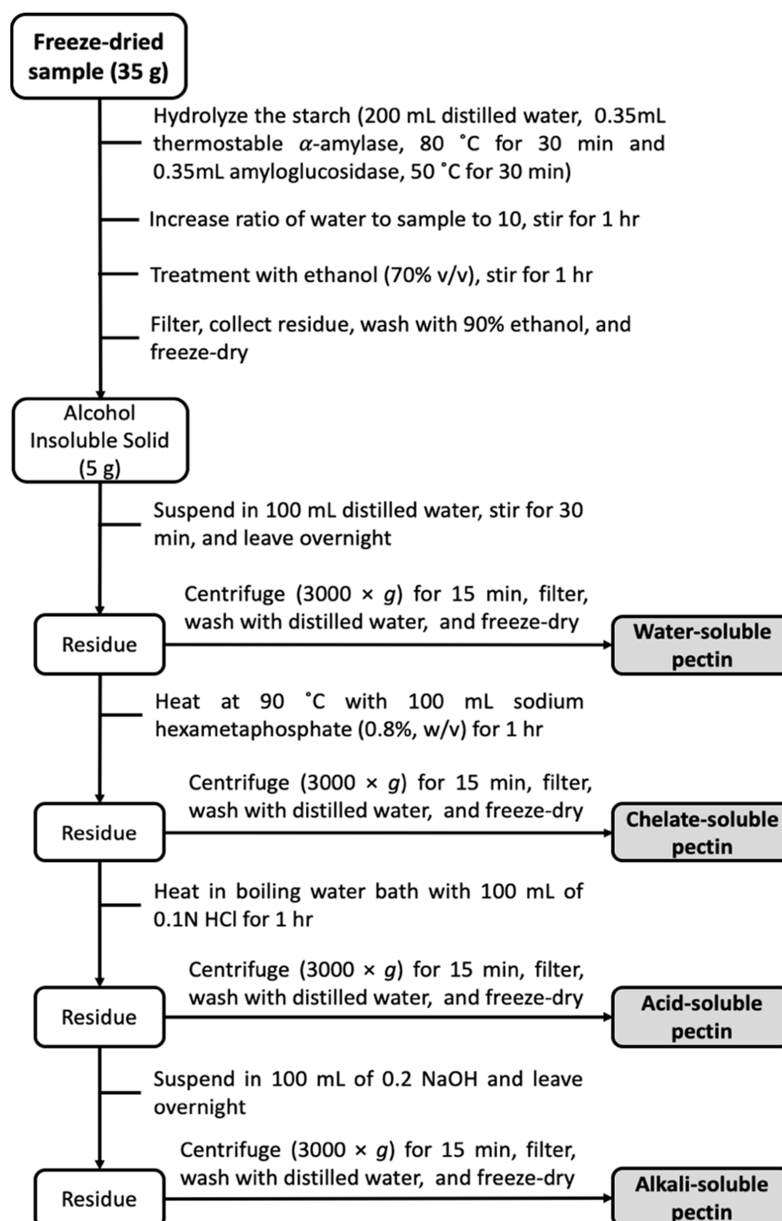

Figure 4.2 Sequential extraction of pectic polysaccharides from Saba banana.

concentration of pectic substances expressed as anhydrogalacturonic acid (%) was read from a standard curve of galacturonic acid monohydrate (0-300 μg/mL) which was treated similarly to the samples subjected to uronic acid measurement.

4.2.7 Measurement of pectin content (%)

The pectin content of different maturity stages of Saba banana was determined by spectrophotometric analysis using two colorimetric agents, 3,5-dimethylphenol and carbazole (Cesaretti, Luppi, Maccari, & Volpi, 2003), and by gravimetric method as calcium pectate

(Rahman, Miaruddin, Chowdhury, Begum, & Islam, 2013). The pectin estimation using 3,5-dimethylphenol was done by determining the anhydrogalacturonic acid (%) and accounting the yield in each fraction. Pectin content was expressed as the ratio of dried pectin extracted to the dry basis weight of Saba banana.

For the estimation of pectin as calcium pectate, freeze-dried Saba banana samples (5 g) were extracted with 40 mL of 0.05N HCl at 85 °C for 2 hr. The volumes were adjusted to 50 mL with distilled water and filtered through No. 4 Whatman paper. A 10 mL aliquot was separated, added with 250 mL distilled water, and neutralized with 1 N NaOH. Additional 1 mL of 1 N NaOH was added in excess and allowed to stand overnight. Then, the samples were added with 5 mL of 1 N acetic acid. After 5 min, 25 mL of 1 N calcium chloride was added with continuous stirring and was allowed to stand for 1 hr. This was followed by boiling for 1–2 min and filtration through previously prepared filter paper (washed with hot water and dried at 102 °C for 2 hr). The residue remained was dried to constant weight and the pectin was calculated using the equation below:

$$\% \text{ calcium pectate} = \frac{\text{weight of calcium pectate } \times 50}{\text{weight sample } \times \text{ mL of aliquot taken for estimation}} \times 100$$

For the carbazole method, a serial dilution of sample (50 μL) was placed in a 96-well plate. A 200 μL of 25 mM sodium tetraborate in sulfuric acid was added. The plate was heated in a conventional air oven for 10 min at 100 °C. After cooling at room temperature for 15 min, 50 μL of 0.125% carbazole in absolute ethanol was carefully added. The samples were further heated at 100 °C for 10 min in an oven and cooled at room temperature for 15 min, the plate was read in a microplate reader at a wavelength of 540 nm. A standard curve of anhydrogalacturonic acid (0-400 μg/mL) was prepared to estimate the pectin content (%) based on the yield of each fraction the same as in 3,5-dimethylphenol.

4.2.8 Re-extraction of pectin for characterization

Excess pectin extracted in different fractions were combined and reprecipitated by suspending in 150 mL distilled water and treating with absolute ethanol until 70% solution was obtained. The pectin was centrifuged at 3000 × *g* for 15 min, washed with 90% ethanol, air-dried inside fumehood, and freeze-dried. The re-extracted pectin was ground to pass 0.5 mm sieve and stored at -40 ˚C until further analysis.

4.2.9 Measurement of the degree of esterification (%)

The degree of esterification was estimated following the methods from L. Liu, Cao, Huang, Cai, and Yao (2010) and modified by Castillo-Israel et al. (2015). The freeze-dried pectin powder (0.5g) was placed in a 250 mL flask, moistened with 5 mL of 95% ethanol and

dissolved in 50 mL of carbon dioxide-free distilled water. Sodium chloride (1 g) was added to sharpen the endpoint with 2-3 drops of phenol red indicator. The mixture was then stirred rapidly to dissolve all pectin substances. The first titration was done with 0.1 N sodium hydroxide until the color of the indicator changed to pink (pH 7.5). The volume of initial titer was recorded (a) and the neutralized solution obtained was added with 5 mL of 0.5 N sodium hydroxide. After allowing to stand for 30 min at room temperature, 5 mL of 0.5 N hydrochloric acid was added. The mixture was titrated with 0.1 N NaOH until the color of the indicator changed to pink (pH 7.5) (b). Degree of esterification (%) was computed using the equation:

$$\text{DE (\%)} = \frac{\text{volume final titer (b)}}{\text{volume initial titer (a)} + \text{volume of final tier (b)}} \times 100$$

4.2.10 Measurement of viscosity

The extracted water-soluble pectin was dissolved in distilled water (0.007% w/v) using a magnetic stirrer at ambient temperature for 15 min. Afterward, sucrose was added following the concentration of sugars present in Saba banana in relation to pectin ratio (0.057–0.73% w/v). Viscosity was measured using a torsional oscillation viscometer (Viscomate VM-10A, Sekonic, Tokyo, Japan) at different conditions and sampling times. The pH of the pectin solution was first adjusted to 1.2 ± 0.1 then changed to 6.8 ± 0.1 to simulate the conditions of *in vitro* digestion at 37 ± 1°C. For acidic conditions, sampling points were at initial (time 0), after 30 min (G30), and 60 min (G60) while after pH adjustment (I0), after 60 min (I60), and 120 min (I120) at near-neutral pH. The percent change in viscosity was computed by dividing the difference between the viscosity values at different sampling points and the viscosity at time 0 by the initial value.

4.2.11 Statistical analysis

All extraction was done in two replications, and the analyses were carried out at least in duplicate. Analysis of variance (ANOVA) was used to analyze the data, and the comparison of means was carried out at 5% significance level using Tukey's test. Statistical analyses were made using R software version 4.0.0 GUI 1.71 Mavericks (R Development Core Team, 2018).

4.3 Results and Discussion

4.3.1. Electrical impedance characteristics

Figure 4.3 shows the electrical impedance data of banana samples in each maturity stage determined by the average resistance and reactance values. At low-frequency domain, polarization happened due to the interaction of charged electrode surface and ions from the sample (Padmaraj, Miller Jr, Wosik, & Zagozdzon-Wosik, 2011) resulting in the occurrence of a linear part in the plot. This was eliminated as the occurrence was not related to the characteristics of cellular structure (Watanabe, Ando, Orikasa, Shiina, & Kohyama, 2017). The resistance of the cell wall and associated extracellular fluid is measured at low frequencies, while the resistance through the entire cell is measured at high frequencies. Since the study only used low frequencies, electric current could not pass through plasma membrane and observation was restricted only to apoplast (Jócsák, Végvári, & Vozáry, 2019) and extracellular environment (Harker & Maindonald, 1994). The decreased resistance observed during ripening might be related to changes in fruit texture. The result was consistent with previous studies about tomato (Varlan & Sansen, 1996), persimmon (Harker & Forbes, 1997), and nectarine (Harker & Maindonald, 1994). In general, electrical impedance of each stage decreased gradually with increasing frequency.

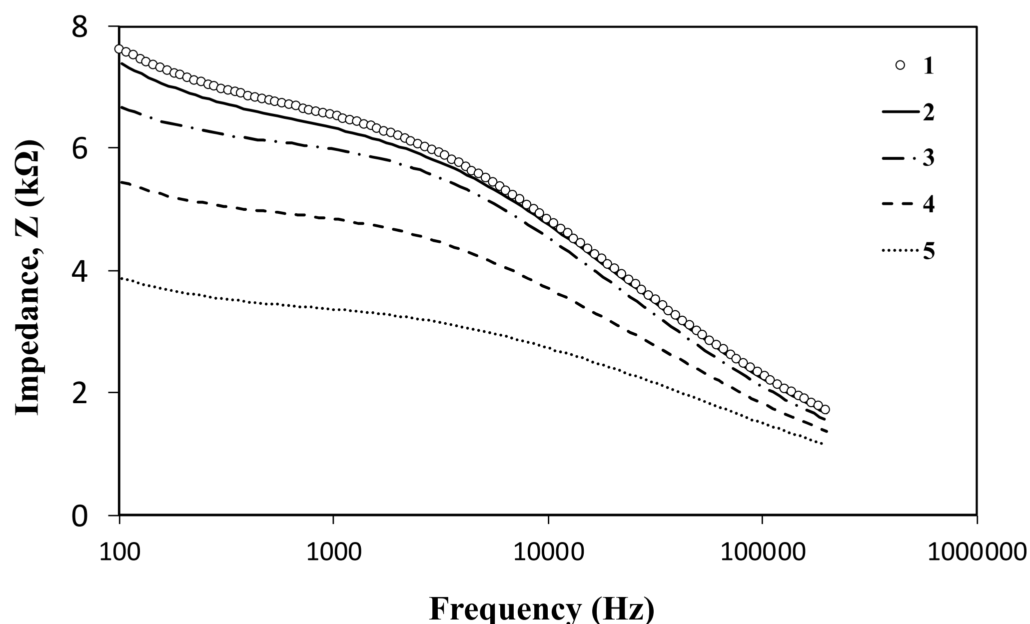

Figure 4.3 Electrical impedance data of different maturity stages of Saba banana.

4.3.2 Pectic polysaccharide fractions

Depending on the solvent used, each pectin fraction was identified as water-soluble pectin (WSP), chelate-soluble pectin (CSP), acid-soluble pectin (ASP), and alkali-soluble pectin (NSP). In Figure 4.4, the conversion of water-insoluble pectin to water-soluble pectin with the action of protopectinases (Godoy, Amorim, Barreto, & Freire, 2018) as ripening proceeded could account for the significant increase in WSP content. This could indicate increased depolymerization and solubilization of pectin (Brummell & Harpster, 2001) as a result of protopectin degradation into lower molecular weight fractions, which reported to have high solubility in water (Erkan & Dogan, 2019). WSP fraction is composed of polymers that are non-ionic and non-covalently bound to cell walls (Gawkowska et al., 2018; Mierczyńska et al., 2015). WSP of Saba banana from stage 1 to 4 increased by more than 2-fold with a slight decrease in the last stage. The result was in accordance with the study of Duan et al. (2008) which obtained higher yield of WSP in ripe than unripe cavendish banana.

Only a portion of pectin becomes soluble in water, as most polymers are still remained associated with the cell wall by ionic bonds such as CSP (Gawkowska et al., 2018). Thus, other extraction conditions are necessary for ease of extractability and solubilization of pectin (Brummell & Harpster, 2001). Among the conditions applied, the highest anhydrogalacturonic acid value (%) was observed in chelate-soluble pectin fraction (CSP) (Figure 4.4). CSP is considered ionically-bound pectin which contains the middle lamella pectin and is responsible for cell cohesion (H. Liu et al., 2009). More than 70% of Saba banana pectin yield was extracted by the use of chelators at high temperature condition. These chelating agents are known to be effective in separating and removing bound calcium ions in the middle lamella of the cell. The ions are responsible for cross-linking de-esterified pectin through ionic bonding which results to gelling of pectin, thereby increasing the rigidity of cell wall (Daher & Braybrook, 2015; H. Liu et al., 2009; Yabe, 2018). In addition, demethylation also causes changes in pH and ionic conditions in the apoplast (Brummell & Harpster, 2001). If calcium is removed by the use of chelators, links between de-esterified pectin will be broken leading to increase solubility of pectin. During maturation of Saba banana, a large percent decrement in CSP was observed accounting to 56% from stage 1 to stage 5.

The extraction of pectin by hot diluted acid method showed the lowest AGalA (%) among the conditions involved. This result was in contrast with a previous study about murta fruit which concluded that extraction at high temperature under acidic medium was the most effective in terms of yield, extractability, and structural composition of pectin (Taboada et al., 2010). Another research reported a significant decrease in ASP content of banana during

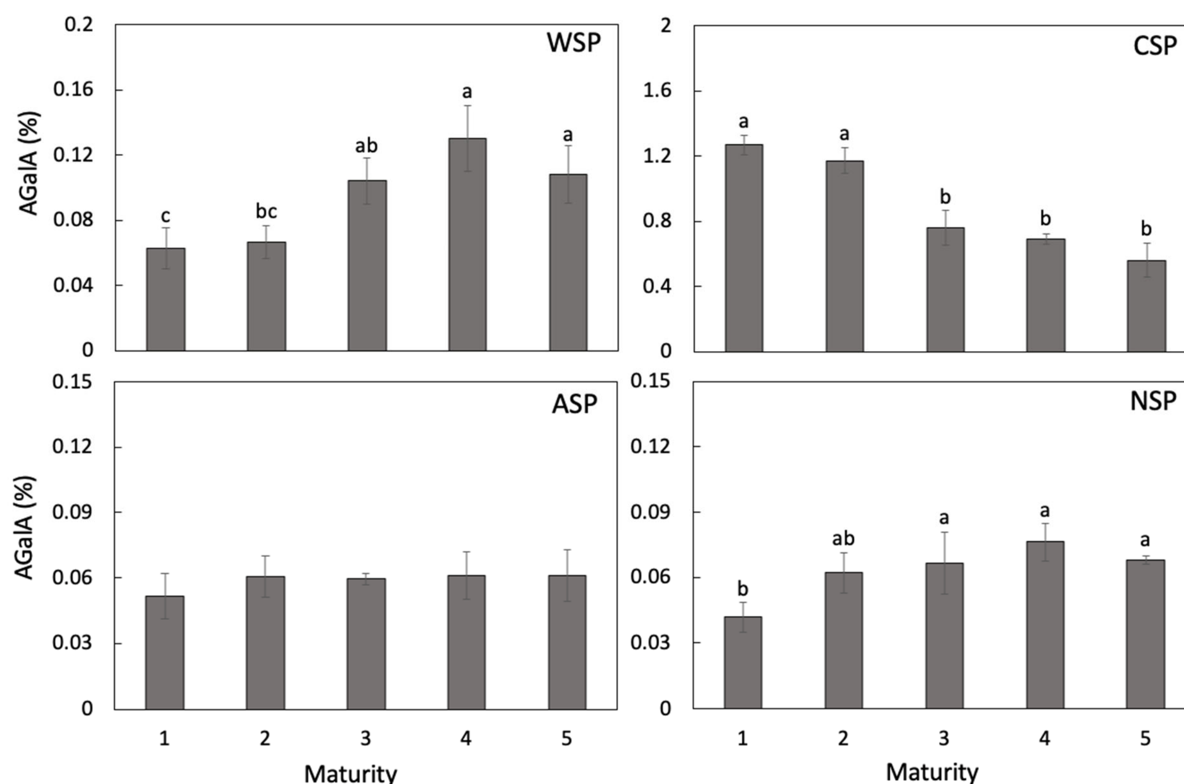

Figure 4.4 Anhydrogalaturonic acid (AGalA, %) content of pectin fractions of different maturity stages of Saba banana. WSP: water-soluble pectin; CSP: chelate-soluble pectin; ASP: acid-soluble pectin; NSP: alkali-soluble pectin. Mean values with different lowercase letters for the same parameter indicate significant differences between maturity stages ($p < 0.05$).

ripening (Duan et al., 2008). The difference may be accounted for the overall low ASP yield obtained during extraction as some acid-soluble fractions were also chelate- and water-soluble. The same reason can be accounted for the result of NSP and that alkali treatment could also result in some degree of degradation of pectin molecules through β-elimination reactions (H. Liu et al., 2009). Both ASP and NSP fractions contain pectin which are bound to the cell wall linked by covalent ester bonds (Gawkowska et al., 2018). In ASP fraction, the obtained values showed no significant differences among the maturity stages. However, increasing values of NSP were observed as maturity proceeded with significant differences between unripe and ripe stages.

4.3.3 Estimated pectin content

In the spectrophotometric quantification of pectin, the carbazole method showed a significantly higher pectin content than the DMP method in all maturity stages (Figure 4.5). This may be accounted for the reaction of non-uronide carbohydrates such as neutral sugars and monosugars (hexoses and pentoses) which happens mainly upon heating in concentrated

H_2SO_4 resulting in the formation of furan compounds by maillard-type reactions (Luzio, 2004; Yapo, 2012). The presence of a substantial amount of neutral sugars decreases the specificity of carbazole method while other monosugars would affect its selectivity towards uronic acid leading to overestimation of pectin content (Kumar et al., 2014). It has been reported that neutral sugar interference can be estimated up to 20-fold excess over the level of uronic acids in the sample (van den Hoogen et al., 1998). In the present study, around this amount of fold difference was also observed when pectin values of the two colorimetric methods were compared. From this data, it was evident that the extracted fractions contained an excess of neutral sugars which caused error in the estimation of pectin content with the use of carbazole. Hence, the values of 3,5-dimethylphenol method could correlate more to the actual value of pectin in Saba banana. DMP method was previously reported to have high specificity for uronic acids, less sensitivity to neutral sugar interference, and higher comparability of results to gas-liquid chromatography than carbazole leading to a more precise estimation of pectin (Kumar et al., 2014). The addition of NaCl and the measurement of the absorbance at 400–415 nm which then deducted to the value obtained from the maximum absorbance of uronic acid at 450 nm (Scott, 1979) could correct the interference caused by neutral sugars. Moreover, a modification was done in the study further minimizing the errors due to unwanted reactions with neutral sugars present in the sample. Such modification was through the use of a microplate assay which could perform a simultaneous rapid reading of multiple samples (Luzio, 2004). The cumulative amounts of pectin extracted by water, chelators, acid, and alkaline (Figure 4.5) as measured by DMP method showed significant decrease during ripening. From 1.43% in initial stage, around 44% was degraded in the last stage with pectin content of 0.80%. Previous published data showed that banana contained lower amount of pectic substances compared to other fruits which accounted for 0.7–1.2% of the fresh weight (Duan et al., 2008; Jayani, Saxena, & Gupta, 2005). The decrease in pectin content during ripening was mainly due to the action of pectin degrading enzymes or pectinases, which are known to depolymerize and de-esterify pectins making the polymers increasingly soluble in water and chelator solutions (Guillon et al., 2008). Pectinases are divided into protopectinases, pectin esterases, and depolymerases (Godoy et al., 2018; Jayani et al., 2005). Among the group of enzymes, polygalacturonases (PG) and polymethylgalacturonases (PMG) catalyze the decomposition of glycosidic bonds in pectic acids and pectin, respectively, by the mechanism of hydrolysis or by ß-elimination in lyases counterparts (polygalacturonases lyase [PGL] and polymethylgalacturonases lyase [PMGL]) (Jayani et al., 2005).

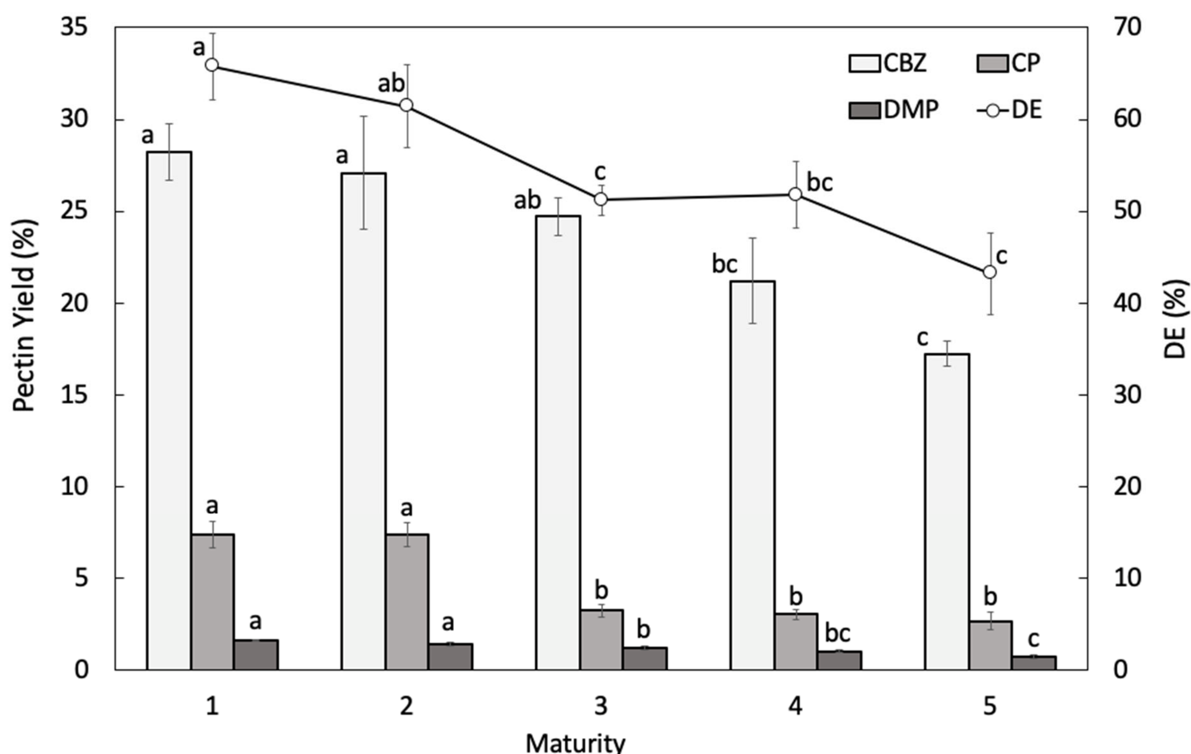

Figure 4.5 Pectin yield (%) and degree of esterification (DE, %) of different maturity stages of Saba banana. CBZ: carbazole; CP: calcium pectate; DMP: 3,5-dimethylphenol. Mean values with different lowercase letters for the same parameter indicate significant differences between maturity stages ($p < 0.05$).

Saba banana pectin extracted as calcium pectate showed the same trend of result as that of the colorimetric quantification. A decreasing amount of calcium pectate was observed as maturity advanced. However, the amount of calcium pectate, which could be an estimation of the amount of pectin, was higher than what was estimated in DMP method. This may be due to the incorporation of non-pectic substances during the extraction of calcium pectate. The possibility of inclusion of starch could give rise to possible contamination. Thus, the removal of non-resistant starch from Saba banana was done before pectin extraction and subsequent quantification using the chromogens mentioned above.

4.3.4 Degree of esterification

Pectin is divided into two groups depending on the degree of esterification. The pectin with DE higher than 50% is known as high methoxyl pectin (HMP) while low methoxyl pectin (LMP) has a DE lower than 50%. This study showed that methylation was altered during ripening. At the mature green stage, the estimated degree of pectin methylation was comparably high (65%) (Figure 4.5) as that of the commercial apple pomace pectin (70%).

This indicated that the pectin in the initial stage of Saba banana was of high-methoxy type. However, as the fruit advanced to the ripening stage, the pectin became increasingly de-esterified with a significant percent decrement of 34% in stage 5. The degree of esterification of pectin declines as a whole throughout the fruit development not only due to loss of methyl groups brought about by the activity of pectin methylesterase (Daher & Braybrook, 2015) but also due to broken inter-pectate ester links in addition to glycosidic bonds (Brummell & Harpster, 2001).

4.3.5 Viscosity of water-soluble pectin solutions

Viscosity of water-soluble pectin extract was investigated simulating the conditions during *in vitro* digestibility reported in previous study (Reginio, Ketnawa, et al., 2020). At pH 1.2, the viscosity of WSP decreased as shown by the negative percent change denoting a decreased gel strength in highly acidic solutions (Figure 4.6). Upon the adjustment of pH to 6.8, a significant increase in viscosity values of all samples was observed. WSP extracts of ripe stages showed a significantly higher percent change than unripe stages. At the end of the measurement, the samples showed a positive percent change indicating that there was an increase in viscosity readings and that pH could differentially affect the viscosity of WSP solutions. This result supports the previous findings that pectin of ripe stages of Saba banana tend to have higher viscosity values than unripe stages. The increase in viscosity could account for the influence of alkaline adjustment, particularly of the reaction of monovalent cations, aside from divalent ions. Monovalent ions, such as Na and K, could induce gelation of enzymatically de-esterified pectin even at near-neutral pH (Wehr, Menzies, & Blamey, 2004; Yoo, Fishman, Savary, & Hotchkiss, 2003). Previous research studies reported that these ions bound to pectin via electrostatic bonds (Malovíková, Rinaudo, & Milas, 1994) without the formation of junction zones like that of divalent cations. With increasing de-esterification during ripening, monovalent ions can react with LMP in ripe stages, neutralizing the pectin charge (Wehr et al., 2004), and improving its solubility in the presence of calcium (Sriamornsak, 2003). In addition, LMP gelation from the reaction with calcium ions was reported to be most compact at alkaline condition (pH 8.5) as it increased the amount of dissociated carboxylic groups leading to increased gel strength and gelling rate (Yang et al., 2018). Sugars contribute in the strength of gel formation as it reduces the amount of calcium required for LMP to form gel (Sriamornsak, 2003). However, the presence of contained traces of Saba banana components in the crude WSP extract may also affect the viscosity of the solutions, possibly due to saponification.

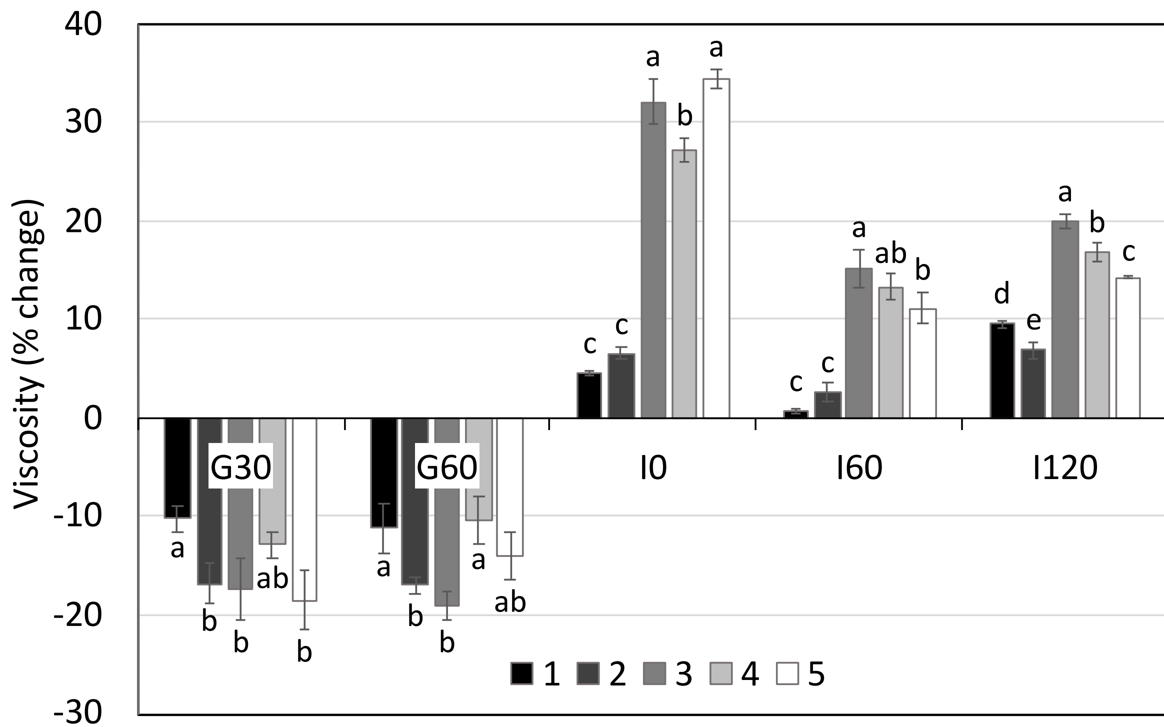

Figure 4.6 Change in viscosity (%) of water-soluble pectin during *in vitro* digestibility simulation. G30, pH 1.2 at 30 min; G60 pH 1.2 at 60 min; I0 pH 6.8 at time 0; I60, pH 6.8 at 60 min; I120, pH 6.8 at 120 min. Mean values with different lowercase letters for the same condition indicate significant differences between maturity stages ($p < 0.05$).

4.3.6 Relationship between changes in pectic polysaccharides and impedance characteristics during maturity

The impedance characteristics of the tissues are greatly affected by the changes in cell wall, membranes, and the composition of the cell contents during ripening (Juansah et al., 2012). The compositional changes would include differences in the concentration of sugars and electrolytes in cells, which are regarded as insulators and conductors, respectively. Therefore, a reduction in electrolytes and an increase in the concentration of sugars could contribute to an increase in resistance (Harker & Forbes, 1997). On the other hand, the decrease in cell wall and membrane resistance may be associated with the loss of cellular structure (Maduwanthi & Marapana, 2019) which is directly related to changes in fruit texture. The reduction in firmness or softening encompasses the degradation of polysaccharide components of the primary cell wall and middle lamella (Brummell, 2006) due to the action of cell wall modifying enzymes (Paniagua et al., 2014). As mentioned above, pectin is one of the major components responsible for cell wall rigidity. Numerous evidences have been reported

attributing the modifications of pectin polysaccharides in fruit softening during ripening. Jiménez-Bermudez et al. (2002) and Atkinson et al. (2012) revealed that downregulation of the enzymes (pectate lyase and polygalacturonase1, respectively) responsible for pectin break down reduces softening in strawberry and apple, respectively. Yun et al. (2019) reported that several pectin-degrading enzyme genes in banana peel were upregulated throughout the softening process. Duan et al. (2008) concluded that the loss of backbone structure in acid-soluble pectin fraction of banana could be a crucial step to result in fruit softening. While H. Liu et al. (2009) examined the morphology changes of CSP in relation to fruit firmness and found out that exogenous treatment of calcium (1%) retarded the physicochemical changes during storage of apricot fruit and depolymerization of CSP. Thus, depolymerization and increased solubilization of pectin could lead to loss of structural integrity of cell wall, which could result to low electrical impedance. The decline in electrical impedance is also associated with the expansion of the volume of apoplast due to swelling of cell walls (Bauchot, Harker, & Arnold, 2000) in fruits that rapidly softened once ripening is initiated, such as banana (Duan et al., 2008). There was a possibility that the weakening of the cell wall structure superseded the effect of those factors that could trigger the increase in resistance. Such increase could be due to the reduction in free calcium ions as it binds to increasing concentration of de-esterified pectin during ripening. However, demethylation also has an effect of making the pectin more susceptible to decomposition by some enzymes such as endopolygalacturonase which eventually softens the cell wall (Yabe, 2018). This could be the case of CSP wherein enhanced depolymerization at increasing maturity could have an effect of having higher concentration of free calcium ions as a result of pectin breakdown, thereby decreasing the resistance.

4.4 Conclusion

Changes in pectic polysaccharide fractions of Saba banana were observed during maturation, particularly in water- and chelate-soluble fractions. The estimation of pectin using carbazole method and as calcium pectate showed significantly higher results than DMP. Generally, the pectin content and the degree of esterification were affected by maturity. The low-methoxyl characteristic of ripe stages could affect the viscosity of water-soluble pectin solutions in near-neutral pH conditions due to its reactions with mono- and divalent ions. The results also gave rise to a possible relation of the changes in pectic substances to electrical impedance characteristics during ripening. Concomitantly, the enhanced depolymerization of pectic substances as ripening proceeded could be responsible for the loss of integrity of cell walls leading to a decreasing electrical impedance. However, the results should still be further

investigated because the study only reported the content of pectin in Saba banana and other factors that could contribute to the degradation of other cell wall components (e.g. hemicellulose polysaccharides) that is also responsible for fruit softening should also be evaluated for future work.

CHAPTER 5

Comparative evaluation of bio-properties of peel and pulp from Saba banana at different stages of maturity

5.1 Introduction

The development of high-value ingredient or novel food products from wastes generated by the food industry in processing fruits and vegetables have been the subject of recent researches, aiming to contribute in creating new food sources. Banana, being one of the most popular tropical fruit crops, generates approximately 36 million tons of peel waste every year. The peel accounts for about 35% of the whole fruit weight and is usually discarded directly into landfill or with general waste (Vu et al., 2018) contributing to massive amounts of organic materials to be managed. However, the environmental effects can be recovered by utilizing its potential as a source of high-added value compounds (Wachirasiri, Julakarangka, & Wanlapa, 2009). This is because banana peel waste has been previously reported to contain various functional properties such as antimicrobial, anti-inflammatory, antidiabetic, and anticancer properties (Vu, Scarlett, & Vuong, 2019). Its potent antioxidant capacity brought about by the presence of bioactive constituents, mainly phenolics (González-Montelongo, Lobo, & González, 2010), makes it a promising raw material for the eventual production of nutraceuticals and other functional products.

Among the large number of banana cultivars in the Philippines that have not yet been fully studied for bio-properties composition, Saba variety [*Musa 'saba'*(*Musa acuminata* × *Musa balbisiana*)], ABB genome group, is the most promising. This is the 2nd most-produced variety next to cavendish accounting for about 28% of the country's banana production. The fruit can either be consumed as a cooking or dessert type and is also regarded as a substitute for staple food such as rice and corn. Our previous research study showed that Saba banana pulp could be an excellent source of resistant starch with around 17% in mature green stage (Reginio, Ketnawa, et al., 2020). Moreover, different maturity stages of Saba banana pulp were reported to have significant amounts of total phenolics and flavonoids content which are known sources of natural antioxidants.

Phenolic compounds, which are secondary metabolites produced in plants, are not only involved in plant defense mechanisms but also known to exert numerous health-promoting effects to humans (Tsamo et al., 2015). The beneficial effects of phenolic compounds have been attributed mainly to their antioxidant activity. The phenolic compounds in food can be

classified according to their solubility features: as soluble and insoluble-bound fractions. The former is typically extracted using organic solvents, whereas the latter, which is covalently bound to the cell-wall matrix, could be released by acid, alkaline, or enzyme hydrolysis (Q. Li et al., 2018). Soluble phenolics also exist in free, esterified, and glycosylated forms. Free phenolics, present as phenolic aglycone (Shahidi & Peng, 2018), are the most extensively studied fraction of phenolics in the past years (Pereira et al., 2018) because it is easily extractable using solvents. Both esterified and glycosylated are conjugated to low-molecular mass components and sugars, which the same as free, are extractable by solvolytic solutions such as water, methanol, ethanol, and acetone (Yu et al., 2019). However, conjugated fractions were extracted under mild saponification conditions to hydrolyze and release the existing phenolics (Salawu et al., 2014). In contrast, insoluble-bound phenolics are covalently bound to indigestible matrices such as polysaccharides (pectin, hemicellulose, cellulose, and arabinoxylan), rod-shaped structural proteins, and highly-polymerized phenolics (condensed tannin and lignin) (Shahidi & Peng, 2018) which play important roles in providing both chemical and physical barrier and protecting against pathogen invasion (Chen et al., 2017). In the body, the bioavailability of insoluble phenolic compounds is facilitated by the intestinal enzymes or colonic microbiota which could transform into small phenolics and metabolites and thereby subsequently absorbed (B. Wang et al., 2016). There is an increasing interest in the determination of bound phenolics as neglecting this could underestimate the amount the real phenolic content of the fruit and their corresponding antioxidant activities. The determination of insoluble-bound phenolics in fruits has been extensively done (Arruda, Pereira, de Morais, Eberlin, & Pastore, 2018; B. Wang et al., 2016). In our previous study, higher TPC was found in bound phenolics of Saba banana than free fractions in all five stages of the fruit (Reginio, Qin, Ketnawa, & Ogawa, 2020). However, the study is lacking the information related to the profile of phenolic compounds. Further fractionation of the soluble-bound fractions into esterified and glycosylated phenolics was not investigated.

This current study evaluated the bio-properties of Saba banana peel and pulp at various stages of maturity in terms of chemical compositions and functional components. The functional components were comprehensively investigated using different fractions of phenolics (free, esterified, glycosylated, and insoluble-bound) and quantified by high-performance liquid chromatography coupled with diode array detector and electrospray ionization, alongside tandem mass spectrometry (HPLC-DAD-ESI-MS). Knowledge of the qualitative and quantitative profile of chemical constituents is of importance to evaluate the functionality of Saba banana fruit.

5.2 Materials and Methods

5.2.1 Materials and chemicals

Bunches of Saba banana fruit at the color index of 1 (mature green) were obtained from Diamond Star Agro-Products Inc., Taguig City, Philippines, and directly brought at the Postharvest and Food Engineering Laboratory, Graduate School of Horticulture, Chiba University for analysis. Resistant starch assay kit (K-RSTAR) was purchased from Megazyme International (Wicklow, Ireland). Sugar standards (sucrose, glucose, and fructose), ultrapure water, $FeCl_2$, $FeSO_4$, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$) , Trolox, and reference standards used in phenolic compounds profiling such as 3,4-dihydroxybenzoic acid (protocatechuic acid), rutin, (-)-epicatechin, (-)epigallocatechin, myricetin, apigenin, naringenin, (-)isoferulic, sinapic acid, chlorogenic acid, and kaempferol were supplied by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Chemicals used in bioactive compounds and antioxidant activity determination which included 2,2-diphenyl-1-picryl hydrazyl ($DPPH^{\bullet}$), 2,4,6-tris(2′-pyridyl)-s-triazine (TPTZ), and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate (FerroZine) and standards such as gallic acid monohydrate, (+)-catechin hydrate, vanillic acid, and *p*-hydroxybenzoic acid were provided by Sigma-Aldrich Ltd. (St. Louis, MO, USA). Other HPLC-grade standards such as ferulic and *p*-coumaric acids were from MP Biomedicals, LLC (Solon, OH, USA), and gallic acid and quercetin from ChromaDex, Inc. (Irvine, CA, USA)

5.2.2 Sample preparation

Saba banana fruits were stored in an incubator (MIR-153, Sanyo Electric Co., Ltd., Japan) maintained at 23 ± 1 °C and allowed to ripen into five different stages. At least 15 fingers of the fruit from each stage were randomly selected which was evaluated using peel color evaluation and impedance characteristics (stage 1, all green; stage 2, green with trace of yellow; stage 3, more yellow than green; stage 4, yellow with green tip; stage 5, yellow with brown flecks) as reported in the previous study. After sampling, fruits were then soaked in 200 ppm of sodium hypochlorite solution for 1 min, rinsed in running water, and allowed to stand on absorbent paper until dry. The peel was separated from the pulp and both were cut into small round sections, immediately frozen with liquid nitrogen, and freeze-dried. After freeze-drying, the samples were ground, passed through a 0.5 mm mesh sieve (Sanpo, Sanyo, Japan), and stored at -40 °C until analyses.

5.2.3 Chemical analyses

The initial moisture content, crude protein, crude fat, crude fiber, and ash were carried out in five maturity stages of Saba banana peel and pulp following the method of AOAC (AOAC International, 2000) with some modifications. Moisture content (MC) in fresh weight (FW) was measured by gravimetric heating using an oven dyer (Oven 8150, Labserv, Longford, Ireland), crude fat (CF) by Soxhlet method using petroleum ether; ash by calcination in a muffle furnace at 550 °C, fiber by acid-base digestion method, and crude protein by Kjeldahl method with a conversion factor of 6.25. Total and resistant starch were analyzed using an assay kit (Megazyme International, 2019) following AOAC Official Method 2002.02. Sugars (sucrose, glucose, and fructose) were determined using reversed-phase high-performance liquid chromatography (RP-HPLC). Freeze-dried samples (1 g) of Saba banana were extracted twice with 80% (v/v) ethanol. After homogenizing the mixture for 1 min, the homogenate was centrifuged and the supernatants for both extractions were combined and collected. The extracts were filtered through a 0.45 um membrane filter (Advantec, Tokyo, Japan) before the chromatographic separation. The HPLC system (Shimadzu, Kyoto, Japan) consisted of pump (LC-20 AD), refractive index detector (RID-20A), column (Shim-pack SCR-101N), oven (CTO-20 AC), and degassing unit (DGU-20A3). Ultrapure water as the mobile phase was pumped at a flow rate of 0.8 mL min^{-1} under isocratic elution at a constant oven temperature of 60 °C. Analytical data were collected and processed by LabSolutions software (Shimadzu, Kyoto, Japan).

5.2.4 Extraction of phenolic fractions

The content of phenolic compounds in free, esterified, glycosylated, and insoluble-bound fractions was determined according to the method of Arruda et al. (2018), with some modifications (Figure 5.1). Briefly, 2 g of freeze-dried unripe (stage 1) and ripe (stage 4) Saba banana peel and pulp were extracted with 15 mL of methanol-acetone-water mixture (7:7:6 v/v/v) using an ultrasonic bath (3510J Bransonic, Branson Ultrasonics Corp., Danbury, USA) for 30 min at room temperature, and then centrifuged at 4000 × g for 5 min at 5 °C. The residues obtained after centrifugation were re-extracted twice under the same conditions and the supernatants were collected and used for the fractionation of soluble phenolic compounds.

5.2.4.1 Free phenolics

Free phenolics were extracted by combining the supernatants recovered after centrifugation and evaporation under vacuum at 35 °C. The resulting aqueous phase was acidified to pH 2 using 6 M HCl and further centrifugation was done at 4000 × g for 5 min at

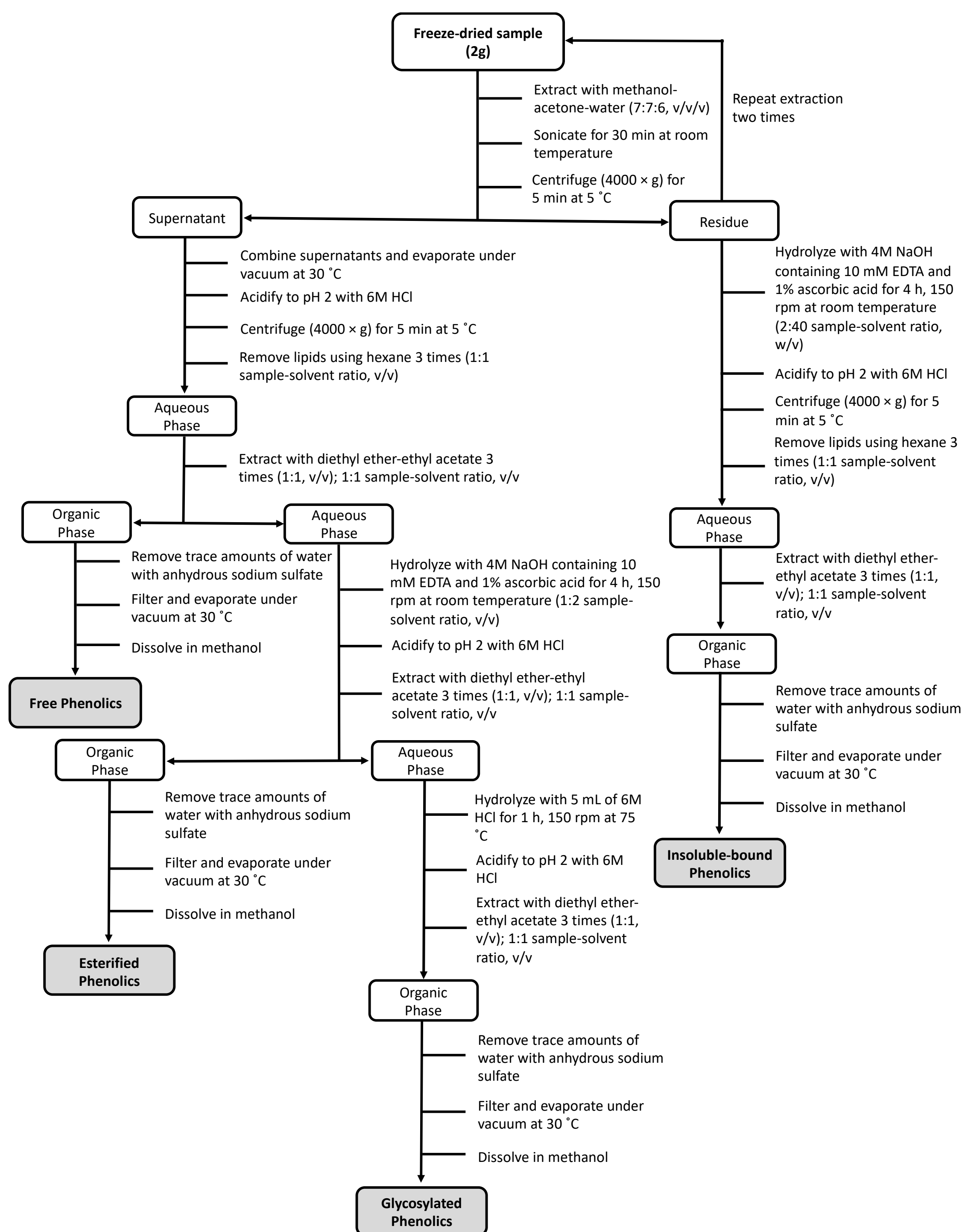

Figure 5.1 Summary of the extraction procedures of different phenolic fractions.

5 °C. Then, the clear supernatant was extracted with an equal volume of hexane three times followed by separation using diethyl ether-ethyl acetate (1:1, v/v) at a solvent to aqueous phase ratio of 1:1 (v/v) initially and then repeated two more times with half amount of solvent. The organic phases extracted were combined, dehydrated with anhydrous sodium sulfate, filtered

using a No. 1 Advantec filter paper, and evaporated to dryness under vacuum at 35 °C. The dry residues of free phenolic fraction were dissolved in 5 mL of HPLC grade methanol.

5.2.4.2 Esterified phenolics

Esterified phenolics were extracted using the aqueous phase remained after extraction of free phenolics. The solution was hydrolyzed with 4 M NaOH containing 10 mM EDTA and 1% (w/v) ascorbic acid in 1:2 v/v ratio of aqueous phase to solvent for 4 h at room temperature using a water bath shaker (150 rpm). The pH was adjusted to 2 using 6 M HCl and the separation of esterified phenolics was done the same as in free fractions using diethyl ether-ethyl acetate.

5.2.4.3 Glycosylated phenolics

Glycosylated phenolics were extracted using the aqueous phase remained after extraction of esterified phenolics. The solution was hydrolyzed with 5 mL of 6 M HCl for 60 min at 75 °C using a water bath shaker (150 rpm). The pH was adjusted to 2 using 6 M HCl and the organic phase recovered was subjected to the same condition as that of esterified phenolics.

5.2.4.4 Insoluble-bound phenolics

Insoluble-bound phenolics were extracted using the solid residue remained after extraction of soluble phenolics. The residue was hydrolyzed with 4 M NaOH containing 10 mM EDTA and 1% ascorbic acid in 1:2 v/v ratio of aqueous phase to solvent for 4 h at room temperature using a water bath shaker (150 rpm). The pH was adjusted to 2 using 6 M HCl, centrifuged at 4000 × g for 5 min at 5 °C and then extracted following the procedures of soluble phenolics.

5.2.5 Determination of bioactive compounds and antioxidant activity

A microplate reader (Multiskan FC, Thermo Fisher Scientific, MA, USA) was used in the spectrophotometric determinations of bioactive compounds and antioxidant activities of unripe and ripe stages of Saba banana peel and pulp. Assays were performed and read in 96-well microplates with 3 repetitions per sample and 5 to 6 levels of standards. On the other hand, condensed tannin content (CTC) was analyzed using UV–Vis spectrophotometer (V-630Bio, Jasco, Tokyo, Japan). All analyses were done according to previous study (Reginio, Qin, et al., 2020), except for CTC.

5.2.5.1 Total phenolic content (TPC)

The diluted extract (25 μL) was mixed with 125 μL of 10% (v/v) Folin-Ciocalteu reagent and 100 μL of 7.5% (w/v) Na_2CO_3. After incubation for 1 hour in the dark at room temperature, the absorbance was measured at 740 nm with blank. Gallic acid was used as a reference

standard for plotting the calibration curve (0–120 ppm). TPC was determined from the linear equation of the standard curve and expressed as mg gallic acid equivalents per gram of dry weight (mg GAE g^{-1} DW).

5.2.5.2 Total flavonoid content (TFC)

The diluted extract (33 µL) was mixed with 10 µL of 5% (w/v) $NaNO_2$. After 5 min, 10 µL of 10% (w/v) $AlCl_3$ was reacted to the mixture and incubated for another 6 min. Then, 67 µL of 1 M NaOH was added and the total volume was adjusted to 333 µL by adding distilled water. The absorbance of the reaction mixture was measured at 520 nm with blank. Catechin was used as a reference standard for plotting the calibration curve (0–100 ppm). TFC was determined from the linear equation of the standard curve and expressed as mg catechin equivalents per gram dry weight (mg CE g^{-1} DW).

5.2.5.3 Condensed tannin content (CTC)

CTC was determined using vanillin-HCl method performed according to Arruda et al. (2018) with minor modifications. Briefly, 30 µL of the diluted extract was mixed with 900 µL of 4% (w/v) vanillin in methanol and 450 µL of concentred HCl. After incubation at room temperature for 20 min, the absorbance was measured at 500 nm with blank on a UV-Vis spectrophotometer. A calibration curve was constructed with standard solutions of catechin (0–300 ppm) and the results were expressed as mg catechin equivalents per gram dry weight (mg CE g^{-1} DW).

5.2.5.4 Ferric-reducing antioxidant power (FRAP)

The diluted extract (20 µL) was mixed with 130 µL FRAP reagent containing acetate buffer (300 mM pH 3.6), TPTZ solution (10 mM), and $FeCl_3$ (20 mM) in a 10:1:1 (v/v/v) ratio. After incubation at 37 °C for 30 min in the dark condition, the absorbance was measured at 595 nm with blank. A calibration curve was constructed with standard solutions of $FeSO_4$ (0–200 µmol/L) and the results were expressed as µmol $FeSO_4$ equivalent per g DW.

5.2.5.5 Metal ion chelating (MIC) activity

The diluted extract (300 µL) was mixed with 5 µL of 2 mM $FeCl_2$ and 10 µL of 5 mM FerroZine. The mixture was gently shaken and left standing at room temperature for 10 min. Absorbance was measured at 560 nm with blank. A standard curve of EDTA (0-15 µmol/L) was prepared and the chelating activity was expressed as µmol EDTA equivalent per g DW.

5.2.5.6 $DPPH^•$ scavenging activity (DPPH)

The diluted extract (5 µL) was mixed with 195 µL methanolic solution of $DPPH^•$ (60 µM). The mixture was shaken and left to stand at room temperature for 30 min in the dark

condition. The absorbance was measured at 520 nm with blank. The $DPPH^{\bullet}$ scavenging activity was calculated based on calibration curves of Trolox (0-1200 μmol/L) and the results were expressed as μmol Trolox equivalent (TE) per g DW.

5.2.5.7 $ABTS^{\bullet+}$ scavenging activity (ABTS)

The $ABTS^{\bullet+}$ solution was prepared by reacting 7 mM $ABTS^{\bullet+}$ dissolved in 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12 h before use. The $ABTS^{\bullet+}$ solution was diluted with distilled water to obtain an absorbance of 0.70±0.02 at 740 nm. The assay was initiated by combining 10 μL of the diluted extract with 320 μL of diluted $ABTS^{\bullet+}$ solution. After 10 min of incubation at 30 °C in the dark, the absorbance was measured at 740 nm with blank. The results were expressed as Vitamin C equivalent (VCE) per g DW.

5.2.6 HPLC-DAD-ESI-MS analysis

The phenolic compounds of unripe and ripe Saba banana peel and pulp were analyzed using high-performance liquid chromatography with diode array detection electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS) system after obtaining the extracts from free, esterified, glycosylated, and insoluble-bound fractions. The mixture was filtered using a 0.45-μm membrane filter and analyzed using ACQUITY UPLC separation module coupled with Waters Micromass Q-micro MS(/MS) system that was controlled with MassLynx software (Waters, Milford, MA, USA). The column used was Xselect® HSS T3 (250 × 4.6 mm, 3.5 μm) with a security guard cartridge (4.0 × 3.0 mm). The solvents used were as follows: (A) 0.1% (v/v) acetic acid in ultrapure water and (B) acetonitrile. The gradient program was initiated with 0% solvent B from 0 to 5 min, and the gradient was slowly shifted to obtain 10% solvent B from 5 to 20 min, 20% B from 20 to 30 min, 35% B from 30 to 40 min, 90% B from 40 to 50 min, and 10% B from 50 to 60 min under a flow rate of 0.5 mL min^{-1} as described by H. Wang et al. (2019) with slight modification. The running temperature was 40 °C, and the injection volume was 20 μL. Mass spectrometer was operated in a negative ion mode to analyze the degraded mixture in the following conditions: capillary voltage of -3.0 kV and nebulizer gas (N_2) temperature of 350 °C (gas flow of 600 L/h). The cone voltage was set to 25 V. Mass spectra were scanned over the m/z range of 100-1000. Analysis in the positive ion mode was also conducted for identification.

5.2.7 Quantification by HPLC-DAD analysis

The quantification of individual phenolic compounds was conducted using HPLC-DAD comprising of Waters Alliance E2695 separation module and 2996 DAD with Empower® 3

software (Waters, Milford, MA, USA) under the same condition mentioned above. Simultaneous monitoring was set at 250, 280, 300, 320, and 350 nm for phenolic acids namely gallic acid, protocatechuic acid, vanillic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, isoferulic acid, and sinapic acid, and flavonoids viz. catechin, epicatechin, epigallocatechin, rutin, myricetin, quercetin, apigenin, naringenin, and kaempferol (Table 5.1). Stock solutions of each standard compound were prepared in either absolute methanol or ultrapure water. Subsequent dilutions were done using ultrapure water to come up with at least five-point standard curve (0–60 μg/mL). Limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the y-intercept and the slope of the calibration curve following ICH guidelines (European Medicines Agency, 2005). Peak identification was done based on the retention times of authentic standards and mass spectra. The quantification of identified phenolic compounds was performed using the constructed calibration curves. Results were expressed as μg per g dry weight (μg g^{-1} DW).

5.2.8 Statistical analysis

All extraction was done in three replications, and the analyses were carried out at least in triplicate. Statistical analyses of the data were performed using R software version 4.0.0 GUI 1.71 (R Development Core Team, 2018). Tukey's test was used for mean value comparisons at 5% significance level. Correlation analysis based on Pearson's method and principal component analysis (PCA) were performed among the variables using R studio version 1.2.1335.

5.3 Results and Discussion

5.3.1 Chemical composition of peel and pulp

The same as other climacteric fruit, the ripening of Saba banana involves a high metabolic activity which could affect its composition. The modifications in the chemical compositions of Saba banana peel and pulp during ripening are presented in Table 5.2. Proximate analysis showed that Saba banana peel and pulp were mostly composed of water accounting for more than half of their composition. The moisture content of the peel was significantly higher than the pulp. The gradually decreasing moisture content found in the peel as maturity advanced was accompanied by a concomitant increase in moisture of the pulp primarily due to osmotic transfer (Mohapatra et al., 2010). Similarly, a relatively higher percentage of crude protein, fat, fiber, and ash was observed in peel than pulp. Only around 1% of the pulp composition was accounted separately for crude fat and protein. While pulp

Table 5.1 Phenolic compounds determined by HPLC-DAD-ESI-MS and parameters used for quantification.

Phenolic compound	RT (min)	λmax (nm)	MS (m/z)	r	Slope	Intercept	LOD (µg/g)	LOQ (µg/g)
Phenolic Acids								
Protocatechuic acid[1,2]	17.2	250	353.90, 153.49, 117.30, 111.28	0.9999	118600	-1450	0.59	1.80
Vanillic acid[1]	18.7	250	287.75, 175.54	0.9991	14945	4668	0.78	2.35
Chlorogenic acid[1]	19.9	320	463.84, 158.59, 125.33	0.9990	125366	-58855	0.55	1.66
p-Hydroxybenzoic acid[1,2]	22.2	250	379.79, 137.42	0.9997	239614	-32163	0.28	0.85
Gallic acid[2]	23.7	280	323.72, 217.63, 169.49, 115.31	0.9996	94923	-13789	0.30	0.90
p-Coumaric acid[1,2]	37.7	300	287.69, 163.51, 119.34	0.9996	297963	-91666	0.36	1.09
Ferulic acid[1,2]	33.9	320	453.89, 193.62, 115.33	0.9974	216480	-244166	2.30	6.97
Sinapic acid[2]	39.4	320	409.91, 223.74	0.9998	250575	1125	0.22	0.68
Isoferulic acid[1,2]	35.1	320	594.01, 193.62, 115.39	0.9997	217186	-31307	0.27	0.82
Flavonoids								
Catechin[1,2]	20.9	280	379.80, 289.70	0.9994	29054	-8557	0.74	2.25
Epicatechin[1,2]	24.2	280	289.76, 102.33	0.9955	31610	5049	0.60	1.82
Epigallocatechin[1]	24.7	250	561.94, 316.79, 169.49	0.9998	105123	20037	0.27	0.81
Rutin[1]	30.2	250	545.94, 273.70	0.9991	97980	-46411	0.60	1.81
Myricetin[1,2]	37.4	250	386.78, 317.68	0.9987	53664	-393	0.45	1.37
Quercetin[1,2]	39.3	250	327.88, 301.67, 137.42	0.9972	35156	29728	0.94	2.86
Apigenin[1]	40.5	320	329.89, 117.20	0.9931	9312	-1941	1.06	3.21
Naringenin[1,2]	40.6	280	331.79, 271.76, 115.31	0.9998	122621	-12926	0.17	0.52
Kaempferol[1,2]	40.7	250	590.10, 285.67, 191.69	0.9953	6236	4239	1.22	3.69

RT, retention time; MS, mass spectrophotometry of parent ion and fragments; LOD, limit of detection; LOQ, limit of quantification

[1]Identification was confirmed with the retention time of an authentic standard.

[2]Identification was confirmed with the molecular formula of the parent ion and fragments.

was shown to have insignificant differences among the maturity stages in the contents of protein, fat, fiber, and ash, the peel displayed varying trends of result. A significant increase in the crude fat content of peel was observed from stage 1 up to stage 4 and then decreased in stage 5 owing to the possible continuous synthesis of fatty acids during metabolism (Khawas & Deka, 2016). The increasing amount of saturated and polyunsaturated fatty acids in the peels during ripening was previously reported in a banana variety that belonged to the same genome group (Emaga, Andrianaivo, Wathelet, Tchango, & Paquot, 2007). As the percentage of dry matter increased in the peel, with respect to maturity, the ash followed the same trend. The findings of previous studies (Hammond, Egg, Diggins, & Coble, 1996; Khawas & Deka, 2016)

Table 5.2 Comparison of the chemical composition of Saba banana peel and pulp.

Stage	MC (% FW)	Crude Protein	Crude Fat	Crude Fiber	Ash	RS	TS	Suc	Gluc	Fruc
Peel										
1	85.39 ± 0.66^{a}	6.96 ± 0.33^{a}	7.14 ± 0.47^{c}	7.23 ± 0.24^{ab}	10.84 ± 0.05^{c}	27.42 ± 0.40^{d}	28.88 ± 0.39^{e}	3.20 ± 0.19^{g}	1.10 ± 0.09^{f}	1.25 ± 0.07^{e}
2	84.15 ± 0.40^{b}	6.30 ± 0.15^{a}	7.20 ± 0.91^{c}	6.83 ± 0.28^{bc}	9.82 ± 0.12^{d}	28.01 ± 0.37^{d}	29.43 ± 0.35^{e}	3.76 ± 0.03^{g}	1.49 ± 0.23^{f}	1.83 ± 0.35^{d}
3	83.85 ± 0.23^{bc}	6.95 ± 0.29^{a}	8.64 ± 0.36^{b}	6.73 ± 0.05^{c}	10.82 ± 0.20^{c}	22.36 ± 0.50^{e}	24.32 ± 0.42^{f}	6.02 ± 0.36^{f}	2.02 ± 0.06^{e}	2.47 ± 0.15^{c}
4	83.66 ± 0.52^{bc}	7.25 ± 0.29^{a}	10.12 ± 0.32^{a}	7.39 ± 0.11^{a}	11.58 ± 0.25^{b}	9.36 ± 0.36^{g}	11.70 ± 0.59^{g}	8.74 ± 0.15^{d}	5.01 ± 0.08^{d}	5.61 ± 0.10^{b}
5	82.90 ± 0.36^{c}	7.45 ± 0.30^{a}	9.87 ± 0.12^{ab}	7.10 ± 0.23^{ac}	12.03 ± 0.02^{a}	5.48 ± 0.12^{h}	6.44 ± 0.48^{h}	10.31 ± 0.51^{c}	8.50 ± 0.26^{b}	9.18 ± 0.25^{a}
Pulp										
1	61.36 ± 0.95^{f}	3.30 ± 0.23^{b}	0.66 ± 0.01^{d}	0.68 ± 0.13^{d}	2.60 ± 0.03^{e}	53.02 ± 0.77^{a}	71.78 ± 0.66^{a}	7.05 ± 0.08^{ef}	0.21 ± 0.01^{g}	0.23 ± 0.02^{f}
2	61.94 ± 0.61^{ef}	3.14 ± 1.16^{b}	0.67 ± 0.09^{d}	0.81 ± 0.09^{d}	2.26 ± 0.19^{f}	52.90 ± 0.97^{a}	70.91 ± 1.05^{a}	7.81 ± 0.15^{de}	0.49 ± 0.02^{g}	0.50 ± 0.02^{f}
3	62.29 ± 0.96^{ef}	2.56 ± 0.01^{b}	0.75 ± 0.09^{d}	0.72 ± 0.06^{d}	2.52 ± 0.05^{ef}	45.33 ± 0.30^{b}	62.11 ± 0.46^{b}	15.41 ± 0.19^{b}	1.36 ± 0.05^{f}	1.30 ± 0.06^{e}
4	62.83 ± 0.22^{de}	3.10 ± 0.56^{b}	0.85 ± 0.06^{d}	0.71 ± 0.10^{d}	2.56 ± 0.03^{e}	30.01 ± 1.09^{c}	42.44 ± 1.09^{c}	28.19 ± 0.96^{a}	5.47 ± 0.10^{c}	5.24 ± 0.19^{b}
5	63.53 ± 0.57^{d}	2.81 ± 0.12^{b}	0.83 ± 0.11^{d}	0.87 ± 0.06^{d}	2.52 ± 0.02^{ef}	20.26 ± 0.77^{f}	32.36 ± 0.78^{d}	29.12 ± 0.67^{a}	9.29 ± 0.22^{a}	9.24 ± 0.22^{a}

MC, moisture content; RS, resistant starch; TS, total starch; Suc, sucrose; Glu, glucose; Fruc, fructose
All parameters are in % DW, except for moisture content which is in % FW basis. Different letters for the same parameter indicate significant differences among the stages of peel and pulp ($p < 0.05$).

also supported our results of increasing amount of ash content during ripening as this was related to the absorption capacity of mineral salts by the plant. Crude protein and fiber of the peel, on the other hand, were not significantly influenced by maturity, but an increasing trend was observed in the former. The increasing protein could be explained by the accumulation of proteins linked to ripening-related processes during maturation as reported by Yun et al. (2019).

Based on the data of proximate composition, the nitrogen-free extract could be estimated which ranged from 63–70% and 92–93% of the dry matter of peel and pulp, respectively. Our previous study showed that starch was the main dry matter component of unripe Saba banana pulp (stages 1 and 2) (Reginio, Ketnawa, et al., 2020). This was also true in the current study wherein more than 70% of the nitrogen-free extract of unripe pulp was from starch (based on DW basis). Though a lesser amount of starch was determined in peel than in pulp, the bulk of which was resistant starch. Resistant starch was accounted for more than 80% of the total starch content of the peel and the values were even higher in the unripe stages. This confirmed that

Saba banana peel could also be an excellent source of resistant starch. Nevertheless, in both peel and pulp, starch decreased considerably during ripening as resistant starch was degraded into free sugars under the action of several starch-degrading enzymes (Emaga et al., 2007). Previous research reported that enzymes involved in starch degradation were observed to have increased activity as proven by the upregulated expression of starch-degradation related genes during maturation (Yun et al., 2019).

The accumulation of soluble sugars, specifically sucrose, in Saba banana pulp during ripening was consistent with our previous study (Reginio, Ketnawa, et al., 2020). The content of sucrose was higher than glucose and fructose in both peel and pulp at all stages of maturity. When compared at the initial stages, the unripe stages (stages 1 and 2) of peel contained significantly higher sugar content than pulp. However, as the maturity advanced, the pulp was shown to surpass the content of sugars in the peel. The more rapid increase in sugar content of pulp than peel during ripening could explain the changes in the osmotic pressure, as discussed earlier, which eventually decreased and increased the moisture content of peel and pulp, respectively.

5.3.2 Total content of phenolic compounds and antioxidant activity

The extracts of Saba banana peel and pulp showed TPC values ranging from 3.7–12.1 mg GAE/g DW, TFC of 1.09–5.78 mg CE/g DW, and CTC of 0.63–4.5 mg CE/g DW (Figure 5.2a). Almost half of the TPC (48–52%) in the peel was from TFC while pulp had 26–33% TFC contribution to TPC. CTC showed a lower contribution to TPC accounting to 17% in pulp and 34% on the average in peel. In all assays conducted, the bioactive compounds in peel were found to be significantly higher than in pulp. About three times more TPC and five times more TFC and CTC were detected in peel than pulp. Comparing the maturity stages, no significant differences were observed in bioactive compounds of the two stages of pulp while peel showed significantly higher TPC and CTC in unripe than ripe counterpart. A percent decrement of 8% and 22% were determined in TPC and CTC, respectively, when Saba banana peel turned from all green stage to yellow with green tip stage. The observed no significance in TPC of pulp was consistent with the result of our previous study (Reginio, Qin, et al., 2020); however, the different trend in TFC could be accounted for the detection of esterified and glycosylated phenolic fractions.

Different fractions of phenolics were determined for the first time in Saba banana fruit. With few exceptions, free phenolics showed the lowest amount of TPC, TFC, and CTC while

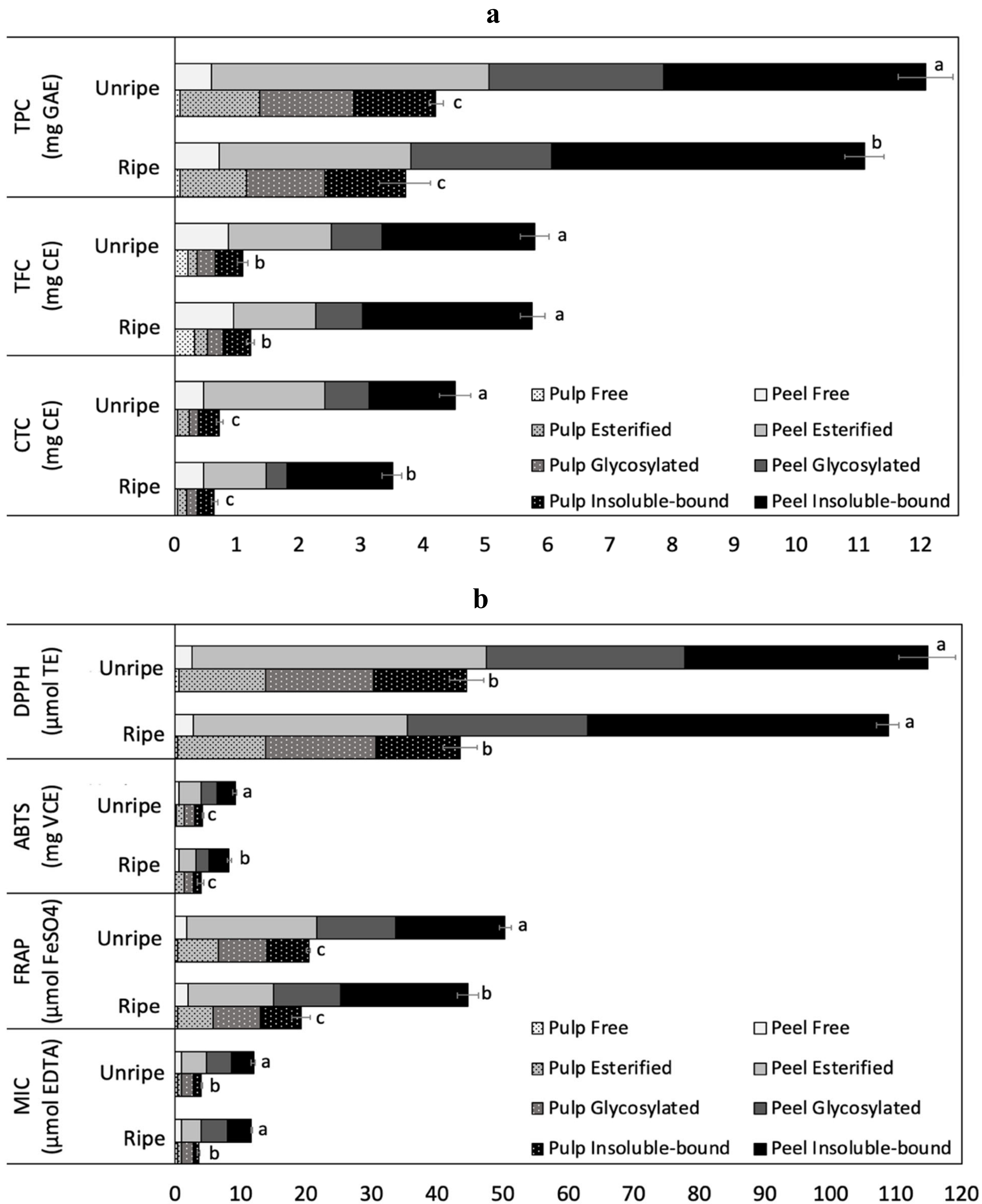

Figure 5.2 Contents of bioactive compounds (a) and antioxidant activities (b) of ripe and unripe stages of Saba banana peel and pulp. Different letters for the same parameter indicate significant differences between the unripe and ripe stages of peel and pulp ($p < 0.05$).

fractions containing esterified and insoluble-bound phenolics were the highest. On average, free fractions contributed to less than 7% of TPC, 14–25% of TFC, and 5–13% of CTC. Esterified and glycosylated contributions ranged from 20–37% for TPC, 12–28% for TFC, and 9–43% in CTC while insoluble-bound phenolics showed more than 30% contribution in the content of all bioactive compounds. This result supported our previous research (Reginio, Qin, et al., 2020) that bound phenolics were the major contributors of phenolic compounds in Saba banana. Higher proportions of esterified and insoluble-bound phenolics were also reported in araticum fruit (Arruda et al., 2018), calabura fruit (Pereira et al., 2018), blackberry, black raspberry, blueberry (Ayoub, de Camargo, & Shahidi, 2016), and leaves of jackfruit and carambola fruits (Chen et al., 2017). Interestingly, there was more contribution of glycosylated phenolics to TPC of pulp than of peel which could be possibly explained by the higher amounts of sugars in the pulp, in addition to the free sugars from starch hydrolysis, that are bound as glycosides.

Generally, the samples with higher bioactive compounds were most effective in scavenging free radicals, chelating ferrous ions, and reducing ferric iron. The antioxidant activities measured in terms of DPPH, ABTS, FRAP, and MIC followed the same trend as that of the contents of bioactive compounds in Saba banana peel having 2–3 fold significantly higher values than pulp (Figure 5.2b). The whole fruit had DPPH values ranging from 43.45–114.69 µmol TE, ABTS of 3.86–9.09 mg VCE, FRAP of 19.12–50.35 µmol $FeSO_4$, and MIC of 3.56–11.57 µmol EDTA/g DW. In terms of maturity, same as in bioactive compounds, no significant differences were found between the two stages of pulp while stages of peel showed only significant differences in ABTS and FRAP.

The antioxidant activities of different phenolic fractions showed that free phenolics still had the lowest contribution in both peel and pulp accounting to less than 9% of the total. In pulp, the highest fraction was observed in glycosylated phenolics which had more than 35% contribution in the antioxidant activity. In contrast, the antioxidant activity in peel in terms of DPPH, ABTS, and FRAP was highest in fractions containing esterified or insoluble-bound phenolics with a contribution of more than 29%. Specifically, the antioxidant activity of unripe peel decreased in the order esterified > insoluble-bound > glycosylated > free while for ripe counterpart insoluble-bound > esterified > glycosylated > free. MIC values of peel showed a different result among others with glycosylated fraction having the highest contribution for both unripe and ripe stages.

The study confirmed that Saba banana peel was also a good source of natural antioxidant compounds aside from its pulp. Numerous researches have reported higher total phenolic

content and stronger antioxidant activities of banana peel than that of pulp (Morais et al., 2015; Sulaiman et al., 2011; Tsamo et al., 2015). However, the amounts of the compounds in the peel have shown to be influenced by maturity (Vu et al., 2018). Based on the current study, maturation significantly affected the phenolic content and antioxidant activities of the peel. As discussed in our previous study (Reginio, Qin, et al., 2020), the decrease could be associated with the presence of polyphenol oxidase which still showed activity even during the late stages of ripening. Another reason could be the usage of phenolics by the cell serving as physical and chemical barriers against pathogenic attack. The changes in the phenolic content and antioxidant activity of banana peel as it turned from green to yellow were also reported in the previous studies (Fatemeh et al., 2012; Sundaram, Anjum, Dwivedi, & Rai, 2011).

5.3.3 Characterization and quantification of phenolic compounds from unripe and ripe stages of peel and pulp

The chromatographic parameters of HPLC-DAD were first optimized to ensure good peaks resolution. The compounds were identified by their retention times as compared to those of authentic standards and molecular formulas of the parent ion and the fragments in comparison with those found in literature. Eighteen phenolic compounds were identified and quantified which included 9 phenolic acids and 9 flavonoids. Phenolic acids present were mostly hydroxycinnamic (ferulic, sinapic, chlorogenic, isoferulic, and *p*-coumaric acids) and hydroxybenzoic acids (gallic, protocatechuic, vanillic, and *p*-hydroxybenzoic acids) (Table 5.3). Flavonoids, on the other hand, were flavanols (catechin, epicatechin, epigallocatechin), flavonols (rutin, quercetin, myricetin, apigenin, and kaempferol), flavanone (naringenin) (Table 5.4).

Esterified and insoluble-bound were the fractions that showed the major diversity of phenolic compounds. In both phenolic acids and flavonoids, the insoluble-bound showed the highest content of phenolic compounds among the fractions. All the phenolic compounds identified in peel and pulp were present and detected in insoluble-bound fraction. This supported the previous results of having higher bioactive compounds in insoluble-bound fractions. The concentration of phenolic compounds in different fractions was in the order insoluble-bound > glycosylated > esterified > free.

From the identified phenolic compounds, epicatechin was the most predominant in free fraction of unripe peel and in esterified fraction of pulp with 26% and around 40% contribution, respectively. Epicatechin is associated with anti-inflammatory, anti-platelet activities, reduction of low-density lipid oxidation, and improvement of endothelial functions in the body

(Anyasi, Jideani, & McHau, 2018). Bennett et al. (2010) analyzed an ABB genome banana and found out that epicatechin was the highest when compared to other flavanols (gallocatechin and catechin). Same as epicatechin, kaempferol has been previously reported to be one of the major components of banana (L. Fu et al., 2011; Shivashankara, 2016). The HPLC-DAD analysis showed high concentration of kaempferol in ripe and both stages of peel in free (30% contribution) and esterified (18–22% contribution) fractions, respectively. Aside from having antioxidant activity, this type of flavonol exhibits antidepressant effect, increases resistance to oxidative stress, and reduces glucose-induced oxidative cell damage and dysfunction in pancreatic cell (Lin et al., 2014). In contrast to the peel, the highest phenolic compounds in free fraction of pulp was vanillic acid with 66–69% contribution. Siriamornpun and Kaewseejan (2017) analyzed various cultivars of Thai banana and quantified vanillic acid at a concentration ranging from 0.08–0.37 mg/g DW. Experimental trials about vanillic acid showed effectiveness in the reduction of DNA damaged cells and against the risk of myocardial dysfunction (Kiokias, Proestos, & Oreopoulou, 2020).

The highest phenolic compound identified in glycosylated fraction was protocatechuic acid with more than 70% contribution. The occurrence of protocatechuic in banana pulp has been reported in the previous study (Borges et al., 2014), thus corroborating the results obtained in this work. Protocatechuic acid is one of biologically active components of some medical plants found to be potent antibacterial, anticancer, antihyperlipidemic, and anti-inflammatory and was proven to possess Fe^{2+} and Cu^{2+} chelating activity. In the current study, glycosylated fraction was found to have the highest contribution to metal chelating activity in both stages of peel and pulp possibly because of the presence of protocatechuic acid.

Among the potent phenolic compounds in insoluble-bound fractions, the highest was ferulic acid accounting to more than 50%, not including its isomer. The presence of ferulic acid in banana has been reported in high amounts, especially in plantains (Tsamo et al., 2015) which corresponds to the result of this study. The high antioxidant activity of insoluble-bound fraction in ripe banana pulp could account for the presence of high amounts of ferulic acid. Previous study reported that ferulic acid was observed to have the strongest action among the hydroxycinnamic acids tested against formation of hydroperoxides in ethanol-buffer solution (Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi, 2002). Based on a number of *in vitro* and *in vivo* studies carried out, ferulic acid is widely known to function as antioxidant and anti-inflammatory compounds which are linked with protective effect against several pathologic conditions such as cancer, cardiovascular diseases, diabetes mellitus, and skin problems (Kiokias et al., 2020). The phenolics liberated from the insoluble-bound fraction comprised

57–64% and 62–72% of the summed values of identified phenolics in Saba banana peel and pulp, respectively. Out of this percentage, 30–43% and 46–60% of the total phenolic composition in peel and pulp, respectively, were accounted for the ferulic acid.

All phenolic compounds detected showed higher values in peel than in pulp. Phenolic compounds that were present in peel but became not detected in pulp in different fractions were chlorogenic acid, *p*-hydroxybenzoic acid, myricetin, quercetin, kaempferol, and apigenin. In terms of maturity, epicatechin and epigallocatechin showed undetectable values in ripe counterparts under free fraction. Interestingly, glycosylated epigallocatechin and free quercetin in pulp became detected in ripe stage which was previously undetected in its unripe counterpart. Among the phenolic compounds, sinapic acid, catechin, and rutin were not detected in glycosylated fraction.

Table 5.3 Content of phenolic acids in different fractions of unripe and ripe stages of Saba banana peel and pulp.

Phenolic acids (ug/g DW)	Free				Esterified				Glycosylated				Bound			
	Peel		Pulp		Peel		Pulp		Peel		Pulp		Peel		Pulp	
	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe
Proto-catechuic acid	4.51 ±1.78^{d}	3.97 ±1.43^{d}	<LOQ	<LOQ	31.64 ±2.61^{d}	20.71 ±1.79^{d}	7.62 ±1.05^{d}	3.59 ±0.45^{d}	960.40 ±68.36^{a}	687.73 ±76.54^{b}	306.46 ±29.53^{c}	270.53 ±40.09^{c}	29.18 ±1.77^{d}	39.45 ±1.41^{d}	12.19 ±0.90^{d}	11.42 ±1.16^{d}
Vanillic acid	23.95 ±3.41d^{e}	20.75 ±1.97^{de}	38.18 ±8.97^{de}	31.20 ±3.03^{de}	51.38 ±8.77^{cd}	36.92 ±4.98^{de}	4.91 ±1.09^{e}	<LOQ	98.24 ±9.54^{b}	75.18 ±5.97^{bc}	31.50 ±3.73^{de}	14.61 ±1.97^{e}	214.53 ±35.68^{a}	105.27 ±20.35^{b}	13.28 ±3.11^{e}	30.96 ±4.86^{de}
Chlorogenic acid	<LOQ	<LOQ	nd	nd	10.73 ±2.20^{a}	9.64 ±2.19^{a}	<LOQ	<LOQ	4.64 ±0.54^{bc}	3.07 ±0.31^{c}	<LOQ	2.28 ±0.33^{c}	4.42 ±0.43^{bc}	7.65 ±1.89^{ab}	<LOQ	<LOQ
Gallic acid	1.14 ±0.04^{e}	0.92 ±0.03^{e}	0.99 ±0.03^{e}	<LOQ	25.54 ±2.03^{a}	18.97 ±2.39^{b}	1.82 ±0.42^{e}	2.07 ±0.57^{e}	1.86 ±0.06^{e}	1.30 ±0.14^{e}	<LOQ	<LOQ	15.91 ±0.81^{c}	21.48 ±0.75^{b}	7.77 ±0.33^{d}	8.09 ±0.19^{d}
p-Hydroxy-benzoic acid	1.01 ±0.14^{d}	1.20 ±0.18^{d}	nd	nd	26.14 ±1.32^{b}	30.68 ±3.69^{a}	<LOQ	0.97 ±0.17^{d}	1.40 ±0.02^{d}	1.32 ±0.14^{d}	<LOQ	<LOQ	21.07 ±1.88^{c}	22.70 ±0.96^{bc}	3.41 ±0.19^{d}	3.77 ±0.50^{d}
p-Coumaric acid	1.96 ±0.06^{e}	12.62 ±1.10^{c}	<LOQ	<LOQ	6.85 ±0.68^{d}	6.50 ±1.08^{d}	2.11 ±0.38^{e}	2.14 ±0.37^{e}	1.56 ±0.04^{e}	1.46 ±0.07^{e}	1.32 ±0.11^{e}	1.24 ±0.08^{e}	68.84 ±2.59^{b}	85.73 ±3.78^{a}	14.76 ±0.37^{c}	14.76 ±0.25^{c}
Ferulic acid	11.86 ±0.78^{e}	14.61 ±1.44^{e}	<LOQ	<LOQ	30.66 ±1.30^{e}	29.10 ±4.37^{e}	20.99 ±2.47^{e}	10.84 ±0.86^{e}	<LOQ	<LOQ	<LOQ	<LOQ	1442.44 ±33.00^{b}	1776.62 ±61.50^{a}	630.64 ±35.37^{d}	847.24 ±21.73^{c}
Sinapic acid	1.09 ±0.20^{b}	<LOQ	<LOQ	<LOQ	24.21 ±0.45^{a}	23.94 ±4.18^{a}	<LOQ	<LOQ	nd	nd	nd	nd	23.53 ±0.44^{a}	28.08 ±1.11^{a}	3.75 ±0.19^{b}	4.45 ±0.54^{b}
Isoferulic acid	1.54 ±0.13^{d}	1.82 ±0.17^{d}	<LOQ	<LOQ	1.89 ±0.06^{d}	1.53 ±0.05^{d}	3.90 ±0.25^{d}	2.01 ±0.29^{d}	1.26 ±0.06^{d}	<LOQ	0.85 ±0.12^{d}	<LOQ	134.92 ±3.50^{a}	100.09 ±1.72^{b}	15.02 ±0.46^{c}	14.34 ±0.75^{c}
ΣPhenolic acids	47.05 ±6.31^{h}	55.88 ±5.99^{h}	39.17 ±9.00^{h}	31.20 ±3.03^{h}	209.04 ±9.74^{g}	177.99 ±18.83^{g}	41.36 ±3.26^{h}	21.61 ±1.04^{h}	1069.36 ±61.25^{c}	770.06 ±82.25^{e}	340.14 ±33.10^{f}	288.66 ±38.90^{fg}	1954.85 ±53.68^{b}	2187.07 ±59.20^{a}	700.82 ±39.38^{e}	935.04 ±16.83^{d}

nd, not detected which is less than limit of detection; <LOQ, detected but less than limit of quantification

Different letters for the same parameter indicate significant differences between the unripe and ripe stages of peel and pulp ($p < 0.05$).

Table 5.4 Content of flavonoids in different fractions of unripe and ripe stages of Saba banana peel and pulp.

Flavonoids (ug/g DW)	Free				Esterified				Glycosylated				Bound			
	Peel		Pulp		Peel		Pulp		Peel		Pulp		Peel		Pulp	
	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe
Catechin	6.57 ±0.99^{ef}	4.94 ±1.40^{f}	<LOQ	<LOQ	50.88 ±4.65^{cd}	28.42 ±12.74^{de}	<LOQ	3.30 ±0.35^{f}	nd	nd	nd	nd	190.73 ±13.98^{b}	228.53 ±13.03^{a}	67.87 ±4.17^{c}	42.79 ±4.08^{d}
Epicatechin	40.96 ±6.44^{bcd}	44.47 ±6.63^{bc}	2.75 ±1.53g	nd	99.69 ±4.88^{a}	51.69 ±14.52^{b}	26.94 ±5.15^{de}	20.01 ±4.11^{ef}	4.38 ±1.33^{g}	5.03 ±1.19^{fg}	1.89 ±0.28^{g}	1.86 ±0.07^{g}	33.41 ±3.34^{ce}	45.43 ±1.42^{bc}	30.77 ±1.96^{ce}	25.15 ±0.37^{e}
Epigallo-catechin	3.46 ±0.17^{dc}	8.94 ±0.64^{b}	<LOQ	nd	8.78 ±1.28^{b}	14.67 ±4.30^{a}	1.21 ±0.51^{d}	1.37 ±0.53^{d}	2.52 ±0.26^{cd}	1.91 ±0.51^{cd}	nd	<LOQ	5.81 ±0.64^{bc}	9.01 ±0.42^{b}	1.22 ±0.07^{d}	1.13 ±0.15^{d}
Rutin	7.13 ±1.58^{bc}	4.70 ±0.56^{def}	10.49 ±0.96^{a}	4.81 ±0.58^{def}	9.23 ±1.25^{ab}	3.39 ±0.35^{f}	<LOQ	<LOQ	nd	nd	nd	nd	6.74 ±0.38^{cd}	4.39 ±0.54^{ef}	5.85 ±0.09^{ce}	5.80 ±0.21^{ce}
Myricetin	1.80 ±0.31^{e}	1.60 ±0.39^{e}	nd	nd	16.56 ±2.19^{d}	18.02 ±1.96^{d}	<LOQ	<LOQ	57.39 ±2.62^{a}	42.92 ±5.84^{b}	7.03 ±1.70^{e}	4.26 ±1.20^{e}	19.44 ±1.36^{d}	27.05 ±1.91^{c}	2.79 ±0.33^{e}	1.92 ±0.24^{e}
Quercetin	2.97 ±0.25^{d}	2.93 ±0.26^{d}	nd	3.59 ±0.87^{d}	54.18 ±0.82^{cd}	61.15 ±9.22^{c}	<LOQ	3.18 ±1.16^{d}	43.61 ±2.85^{cd}	34.44 ±4.35^{cd}	nd	nd	443.44 ±58.23^{a}	147.53 ±22.47^{b}	4.20 ±0.82^{d}	4.56 ±1.44^{d}
Apigenin	16.14 ±1.26^{d}	14.19 ±1.94^{d}	5.19 ±0.69^{e}	5.06 ±0.69^{e}	<LOQ	<LOQ	nd	nd	3.61 ±0.67^{e}	<LOQ	3.38 ±0.26^{e}	<LOQ	37.49 ±2.32^{a}	30.64 ±0.63^{b}	26.48 ±2.03^{bc}	25.02 ±2.65^{c}
Naringenin	1.02 ±0.10^{c}	0.85 ±0.12^{c}	<LOQ	0.54 ±0.03^{c}	0.61 ±0.04^{c}	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	4.78 ±0.47^{b}	8.06 ±0.58^{a}	1.18 ±0.04^{c}	1.25 ±0.17^{c}
Kaempferol	31.01 ±4.05^{e}	60.74 ±7.29^{d}	nd	nd	129.32 ±9.09^{a}	77.39 ±4.57^{cd}	nd	nd	90.97 ±4.55^{c}	72.62 ±11.06^{cd}	26.87 ±2.28^{e}	25.82 ±3.14^{ef}	93.34 ±13.21^{bc}	113.60 ±12.48^{ab}	3.78 ±0.13^{g}	4.33 ±0.69^{gf}
ΣFlavonoids	111.05 ±6.12^{fg}	143.37 ±16.36^{ef}	18.43 ±1.93^{h}	14.00 ±2.14^{h}	369.24 ±17.64^{c}	254.73 ±45.89^{d}	28.15 ±5.55^{h}	27.86 ±2.51^{h}	202.48 ±10.31^{de}	156.92 ±22.73^{ef}	39.16 ±3.97^{gh}	31.94 ±2.76^{h}	835.18 ±65.43^{a}	614.24 ±11.74^{b}	144.14 ±5.52^{ef}	111.94 ±6.69^{f}

nd, not detected which is less than limit of detection; <LOQ, detected but less than limit of quantification

Different letters for the same parameter indicate significant differences between the unripe and ripe stages of peel and pulp ($p < 0.05$)

5.3.4 Correlation and principal component analyses

To summarize the results, a descriptive model showing the grouping of the samples according to their bio-properties (chemical compositions, total contents of bioactive compounds, antioxidant activity, and individual phenolics) was established using principal component analysis (Figure 5.3). The ggbiplot clearly showed the dispersion of the samples according to PC1 and PC2 which both explained 97.2% of the variance. PC1 was able to distinguish the peel from the pulp mainly because of the bioactive compounds, antioxidant activity, chemical compositions except for starch and sugars, and individual phenolics except for rutin. The peel was observed to have higher amounts of those variables than pulp resulting in their separation. It can also be deduced from the plot the close relationship existing between individual phenolic compounds and their antioxidant activities. PC2, on the other hand, showed the separation between unripe and ripe stages of Saba banana peel and pulp. This was mainly explained by the contents of starch, sugars, and rutin. As mentioned above, the amounts of starch and sugars varied considerably during ripening, the reason for their high correlation in PC2. Likewise, the decrease in the amount of rutin in the peel by around 50% during ripening resulting in its insignificant values when compared to pulp could account for its slight separation from the other phenolics. This PCA data showed that these variables could be markers for the determination of maturity of Saba banana. Other principal components showed less than 6% variability indicating that no additional common pattern exist among bio-properties.

The correlation analysis supported the results of the PCA (Figure 5.4). All variables had strong correlations to each other (>0.9), except for rutin, starch, and sugars which previously reported to have high correlation to PC2. Both starch and sugars showed negative correlations to other variables. Sugars had a different trend of result which increased during ripening, whereas starch showed significant dropped in its content for both peel and pulp. Moreover, the peel, which was found to contain high amounts of phenolic compounds and antioxidant activity, was reported to have lower starch contents than pulp. This could be the reason for the strong negative correlation of starch content to phenolic compounds.

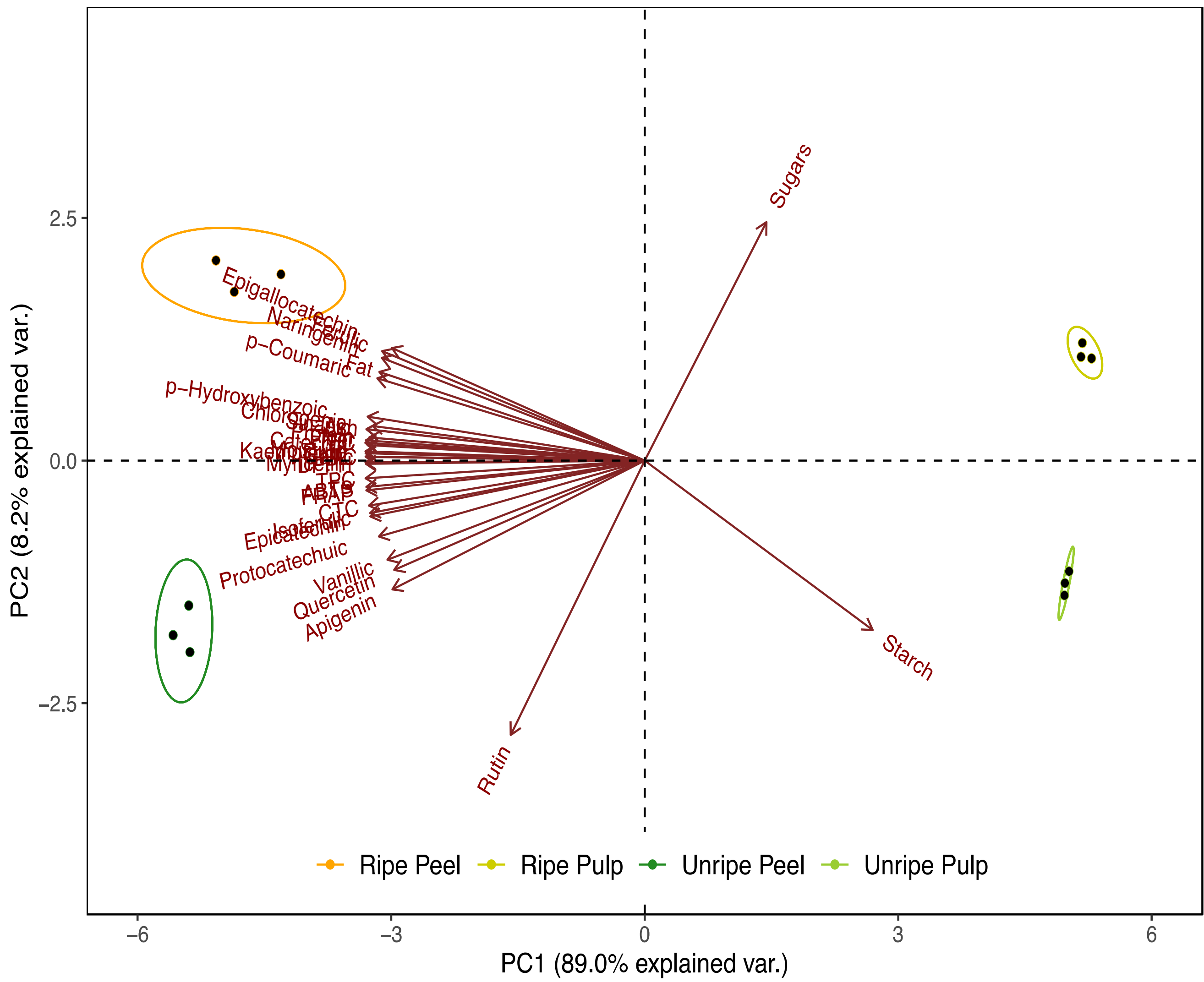

Figure 5.3 Principal component analysis of Saba banana peel and pulp based on their bio-properties.

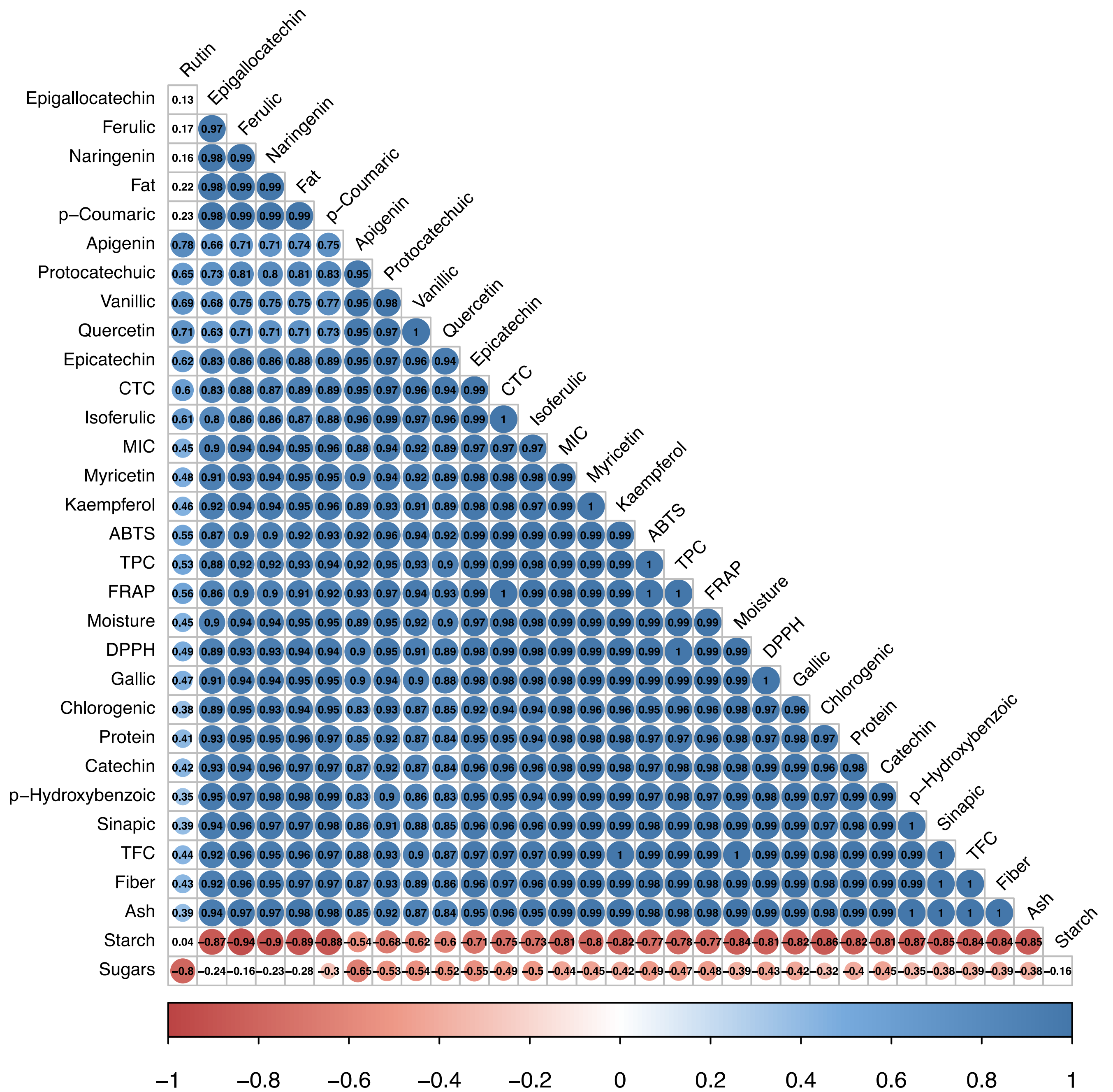

Figure 5.4 Correlation matrix of the bio-properties of Saba banana peel and pulp.

5.4 Conclusion

Evaluation of the different stages of Saba banana peel and pulp showed great diversity in terms of chemical composition, bioactive components, antioxidant activities, and individual phenolics. The peel showed the same trend as that of pulp in terms of starch and sugar contents. Two stages representing unripe and ripe peel and pulp of Saba banana were fractioned into free, esterified, glycosylated, and insoluble-bound forms. The high contents of bioactive compounds in esterified and insoluble-bound fractions were consistent in the results of antioxidant activity; however, glycosylated phenolics dominated both fractions by having higher antioxidant activity in pulp. HPLC-DAD-ESI-MS showed that phenolic acids had the highest contribution of bioactive compounds in both peel and pulp. Specifically, ferulic acid was the main phenolic compound being present mostly in insoluble-bound fraction followed by protocatechuic acid in glycosylated fraction. Eighteen individual phenolic compounds were identified in this study, but further evaluation is needed to confirm and characterize other unknown chromatographic peaks. Maturation was found to have significant impact on the peel which also showed consistently higher phenolic compounds and antioxidant activities than the pulp. The study suggests that the whole Saba banana fruit is not only a good source of nutritional components, but also contains natural antioxidant compounds which could be used in the development of functional ingredients that could later on add higher value to this particular variety of banana.

CHAPTER 6

General Conclusion

Maturation influenced the rate of starch hydrolysis and the release of bioactive components during *in vitro* digestibility of Saba banana. The changes in the composition of the fruit accompanying maturation in combination with the physical properties of the digesta could account for the decreasing trend of starch hydrolysis as ripening proceeded and the slow release of bioactive compounds in ripe fruits during *in vitro* digestion. The highly viscous digesta was one of the physical properties observed to have an impact on the release of substances due to the immobilization of digestive components, inhibiting substrate-enzyme catalyzed reactions. The weak gel-like behavior of the digesta in ripe stages which might be due to interactions of fruit components, possibly water-soluble pectins, moisture, sugar, and acid, increased the bulk viscosity of the sample, and thus reduce the rate of starch hydrolysis and the release of bioactive components. The same effect was observed in the presence of intact cell structure as this acted as a barrier to the action of enzymes making the penetration effects of digestion slower than structure-less samples. Both of these physical properties of digesta could offer physiological advantages in preventing the sudden surge in glucose level upon ingestion of banana. Moreover, together with the physical properties, the action of digestive enzymes and the changing pH conditions in the gastrointestinal tract could contribute to the release and stability of bioactive compounds from the food matrix, which in turn may have an impact on their bioaccessibility and bioavailability.

Maturation brought significant modifications in the pectic polysaccharides of Saba banana. As water-soluble pectin increased, chelate-soluble pectin decreased during ripening. The estimation of pectin yield obtained through spectrophotometric and gravimetric methods showed the same trend of decreasing values as maturity proceeded. However, the values were incomparable and significantly different possibly due to the effect of neutral sugars and starch contamination. The decreasing degree of esterification during maturity led to the characterization of ripe stages as low methoxyl pectin. This finding supported the assumption that pectin in ripe stages affected the viscosity of the digesta. However, the increase in viscosity was not associated to the effect of acid but due to the reaction of LMP with mono- and divalent ions under alkaline conditions. On the other hand, electrical impedance also showed a decreasing trend of values as pectin content decreased. With these results, an attempt to correlate the changes in impedance characteristics and pectic polysaccharides during

maturation was done suggesting a relationship associated with softening brought about by the loss of cell wall structure.

Maturation significantly affected the chemical compositions, total contents of bioactive compounds, and antioxidant activities of Saba banana, especially of the peel. Bio-properties of pulp followed the same trend as observed in peel by having highest values in unripe stage and then decreased slightly as ripening proceeded with some exceptions; however, the results were mostly insignificant. The observed bio-properties were found to be significantly higher in peel than in pulp. In general, bound phenolics showed higher contents of bioactive compounds and antioxidant activities than free form in both peel and pulp. The results suggest that full exploitation of Saba banana fruit could be a good source of natural antioxidant compounds for preparing functional food ingredients necessary to promote human health.

In general, the study was able to investigate the maturity effect on the bio-properties of Saba banana. This was the first report about the digestibility of starch and bioactive compounds in fresh form of banana. Previous data were available about starch digestibility; however, most *in vitro* studies used the raw starch form and not taking into account the overall composition of the food matrix which could affect the digestibility of different compounds. The results could be a source of basic information for a more complex analysis in the future, e.g. *in vivo* studies. It also implied that foodstuffs could be structurally modified to have lesser effect on postprandial blood glucose level and for the immediate release of bioactive components. Based on the *in vitro* simulation studies, it can be suggested that when eating ripe bananas, processing conditions (e.g. crushing or blending) or by thorough mastication of food with less dilution would result to a highly viscous product that could have low impact on starch digestibility. On the other hand, slight processing is necessary for the green mature bananas, (e.g. cutting or by chewing into small pieces) to maintain the intact cell structure which could prevent the sudden surge in the blood glucose level upon ingestion.

The phenolic compositions of peel and pulp from Saba banana in different fractions were also evaluated for the first time. Given the lack of published data about this variety, Saba banana could be used as a model for evaluating other indigenous fruit crops that have the potential to provide a healthy food source. Though some published articles included the varieties in the same genome group, those studies were not able to determine other phenolic fractions which could lead to underestimation of the biological activities of this fruit crop. For a better bioaccessibility of bioactive compounds, immediate release by disrupting the cell structure through physical processing could be applied. Moreover, to liberate bound phenolic

compounds from the cell wall matrix of Saba banana, processing procedures such as fermentation or cooking could be done.

For future investigations, the effect of other non-polysaccharide components of fruit in starch digestibility and other factors that contribute to the degradation of cell wall components, aside from pectin polysaccharides, required for fruit softening should be further investigated. Correlation of the obtained results using *in vivo* methods is also necessary to better model the digestive fate of bioactive compounds in Saba banana as complex reactions and transformations are simultaneously occurring during the gastrointestinal digestion process. Moreover, the used of equipment with increased sensitivity to gain more structural information of the analytes based on fragmentation pattern is recommended to characterize other unknown chromatographic peaks.

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